Monograph on Zinc Salts-Volume 2

9/11/72

J34

J3c

MONOGRAPH

ON

ZINC SALTS

VOLUME II

TR-72-1552-11

Submitted Under:
Contract No. FDA 72-104

September 11, 1972

INFORMATICS INC.
6000 Executive Boulevard
Rockville, Maryland 20852

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 97, 309-320 (1962)

Observations on the Role of Sulfhydryl Groups in the Enzymatic Activity of Myosin

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Received October 17, 1961

Low concentrations of *p*-chloromercuribenzoate (PCMB), Cu^{++}, Cd^{++}, and Zn^{++} enhance the ATPase activity of myosin while high concentrations inhibit. The presence of ATP reduces the rate at which high concentrations of PCMB, Cu^{++}, Cd^{++}, or Zn^{++} inactivate myosin. The presence of ATP also reduces the rate at which low concentrations of PCMB or *N*-ethylmaleimide activate myosin ATPase. The activating effects of low concentrations of Cu^{++} and PCMB are additive, as are the inactivating effects of high concentrations of Cu^{++} and PCMB. The effects of Zn^{++} and Cd^{++} but not Cu^{++} can be partially reversed by dialysis against cysteine, AET, or EDTA. With increasing time of exposure at 0 C., Cu^{++}, Cd^{++}, and Zn^{++} all become more potent inhibitors. At high pH, Cd^{++} and Zn^{++} become more potent inhibitors of myosin, but Cu^{++} becomes a very weak inhibitor. At low pH, Cu^{++} only inhibits myosin ATPase. The curve of ATPase versus pH in the presence of Cu^{++} resembles a titration curve with a "pK" near 7.5. Myosin that has been extensively treated with trypsin shows no change in the pH dependence of its ATPase activity either in the presence or in the absence of PCMB. It is suggested that two —SH groups, with different effects on ATPase activity and different reactivities toward —SH reagents, are involved in the functioning of the active site.

INTRODUCTION

The —SH groups of myosin are important both in the enzymic and in the contractile processes (1, 2). Of the wide variety of —SH reagents which have been studied, some, such as salyrgan, oxarsan, and iodoacetamide only inhibit these processes (3). Others, such as AET[1] and CEE appear only to accelerate ATPase activity (4), while their effects on contractile models are as yet scarcely explored (5). A third category consists of substances which in low concentrations enhance ATPase activity while in high concentrations they inhibit. In this category are PCMB (6), PMA (7), NEM (6), BAL arsenite (8), Zn^{++} (9), Cu^{++}, and Cd^{++} (10). From these studies it has become clear that the enzymic site of myosin has at least two —SH groups (6, 10) and that these groups have different effects on the ATPase activity. In this paper we have studied the effects of several of these reagents on the enzymic activity of myosin under conditions which permit partial specification of the role of the —SH groups in the active site. A preliminary report of some of these findings has already appeared (11).

[1] The abbreviations used are: PCMB, *p*-chloromercuribenzoate; AET, *S*-aminoethylisothiouronium bromide; CEE, cysteine ethyl ester; NEM, *N*-ethylmaleimide; PMA, phenylmercuric acetate; Tris, tris(hydroxymethyl)aminomethane; EDTA disodium ethylenediamine tetraacetate; ATP, adenosine triphosphate; ATPase, adenosinetriphosphatase; ITP, inosine triphosphate; ITPase, inosinetriphosphatase; NTP, nucleoside triphosphate; BAL, 2,3-dimercaptopropanol. According to the Sigma Chemical Co. PCMB may actually be *p*-hydroxymercuribenzoate, but we shall continue to designate it as PCMB in this paper.

EXPERIMENTAL PROCEDURE

Myosin A was prepared from 15-min. extracts

of rabbit muscle using the KCl-PO_4-EDTA extraction fluid and procedure described by Mommaerts (12). After three purification cycles of precipitation at 0.051 *M* KCl and resolution in 0.6 *M* KCl, it was freed of myosin B by discarding the precipitate resulting from centrifugation at 27,000 × *g* in 0.29 *M* KCl.

Measurements of pH were made with a Radiometer pH meter, which was also used in conjunction with the Radiometer titrimeter for pH-stat work. Reactions studied with the pH-stat were carried out in a thermostated glass cell, with water-saturated nitrogen gas flowing over the surface. Trypsin and trypsin soybean inhibitor were purchased from the Worthington Biochemical Co. AET, CEE, and NEM were purchased from the Schwarz Biochemical Co. Biquinoline was purchased from the Eastman Chemical Co. ATP, ITP, PCMB, and Tris were purchased from the Sigma Chemical Co. PCMB was purified by the method of Whitmore and Woodward (13). All other chemicals were reagent grade.

Phosphate was measured using modifications of the Fiske-SubbaRow technique that have been described in detail (10). Protein concentration was measured by the Kjeldahl technique. Copper concentration was measured by the method of Felsenfeld (14), using excess cysteine to insure the reaction of all the copper, and employing solutions of known copper concentration in the presence of appropriate amounts of myosin as standards.

Except when otherwise noted, the final concentration of ATP was 10^{-3} *M*. All activities are expressed in μmoles P/sec./g.

RESULTS

Activation and Inhibition by PCMB

In previous experiments (6, 10, 15) it has been found that myosin exposed to high concentrations of PCMB exhibits a greatly inhibited rate of NTP hydrolysis. If, however, PCMB and ATP are added to myosin at the same time and the rates are measured within 200 sec., it is found that even 70 moles/10^5 g. myosin does not cause a net inhibition of the ATPase activity, although the activation is entirely abolished (Fig. 1). If the myosin is preincubated with PCMB in the absence of ATP for 50 sec., the familiar inhibitory action of high concentrations of PCMB is observed.[2] With increasing time of preincubation, the concentration of PCMB required to cause almost complete inhibition decreases. It is evident from these data that the presence of ATP greatly reduces the rate at which high concentrations of PCMB inactivate myosin. That ATP also has an effect on the activation process can be seen by comparing the activation at 4 moles/10^5 g. when the PCMB and ATP are added together with the activation when the PCMB has been preincubated with the myosin for 50 sec. or more (Fig. 1). Since the ATPase activity measurements in all the experiments shown in Fig. 1 took more than 50 sec., it is clear that ATP partially prevents the activation process. The effect of the substrate on the —SH groups involved in the activation process is more easily observed by choosing different ionic conditions and using NEM as the sulfhydryl reactive reagent.

[2] Observations made upon the course of the PCMB inactivation of myosin using a pH-stat show that even when the PCMB is added to myosin in the presence of ATP the PCMB will, after sufficient time (several hundred seconds), cause total loss of ATPase activity.

Effects of ATP and ITP on Myosin Reacting with NEM

When myosin reacts with AET or CEE, the sensitivity of its ATPase activity to the inhibiting influence of Mg^{++} is reduced while its sensitivity to the accelerating action of Ca^{++} is increased (16). It therefore seemed likely that the reaction of NEM with the —SH groups of myosin involved in the activation of ATPase activity would be more sensitive to the presence of ATP in a mixture of Mg^{++} and Ca^{++}. If the ATP or ITP is added after the myosin and NEM have reacted for various times, it is found that ATPase activity rapidly increases, while the ITPase activity decreases (Fig. 2). If, however, ATP is present during the NEM-myosin incubation period, the rate at which ATPase activity increases is considerably reduced. If ITP is present during the NEM-myosin incubation period, there appears to be a more rapid loss of activity (Fig. 2), thus indicating that ITP and ATP interact differently with the —SH groups at the active site.

Activation and Inhibition by Metal Ions

When myosin is incubated with Ni^{++}, Co^{++}, Ag^{+}, or Hg^{++}, only inhibition of ATPase activity is observed. With Cu^{++},

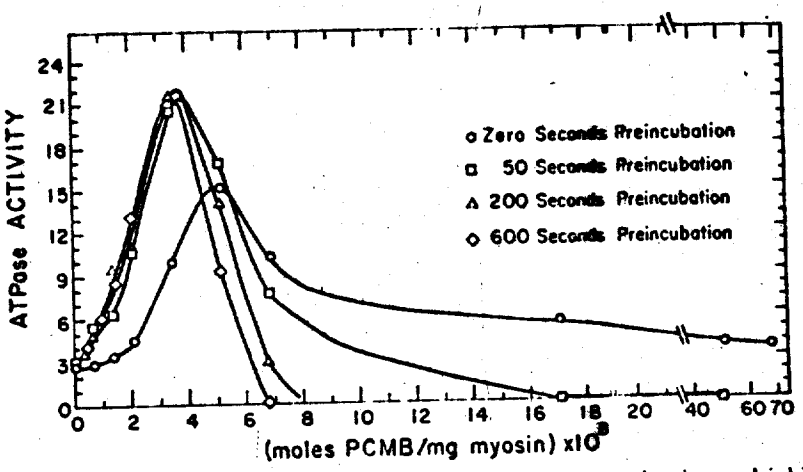

FIG. 1. Effect of time of incubation of myosin A with PCMB on activation and inhibition. The zero seconds preincubation curve was obtained by adding 2 ml. myosin (0.365 mg./ml.) to 6 ml. of a mixture of ATP and PCMB. The other curves were obtained by preincubating a mixture of 2 ml. of the myosin with 4 ml. PCMB for the time shown in the graph and then adding 2 ml. ATP. Conditions: 25.5°C., 0.3 *M* KCl, 0.05 *M* Tris, 5×10^{-3} *M* Ca^{++}, pH 7.5.

Cd^{++}, and Zn^{++}, however, there is a pattern of activation and inhibition that is similar to the pattern observed with PCMB.[3] Thus, at low concentrations these heavy-metal ions enhance and at high concentrations they inhibit ATPase activity, while at all concentrations they inhibit ITPase activity[4] (10). Unlike PCMB and NEM, the activation process is so fast that the influence, if any, of ATP on it is unobservable. This is shown only for Cd^{++} in Fig. 3, but the curves for Zn^{++} and Cu^{++} closely resemble those for Cd^{++}.

The rate at which high concentrations of Cd^{++} (and of Cu^{++} or Zn^{++}) inactivate the ATPase activity of myosin is comparable to the rate at which PCMB inactivates the ATPase activity (Fig. 1). ATP is, however, less effective in decreasing the rate at which these metal ions inactivate myosin, and the activity vanishes at sufficiently high metal-ion concentration even in the presence of ATP. Nevertheless it can be inferred from the data shown in Fig. 3 and from similar data obtained for Cu^{++} and Zn^{++} that ATP does confer partial protection against the inactivating effects of high concentrations of these cations.

[3] There is no appreciable difference between the effects of Cu^{++}, Co^{++}, Ni^{++}, Cd^{++}, or Zn^{++} on the rate of hydrolysis of ATP or ITP at 100°C. in 0.3 *M* KCl, 5×10^{-3} *M* Ca^{++}, 0.05 *M* Tris, pH 7.5.

[4] Activation of ATPase by these cations can only be observed in certain ranges of temperature, pH, and ionic strength. Cu^{++}, for example, only inhibits ATPase at 0.1 *M* KCl.

Activation and Inhibition by Pairs of Modifiers

When the effect of pairs of modifiers which singly caused activation of ATPase was first examined, it was found that these modifiers caused inhibition when added in pairs (10). In view of the similarities between PCMB, Cu^{++}, Cd^{++}, and Zn^{++}, however, one would expect their effects to be additive provided that the concentrations are chosen correctly and that the myosin is not preincubated with either of the pair. When such an experiment was performed using PCMB and Cu^{++} as the pair, it was found (Fig. 4) that the activation and inhibition effects were additive at low levels of Cu^{++} (10^{-5} *M*), while at high Cu^{++} (10^{-4} *M*, just past the peak of the curve of ATPase versus Cu^{++} concentration) net inhibition is observed at levels of PCMB which were still activating in the presence of lower concentrations of Cu^{++}. We have also observed that the activation effects of low levels of Cu^{++} and Zn^{++} are additive.

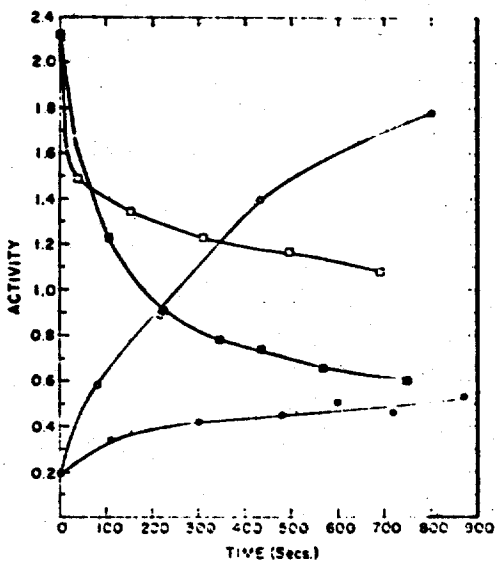

FIG. 2. Influence of ATP and ITP on reaction of NEM with myosin A. Curves with open symbols (○, ATP; □, ITP) were obtained by incubating myosin with NEM for the time shown on the abscissa, and then adding 2 ml. of the NEM–myosin mixture to 6 ml. ATP or ITP. The rate of hydrolysis was measured within the next 100 sec. After mixing, the concentration of ATP or ITP was 10^{-3} *M*. The NEM to myosin ratio was 0.0172 μmole/mg. in the ATP experiment and 0.138 μmole/mg. in the ITP experiment. Curves with closed symbols (●, ATP; ■, ITP) were obtained by adding the same concentrations of NEM to a mixture of myosin and ATP or ITP reacting in the pH-stat. The initial ATP or ITP concentration was 1×10^{-3} *M*. After about 200 sec. of steady-state ATPase or ITPase activity, NEM was added. Activities were computed from the slope of the recording of volume of base added versus time. Conditions: 25.5 C., 0.6 *M* KCl, 5×10^{-3} *M* Ca^{++}, 5×10^{-4} *M* Mg^{++}, pH 7.50, 0.05 *M* Tris, except that Tris was omitted in the pH-stat experiments.

Effects of pH on Metal-Ion Activation and Inhibition

The effects of pH on the ATPase and ITPase activities of myosin have recently been studied in some detail by Gilmour (15) and by Morales and Botta (16). These workers have shown that after treatment of myosin with PCMB (15) or AET (16), the pH versus activity curve of ATPase changes in such a way that it resembles the ITPase curve. They have also suggested that the active site has a group which ionizes at a "pK" near 7. Since Cu^{++}, Cd^{++}, and Zn^{++} interact with the groups causing activation and inhibition, it was of interest to examine the effects of these cations as a function of pH.

When the pH is increased from 7.5 to 8.5, Cd^{++} becomes more potent; i.e., the concentration required for peak activation decreases (Fig. 5). Zn^{++} also becomes more potent at pH 8.5 but the activation, which is small for Zn^{++} even at pH 7.5, becomes so small at pH 8.5 as to be within experimental error of the control rate, and it is, therefore difficult to say whether the curve for Zn^{++} at pH 8.5 has, in fact, become a sigmoid inhibition curve. For Cu^{++}, however, the increase in pH increases the concentration required to get peak activation, increases the activity at the peak, and greatly decreases the sensitivity of myosin to the inactivation process, so that complete inhibition is not observed until concentrations greater than 10^{-3} *M* are attained (Fig. 5).

Activity versus pH in the Presence of Cu^{++}

The change in behavior of Cu^{++} at pH 8.5 as compared to pH 7.5 suggested a more detailed study of the pH dependence of myosin ATPase and ITPase at several concentrations of Cu^{++}. In the absence of Cu^{++}, as is well known, there is a shallow minimum at about pH 7.4 in the curve of ATPase activity versus pH (Fig. 6). Below about pH 6.7 Cu^{++} only inhibits the ATPase activity, the inhibition increasing as the pH decreases. Above pH 6.7 low concentrations of Cu^{++} activate while sufficiently high concentrations of Cu^{++} inhibit. It can be seen that after enough Cu^{++} has been added to suppress most of the activity at low pH, the curves take on the appearance of titration curves with an apparent "pK" that increases from about 7.3 to 7.6 as the Cu^{++} increases from 1.2×10^{-4} to 3.6×10^{-4} *M*. With ITP as a substrate we have found under our ionic conditions that there is a shallow maximum at about pH 6.5 in the absence of Cu^{++}, although Gilmour (15) working at low KCl,

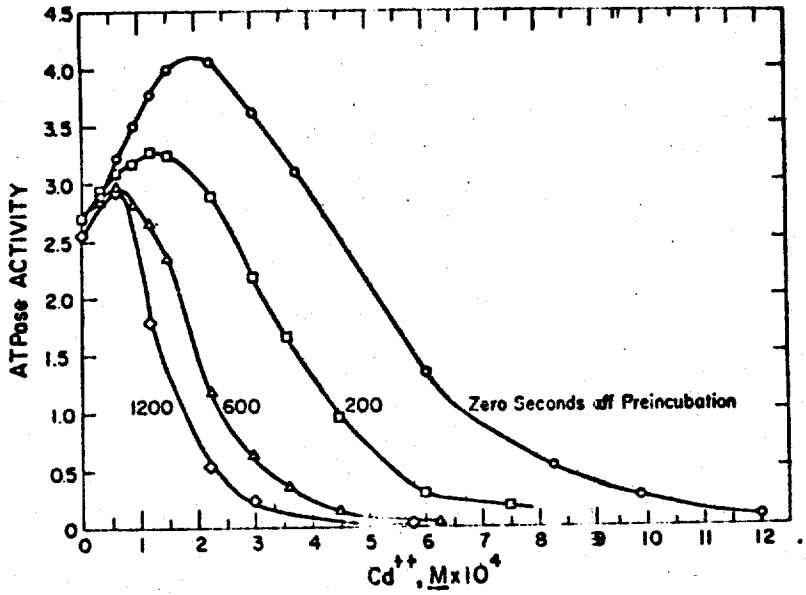

FIG. 3. Effect of time of incubation of myosin A with Cd^{++} on ATPase activity. For conditions, see legend for Fig. 1. The Cd^{++} concentration during the preincubation period was 1.33 times the final concentration shown on the abscissa.

and Nihei and Tonomura (17) working at high KCl but in the absence of Ca^{++}, observed a saddle point at about this pH, with the activity decreasing as the pH decreased below pH 6.5. The addition of Cu^{++} at any pH only inhibits the ITPase activity, and it is difficult to say whether there is any evidence for a group with a "p*K*" in the neighborhood of 7.4.

ACTIVITY VERSUS pH IN THE PRESENCE OF PCMB

When myosin is treated with PCMB (with no preincubation), the general shape of the ATPase activity versus pH curve is insensitive to pH and there is no suggestion of the influence of an ionizing group (Fig. 7). The activity is accelerated at low pH, contrary to the results obtained with Cu^{++} (Fig. 6) and contrary to the results that Gilmour (15) obtained with PCMB. The difference between the present results and those of Gilmour may be due to the much lower KCl concentration at which Gilmour studied these phenomena, but is more likely due to the fact that his myosin was preincubated for long periods of time with PCMB.

EFFECT OF TRYPSIN ON pH DEPENDENCE OF ATPASE ACTIVITY

It is well known that myosin treated with trypsin undergoes large changes in molecular structure (18). Mihalyi and Harrington (19) have carefully studied this process and have shown that there occurs simultaneously a fast reaction, presumably localized to certain regions which are nonhelical in character and leading to the formation of large fragments of characteristic size distributions, and a slow reaction occurring in the remaining triple-stranded regions (20). If the pattern of pH activity in the presence and absence of PCMB depends on interactions between relatively distant parts of the native myosin molecule, then one would expect trypsin-digested myosin to change its pattern of activity versus pH in the presence and or in the absence of PCMB. One milligram of trypsin was added to 117 mg. myosin A in a pH-stat at 25.5°C. After 42 min., during which time 19.1 μmoles KOH was required to maintain the pH at 8.00, the reaction was stopped by adding 3 mg. of trypsin soybean inhibitor. (This amount of soybean inhibitor had been shown in control experiments to completely

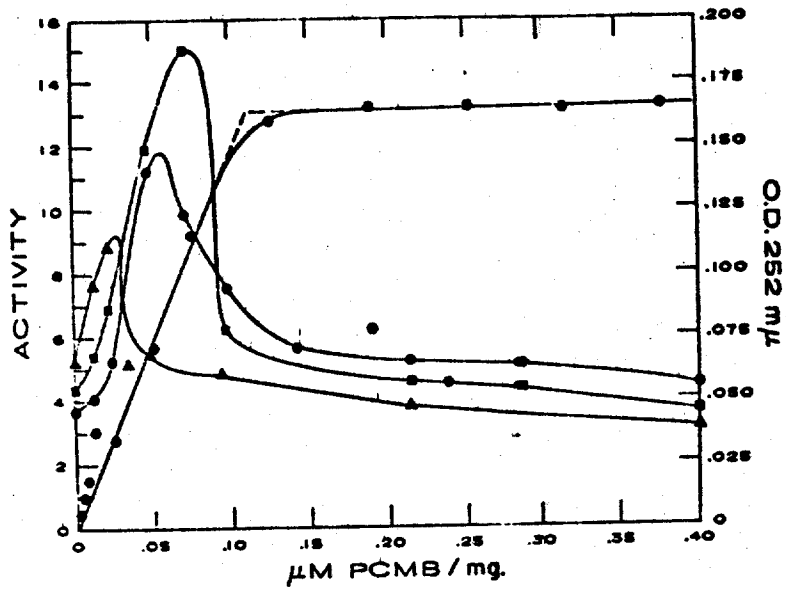

FIG. 4. Effect of Cu^{++} on the PCMB-modified ATPase activity of myosin A. Varying amounts of PCMB, as shown on the abscissa, were added with fixed amounts of ATP (final concentration, 10^{-3} *M*) and Cu^{++} to myosin. The final Cu^{++} concentration was: ○——●, no Cu^{++}; □——□, 10^{-5} *M*; △——▲, 10^{-4} *M*. The curve with hexagonal symbols refers to the right hand ordinate and shows the titration of the —SH groups by PCMB. Conditions: 0.6 *M* KCl, 5×10^{-3} *M* Ca^{++}, 0.05 *M* Tris, pH 7.50, 25.5°C., 0.225 mg. myosin/ml.

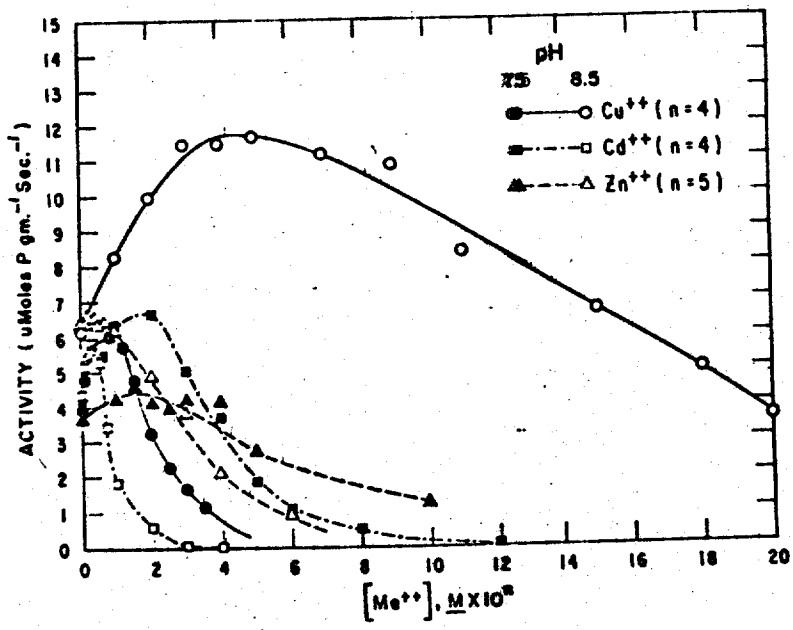

FIG. 5. Effect of pH on ATPase activation and inhibition by Cu^{++}, Cd^{++}, and Zn^{++}. Myosin A was added to a mixture of ATP and the metal ion, and the ATPase activity was measured within 2 min. The final concentrations of ATP and myosin were 10^{-3} *M* and 0.176 mg./ml., respectively. Conditions: 0.6 *M* KCl, 0.05 *M* Tris, 5×10^{-3} *M* Ca^{++}, 25.5°C.

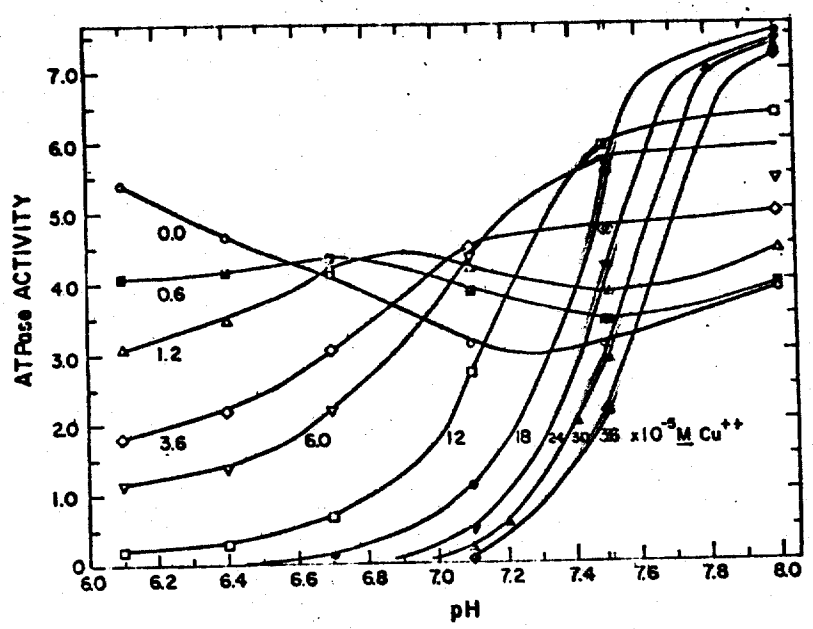

FIG. 6. ATPase activity versus pH in the presence of Cu^{++}. Myosin A was added to a mixture of ATP (final concentrations 0.118 mg./ml. and 10^{-3} M, respectively) and Cu^{++} (final concentration shown in graph). Both the myosin and the Cu^{++}–ATP solution were made up in a buffer of the following composition: 0.3 M KCl, 5×10^{-3} M Ca^{++}, 0.05 M Tris, 0.05 M acetic acid, adjusted to the indicated pH. The temperature was 25.5°C.

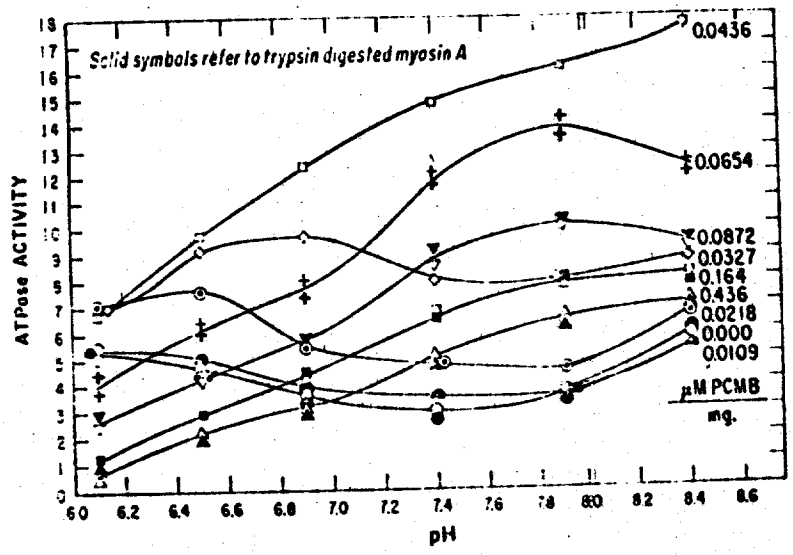

FIG. 7. Effect of pH on ATPase activity at several concentrations of PCMB. Data shown with solid symbols were obtained from myosin treated with trypsin as discussed in the text. Open symbols refer to myosin not treated with trypsin. Two milliliters of myosin A was added to 6 ml. of a mixture of ATP and PCMB such that the final concentrations of ATP and myosin were 10^{-3} M and 0.117 mg./ml., respectively. Conditions: 0.6 M KCl, 0.05 M Tris, 0.05 M acetic acid, 5×10^{-3} M Ca^{++}, 25.5°C.

stop the trypsin digestion.) According to Mihalyi and Harrington (19), the effective pK of the amino groups liberated from peptide bonds is 7.85, so that each mole of KOH corresponds to about 2.4 moles of peptide bonds hydrolyzed. Assuming a molecular weight of 6.29×10^5 (20), one computes that about 4% of the peptide bonds of the myosin were hydrolyzed in the reaction. When the pH dependence of the ATPase activity was studied, it was found (Fig. 7) that there was no discernible effect of the trypsinization. It can be concluded that the ATPase activity resides in a trypsin-resistant part of the molecule and that there is no important interaction in this pH range (in the presence or absence of PCMB) between the part of the molecule where the active site(s) is and the rest of the myosin molecule.

Effects of Temperature

When the temperature is lowered to 0°C., both the ATPase and the ITPase activities of myosin are inhibited by Zn^{++}, Cu^{++}, and Cd^{++} (Fig. 8) as well as by PCMB (9). If myosin is incubated with any of these cations at 0°C. and then assayed for ATPase activity at 25°C., it is found that with increasing time of incubation all the metals become more potent inhibitors, with Cu^{++} undergoing the most extensive reaction. In the experiment shown in Fig. 8, almost 2×10^{-4} *M* Cu^{++} was required to give 50% inhibition at zero time, whereas 50% inhibition was attained when 8×10^{-7} *M* Cu^{++} was incubated with myosin at 0° for 71 hr. Since this amount of Cu^{++} was nearly equimolar with the amount of myosin present, an experiment was performed to measure the amount of Cu^{++}

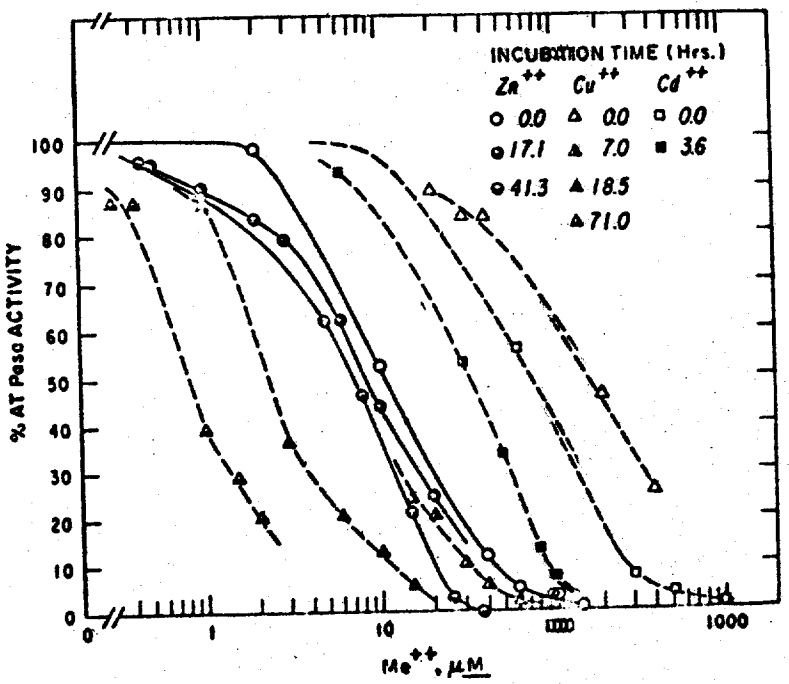

Fig. 8. Effect of time of incubation at 0° upon metal inactivation of myosin ATPase. Myosin A was mixed with Cu^{++}, Cd^{++}, or Zn^{++} and incubated at 0°C. for the time shown in the graph. Aliquots were then incubated at 25.5°C. for 100 sec., added to ATP at 25.5°C., and the ATPase activity was measured within 2 min. Conditions: 0.6 *M* KCl, 5×10^{-3} *M* Ca^{++}, 0.05 *M* Tris, pH 7.5. The myosin concentration during the incubation was 0.752 mg./ml. for the experiments with Cu^{++} and Cd^{++}, and 0.991 mg./ml. for the experiments with Zn^{++}.

bound on the protein after extensive reaction at 0°C.

Ten milliliters of myosin A (4.23 mg./ml.) was dialyzed against 500 ml. buffer (0.6 M KCl, 5×10^{-3} M Ca^{++}, 0.05 M Tris, pH 7.5), and another 10 ml., containing 5.6×10^{-6} M Cu^{++}, was dialyzed against the same volume of buffer containing 5.6×10^{-6} M Cu^{++}. After 70 hr. of dialysis at 4°C., the dialysis sacs were separately dialyzed for 24 hr. against a total of 2 l. buffer containing 5×10^{-3} M cysteine hydrochloride. Aliquots were then assayed for ATPase activity at 0°C., and it was found that the sample treated with Cu^{++} had no measurable ATPase activity while the control had an activity of 0.98 μmole P/sec./g. Four 1.5-ml. aliquots of each protein were taken for Cu^{++} analysis (14). To one pair of aliquots, no Cu^{++} was added. To the other three pairs increasing amounts of Cu^{++} were added. The differences in absorbance at 540 mμ were 0.056, 0.063, 0.062, and 0.060, indicating that the turbidity caused by the precipitated myosin was not causing any problem. From these data it was computed that 3.25 moles Cu^{++} was bound/mole myosin, or approximately one per chain (20).

Reversibility of Metal-Ion Inhibition

Since dialysis against cysteine did not remove all the Cu^{++} from myosin and did not restore the ATPase activity, it was of interest to see whether this irreversibility with Cu^{++} was also true of Cd^{++} and Zn^{++}. Myosin was inactivated by reacting it with a concentration of metal that was expected to give almost complete loss of ATPase activity after 600 sec. at 25.5°C. Aliquots were then dialyzed in the cold against AET, cysteine, or EDTA, and were assayed for ATPase activity at 25.5°C. Dialysis against buffer alone restored about 8% of the activity of the myosin that had been inactivated by Zn^{++} or Cd^{++}, but did not restore any activity to myosin inactivated by treatment with Cu^{++} (Table I). Dialysis against EDTA or cysteine restored about 33% of the activity of Cd^{++}-treated myosin and about 50% of the activity of the Zn^{++}-treated myosin, but did not restore any significant activity to the Cu^{++}-treated myosin. Dialysis against AET restored about the same activity to the Cd^{++}-treated myosin as did cysteine or EDTA, but was more effective than either cysteine or EDTA with respect to the Cu^{++}-inactivated myosin. Thus the effects of Zn^{++}, which is a more potent inhibitor of ATPase activity at 25° than Cu^{++} (see Fig. 5), are much more easily reversed than are the effects of Cu^{++}.

TABLE I

Activity of Myosin Inactivated by Treatment with Cu^{++}, Cd^{++} or Zn^{++} at 25.5°C. and then Dialyzed against Cysteine, EDTA or AET

Myosin A (1 mg./ml.) was treated with 6.9×10^{-4} M Cu^{++}, Cd^{++}, or Zn^{++} for 600 sec. at 25.5°C. Aliquots were then withdrawn and either added to ATP to determine the residual activity (col. 2) or were chilled in ice and transferred to individual dialysis sacs for dialysis against buffer (0.6 M KCl, 5×10^{-3} M Ca^{++}, 0.05 M Tris, pH 7.50), buffer $+10^{-2}$ M cysteine, buffer $+ 10^{-2}$ M EDTA, or buffer $+ 5 \times 10^{-3}$ M AET. The dialysis sacs were agitated for 55 hr. at 4°C. against a total of 2 l. of the indicated solution and then for 16 hr. against buffer only. The contents of each sac were then assayed at 25.5°C. for ATPase activity (col. 3-6). In certain of the Zn^{++}-treated myosin tubes, indicated by an asterisk (*), there was a white precipitate.

Treated with buffer	Activity after treatment	Activity after dialysis against buffer containing:			
		—	Cysteine	EDTA	AET
	%	%	%	%	%
Buffer	100	132	116	126	297
Cu^{++}	0	0	4	0.3	27
Cd^{++}	3	10	44	36	111
Zn^{++}	0*	10*	60	57	—

DISCUSSION

The effect of PCMB on myosin NTPase activity has suggested to several workers that at least two —SH groups are involved in the hydrolytic process (6, 10, 15). Since the active sites for NTPase activity is located in a portion of the molecule which is highly resistant to the action of trypsin, and since the effects of PCMB and of pH are unaltered by trypsinization (Fig. 7), it appears that the active site does not interact

appreciably with the rest of the molecule. One may, therefore, study the effects of modifiers such as PCMB, Cu^{++}, Cd^{++}, and Zn^{++}, with the hope that the observed effects will primarily reflect the behavior of a small region containing the active site.

The effect of PCMB on the ATPase activity is particularly useful as an aid to analyzing the number of —SH groups at the site and as a guide to the interpretation of the effects of Cu^{++}, Cd^{++}, and Zn^{++} on the active site. Under our conditions (high salt, Ca^{++}), low concentrations of PCMB interact preferentially with an —SH group that retards ATPase activity. This reaction is still incomplete after 100 sec. in the presence of ATP, but is complete in less than 50 sec. in the absence of ATP (Fig. 1). As the concentration of PCMB is raised (in the presence of ATP), full activation is reached and then the activation itself is inhibited until, as the PCMB concentration becomes sufficiently high, the ATPase activity appears to return to its control value asymptotically. This suggests that two —SH groups are involved in the hydrolytic activity of myosin. We shall designate $—SH_1$ as the group responsible for the activation at low PCMB levels, and $—SH_2$ as the group involved in the loss of this activation and, perhaps, in the loss of all hydrolytic activity. Since high levels of PCMB in the presence of ATP do not cause a net inhibition of ATPase (Fig. 1), it is possible that the reaction of $—SH_2$ with PCMB merely reverses the activation caused by reaction of $—SH_1$ with PCMB, and that the complete and essentially irreversible loss of ATPase activity at high PCMB results from a secondary configurational change involving $—SH_2$ which is impeded by the presence of ATP (10).

Gilmour and Gellert (21), in a detailed study of the interaction between myosin and PCMB at low KCl, found that the reaction depended on the concentrations both of myosin and of PCMB. The exact pattern of activation and inhibition will depend on what proportions of the —SH groups at the active site have reacted. Gilmour and Gellert (21) also found that the activation process was much faster than the inhibition process and that the presence of ATP or ITP would affect the rate at which activation or inhibition proceeded. In their system, the presence of ATP increased the rate of development of inhibition when the PCMB concentration was 6.4 moles/10^5 g., suggesting that under their conditions ATP enhanced the reaction between $—SH_2$ and PCMB. From the present data, it is clear that the presence of ATP decreases the rate at which low concentrations of PCMB (Fig. 1) and NEM (Fig. 2) activate myosin ATPase, suggesting that ATP partially blocks the access of PCMB to $—SH_1$. Gilmour and Gellert (21) noted that the presence of ITP appeared to partially reverse the inhibition of ITPase activity caused by PCMB. Another indication that ITP interacts differently than ATP with the —SH groups at the active site is shown in Fig. 2, where it is seen that the presence of ITP appears to increase the rate of loss of activity at high concentrations of NEM.

In most enzyme systems studied, Cu^{++} Cd^{++}, and Zn^{++} are inhibitors. These ions, however, resemble PCMB in many ways, e.g., their ability to activate ATPase and inhibit ITPase at low concentrations, the rate at which high concentrations inactivate myosin, and the inhibition of ATPase at low temperatures. An important difference between PCMB and Cu^{++} is seen by comparing their effects on the pH dependence of ATPase activity. Low concentrations of PCMB change the shape of the curves of ATPase activity versus pH by first increasing the activity at pH values between 6 and 7. As the concentration of PCMB increases, the activity at pH values between 7 and 8.5 increases more rapidly, thus eliminating the minimum at pH 7.4 (Fig. 7). At a PCMB concentration sufficient to give maximal activation, the ATPase activity increases smoothly with increasing pH (Fig. 7). These results indicate that reaction of the —SH groups with different amounts of PCMB can cause considerable changes in the pH dependence of ATPase activity. Similar conclusions concerning the heterogeneity of the —SH groups have been reached by Tonomura and Furuya (22), who also noted that treatment with PCMB

removed the minimum at neutral pH. Tonomura and Furuya (22) observed that if the ATPase activity was assayed below pH 6, the effect of low concentrations of PCMB was inhibitory. The effect of low concentrations of Cu^{++} is also inhibitory below pH 6.5 (Fig. 6), and it is possible that —SH_1 may be an activator of ATPase activity in untreated myosin at low pH. At concentrations of Cu^{++} above 10^{-4} *M*, the dependence of ATPase activity on pH is not similar to that of PCMB-treated myosin, but is roughly in the shape of a titration curve (Fig. 6). Edman (23) found that in the presence of Zn^{++} the graph of the isometric tension of glycerol-treated muscle fibers versus pH took on the shape of a titration curve with a "p*K*" near 7, which he tentatively interpreted as reflecting the participation of an imidazole group in the contractile process. In view of the similarities between Zn^{++}, Cu^{++}, and Cd^{++}, it is probable that the group found by Edman in his studies on tension is the same group that is revealed by Cu^{++}.

Although it is generally considered that Cu^{++} is a potent inhibitor of myosin, this is only partly true. As the pH rises, Cu^{++} becomes a weaker and weaker inhibitor, until at pH 8.5 the ATPase activity can be measured in a solution of 10^{-3} *M* or more.[5] Zn^{++} and Cd^{++}, however, become more potent inhibitors as the pH increases. With lactic dehydrogenase, also, Cu^{++} becomes a very weak inhibitor as the pH increases from 7.4 to 8.4 (11). It remains to be established whether there is a change in the nature of the Cu^{++} ion as the pH increases (presumably because of strong binding by an OH^- group or by the amino group of Tris) or whether there is some common feature of the environment of the —SH groups in both enzymes which is responsible for the decreased effectiveness of Cu^{++} at high pH.

At low temperatures, myosin no longer makes a major distinction between ATP and ITP. If the enzyme is allowed to react with Cu^{++}, Cd^{++}, or Zn^{++} at low temperatures, only inhibition is observed. If sufficient time is allowed at 0°C., very small amounts of Cd^{++}, Zn^{++}, and, especially, Cu^{++}, suffice to inactivate the myosin. Gilmour and Gellert (21) have proposed that at low temperatures PCMB can migrate from one —SH group to another in the myosin molecule, and it may be that a similar migration occurs with these metal ions. With Cu^{++}, approximately one atom per chain is sufficient to inhibit the ATPase activity, suggesting the possibility of three identical ATPase sites each containing one group that binds Cu^{++} more tightly than do similar groups in any other part of the enzyme. That Cu^{++} is bound very tightly to the enzyme is also evidenced by the relative inability of extensive dialysis against EDTA, AET, or cysteine to reverse the inactivation (Table I), whereas the effects of Cd^{++} and Zn^{++} are partially reversed by such dialysis.[6]

Tonomura and Furuya (22) found that the pattern of activation or inhibition of ATPase activity caused by PCMB depended both on the temperature at which the myosin reacted with PCMB and the temperature at which the ATPase activity was assayed. A low temperature for the PCMB-myosin reaction changed the activation observed to inhibition as the temperature of the ATPase assay was decreased. Similar effects are observed with Cu^{++}, Cd^{++}, or Zn^{++}, since low concentrations of these cations failed to activate myosin ATPase after reaction at 0° even when the ATPase activity was assayed at 25.5°C. (Fig. 8). These results are compatible with the suggestion (10) that the —SH groups are involved in a conformation change at the active site. More specifically, it would appear that low temperatures favor a conformation which prevents —SH_1 from reacting rapidly with these reagents and from exerting an activating influence on ATPase activity. Kitagawa and Tonomura (24) have discussed other evidence supporting the idea of a sulfhydryl-dependent conformation change.

[5] At pH 8.5 Cu^{++} is also a much weaker inhibitor of ITPase activity than at pH 7.5.

[6] Kalamkarova and Muzheev (26) report that they obtained complete reversal of the Cd^{++} inhibition of myosin ATPase by dialysis against cysteine.

The —SH groups of myosin are heterogeneous not only with respect to the ATPase activity of myosin, but, as has been stressed by Bárány and Bárány (25), are also heterogeneous with respect to their influence on the actin-combining ability of myosin. The role of —SH groups in superprecipitation is discussed in an accompanying paper (27).

ACKNOWLEDGMENT

In all phases of this work the author has had the benefit of the highly skilled technical assistance of Mr. P. J. Buchanan.

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WORK IN PROGRESS

LONG-TERM CARCINOGENICITY TESTS OF ORALLY ADMINISTERED ZINC AND TIN

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Rats

No tumours have so far been observed in 2 groups of 40 rats fed continuously with a 20% protein diet containing 5% sodium chlorostannate or 0.83% stannous-2-ethyl hexoate, nor in a similar control group fed the same diet without the additive. The experiment has been in progress for approximately 8 months.

Mice

Groups of pregnant mice, and the litters subsequently born to them, were exposed to the following:

(1) Sodium chlorostannate in the drinking water (either 15 g/l equivalent to 5000 ppm tin, or 3 g/l equivalent to 1000 ppm tin).
(2) Stannous oleate in 20% protein diet (31.25 g/kg equivalent to 5000 ppm tin).
(3) Zinc sulphate in the drinking water (22 g/l, equivalent to 5000 ppm zinc or 4.4 g/l equivalent to 1000 ppm zinc).
(4) Zinc oleate (50 g/kg reducing because of anaemia to 12.5 g/kg equivalent to 1250 ppm zinc).
(5) 20% protein diet and water without the added chemical.

These experiments have been continued for over a year and many animals from each group have been killed (because of an intercurrent infection) and examined *post mortem*. A few cases of malignant lymphoma have occurred, one in the control group and not more than one in any other group. One hepatoma was seen in each of the two groups treated with zinc sulphate in the drinking water. Although no such tumours have been seen in the control group it is known that untreated animals of the same stock strain not infrequently develop tumours of this type.

So far, therefore, there is no evidence that high levels of dietary zinc or tin increase the incidence of cancer in rats and mice.

These experiments, which will be continued, were undertaken after certain misleading statements, concerning the hazard from canned foods, that had been made in the House of Lords (in Hansard, June 15th, 1961) and the various letters to *The Times*, between the 28th June and 9th July, 1961, inclusive.

PROC. ANN. CLIN. SPINAL CORD INJ. CONF. 16: 70-72. (1967)

THE EFFECT OF ORAL ZINC SULFATE ON THE HEALING OF DECUBITUS ULCERS IN SPINAL CORD INJURED PATIENTS

Robert D. Brewer, Jr., M.D., Nikola Mihaldzic, M.D., and Albert Dietz, Ph.D.

Last year at the Fifteenth Annual Spinal Cord Injury Conference, I presented preliminary observations on the healing effect of oral zinc sulfate 200 mgs. once daily on decubitus ulcers. Our results seemed sufficiently gratifying to warrant a formal double blind study increasing the test dosage to 220 mgs. t.i.d.

First, to review the background of zinc and the rationale for its use: For years it has been known that traces of zinc are essential for the growth and development of crops. Subsequently, it was shown that supplementary zinc is required in the feed of livestock, i.e., poultry, swine, and cattle, to effect adequate growth and reproduction and to prevent diseases of the skin and mucous membrane. (Cattle require 30 to 50 mg. per 100 pounds animal weight per day; pigs need even more--about 300 mgs. zinc per day or twice the dose found most effective in humans.)

The need for zinc in humans and the beneficial effects of supplementary zinc was shown in Egyptian males with dwarfism and hypogonadism (Prasad, et al); in chronic liver disease (Mayer, et al); and in the past two years in surgical wound healing (Pories, et al). Most recently indication has been offered of considerable benefit in occlusive vascular disease (Pories, et al). This latter work in particular has aroused the interest of both researchers and clinicians.

As for the mode of action: Zinc appears to be an essential part of many enzymes and co-enzymes--particularly those involved in tissue respiration and in synthesis of protein and nucleic acids. Thus, its presence in adequate amounts may promote formation of granulation tissue. In the case of vascular disease, it may prevent delay in healing of damaged arterial walls. This delay "probably predisposes to necrosis, intimal proliferation and cholesterol deposition" (Pories). Since low levels of serum zinc are found in patients with acute and chronic infections, liver disease, atherosclerotic conditions, after surgery or trauma, and in many other pathological states, a supplementary intake of zinc could correct deficiency and hasten healing of many types of damaged tissue.

The study we have undertaken was delayed in the start because the Research Subcommittee at Hines justifiably required evidence of minimal toxicity of zinc sulfate in the dosage of 220 mgs. t.i.d. Literature and pharmacology tests indicated safety in animal experimentation, except where very large amounts of zinc are administered. There is, however, very little documented work on human beings and we had to correspond with the pioneers in the field in order to be satisfied that dangerous side effects were unlikely. The project was finally formally approved and the study undertaken early in June. It was conducted double blind utilizing two types of capsules prepared by the Pharmacy: One containing zinc sulfate 220 mgs. (50 mgs. zinc) and the other an inert substance (Lactose). Fourteen patients with poor healing decubitus ulcers of various sizes, types, locations, and duration (5 months to over 2 years), were chosen during the month of June. Selection of the type of capsule (zinc #1 or zinc #2) was made on a random basis and one capsule was administered t.i.d. P.C. A formal consent for the study was obtained in each case. During the study period, routine medical care was continued as before.

Prior to treatment, serum and urine zinc determinations were made by our Research Chemist, Dr. Albert Dietz, using an atomic absorption spectrometer. These were repeated at monthly intervals until September 8, 1967, when the blind aspect of the study was discontinued and results were made known. Urinalyses, blood counts, and blood chemistries were also done before, during, and after the study.

The results to date from the standpoint of healing have not been convincing. Of the seven patients on the control capsules, two healed their decubiti, two definitely improved and three remained essentially unchanged. Of the six who were able to remain on zinc sulfate, one patient healed his decubiti, four showed definite signs of improvement, and one patient showed no change in his decubitus ulcer. In all probability, 2 to 3 months' time is too short a period to expect dramatic healing of decubiti of five months to over two years' duration.

Aside from the equivocal healing results, several other points were brought to light which would further support the safety of employing oral zinc sulfate: 1) There were an equal number of transient gastrointestinal upsets (nausea and loose stools) among the control group and the group receiving zinc; 2) only one patient on zinc had to be discontinued because of upper gastrointestinal distress and that patient was noted to have x-ray evidence of a pre-existing prolapse of gastric mucosa into the duodenum; and 3) there were no significant changes in white blood counts, hemoglobins, hematocrits, total proteins, albumins, BUN, or creatinine before, during and after zinc sulfate.

The results of serum and urine zinc determinations are of interest from a comparative standpoint rather than in terms of actual values obtained. When dealing with trace elements in micrograms there are multiple sources of contamination and, hence, error: 1) Collecting utensils rinsed with tap water coming from galvanized iron pipes can be coated with a film of zinc salts; 2) any hemolysis of the whole blood drawn for serum may give a falsely high serum value because of the high content of zinc in RBC's and WBC's; and 3) it was found at the very end of the study that the rubber stoppers used in the vacuum test tubes have a high zinc content (zinc oxide and zinc chloride).

Thus the figures I present are much higher than the laboratory controlled normal range of values:

1. Serum Zinc (normal average range considered 80-120 micrograms %; pooled serum of Hines' patients=75 micrograms).

   a. Patients on zinc (before treatment average 127.6 micrograms %). (two and one-half months after zinc=157 micrograms %).

   b. Patients on control (before treatment average 129.1 micrograms %). (and two and one-half months later, 134.7 micrograms % average).

2. Urine Zinc (24 hour excretion in urine .4 mgs. per day - can rise to 2 mg. per 24 hours in the presence of albuminuria.)

   a. Patients on zinc (before treatment 1.14 mgs. per 24 hours) (two and one-half months later 3.50 mgs. per 24 hours).

   b. Patients on control (before treatment=.86 mgs. per 24 hours) (two and one-half months later averaged .82 mgs. per 24 hours).

These figures show a moderate increase of zinc in serum but a three-fold increase in urine excretion. This would suggest a good margin of safety.

Conclusions from this Study to Date

1. No objective evidence that zinc sulfate orally will effect healing of chronic decubiti in spinal cord injured patients within 2 to 3 months' time.

2. Logical reasons for continuing zinc sulfate for a longer period and in a larger group of patients before assessing its value.

3. No evidence of general toxicity or irreversible gastrointestinal disturbances from zinc sulfate 220 mgs. t.i.d. in a small series of spinal cord injured patients.

4. Necessity for laboratory controlled preparation of specimens and containers before zinc values are determined.

We are indebted to Major Walter Pories, Dr. William Strain, Dr. Bert Vallee, and Dr. James A. Halsted for their interest and help in providing us directly with useful information on zinc: Its toxicity in a large series of cases and the optimum dosage and as so far ascertained.

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MR. MOELLER: The next presentation is by Dr. Philip Oden, Chief, Spinal Cord Injury Service, Veterans Administration Hospital, Richmond, whose paper is "Gold Leaf Occlusive Therapy in the Management of Decubitus Ulcers."

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 242, No. 9, Issue of May 10, pp. 2192-2198, 1967
Printed in U.S.A.

Ion Transport by Heart Mitochondria

VIII. ACTIVATION OF THE ADENOSINE TRIPHOSPHATE-SUPPORTED ACCUMULATION OF Mg^{++} BY Zn^{++} AND BY *p*-CHLOROMERCURIPHENYLSULFONATE*

(Received for publication, December 12, 1966)

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SUMMARY

The energy-linked accumulation of Mg^{++} by isolated heart mitochondria has been studied with the use of exogenous adenosine triphosphate as the source of energy. In common with the substrate-supported accumulation of Mg^{++}, the ATP-supported reaction is activated by the addition of Zn^{++}, Cd^{++}, and certain other heavy metal cations under the appropriate conditions. In sharp contrast with the substrate reaction, however, it was found that the ATP-supported reaction is also strongly activated by *p*-chloromercuriphenylsulfonate (CMS). The combination of Zn^{++} and CMS strongly inhibits the substrate-dependent accumulation of Mg^{++}, but produces a remarkable enhancement of Mg^{++} uptake in the presence of ATP. The increased accumulation of Mg^{++} and the increased ATPase activity which accompanied the accumulation are both inhibited by oligomycin. The accumulation of Mg^{++} by this pathway is much less sensitive to low levels of dinitrophenol than is the substrate-dependent reaction.

When both substrate and ATP are available as energy sources for Mg^{++} accumulation, and the reaction is activated by Zn^{++}, the uptake of Mg^{++} is sensitive to dinitrophenol and insensitive to oligomycin. Addition of CMS under these conditions reverses this pattern of inhibition so that the accumulation becomes sensitive to oligomycin but insensitive to dinitrophenol.

The available evidence indicates that Zn^{++} may act directly on the process responsible for the uptake of Mg^{++}, perhaps by altering the permeability of the membrane. The mercurial (CMS), however, appears to have its primary effect on the energy transfer process. Evidence is presented that this reagent blocks energy transfer between the dinitrophenol-sensitive site and the site which energizes the Mg^{++} uptake process. In addition, CMS may activate mitochondrial ATPase in such a way that more energy is available for the accumulation of Mg^{++}.

* These studies were supported in part by Grant HE-08364 from the National Institutes of Health, United States Public Health Service, by a grant in aid from the American Heart Association, and by the General Research Support Grant of the Ohio State University College of Medicine. A preliminary report of a portion of this work has been presented (1). For the previous communication in this series, see Reference 2.

‡ Established Investigator of the American Heart Association.

Recent studies in our laboratory have established that Zn^{++}, Cd^{++}, and certain other divalent cations strongly activate the energy-linked accumulation of Mg^{++} and K^{+} in isolated bovine heart mitochondria when the reaction is supported by the oxidation of ascorbate (1-4). This activation of mitochondrial ion transport resembles the activity reported for parathyroid hormone (5, 6) and for the toxic antibiotics valinomycin (7–10) and gramicidin (7, 11–12). These reagents appear to act directly on the process of mitochondrial ion transport, perhaps by increasing permeability to the ions involved (6, 12), perhaps by a combination of permeability effects and more direct effects on the energy-linked ion uptake (9). The simplicity of the Zn^{++} ion compared to the hormone and antibiotics suggested that important information on the chemical processes involved in the induction of ion transport could be gained by a study of the Zn^{++}-activated reaction. The present communication reports a study of the effects of Zn^{++} and other cations on the accumulation of Mg^{++} supported by exogenous adenosine triphosphate.

Since Zn^{++} and the other ions effective in the activation of mitochondrial ion transport share the ability to react with thiols, the activity of a number of —SH group reagents on the ATP-supported uptake of Mg^{++} was also examined. It was found that, in sharp contrast to the substrate-dependent accumulation of Mg^{++} which is inhibited by *p*-chloromercuriphenylsulfonate, the ATP-supported Mg^{++} uptake is activated by this reagent. CMS[1] in combination with Zn^{++} causes the normally weak accumulation of Mg^{++} supported by ATP to approach the magnitude of the uptake in the presence of substrate. These results have been summarized briefly in a preliminary communication (1) and are presented in detail below.

[1] The abbreviations used are: CMS, *p*-chloromercuriphenylsulfonate; HMB, *p*-hydroxymercuribenzoate; TMPD, *N,N,N',N'*-tetramethylphenylenediamine.

METHODS

Bovine heart mitochondria were prepared by a slight modification (2) of the Nagarse procedure of Hatefi, Jurtshuk, and Haavik (13). Mg^{++} and Zn^{++} were determined by atomic absorption spectroscopy of acid extracts as previously described (2). The procedures used for monitoring oxygen uptake, pH changes, and P:O ratios have also been published (2). ATPase was estimated by the increase in inorganic phosphate (14). Disodium ATP (Sigma) neutralized with Tris was used unless specified. The conditions of incubation and the composition of the incubation medium are detailed for the individual experiments reported.

RESULTS

Isolated heart mitochondria accumulate only small amounts of Mg^{++} when supplemented with ATP in the absence of respiration (15). The data presented in Table I establish that this rather weak reaction can be stimulated 2-fold or more by a number of heavy metal cations. High concentrations of Zn^{++} (relative to the levels required to activate the accumulation of Mg^{++} by the substrate-supported reaction) consistently result in up to twice the accumulation seen in the absence of Zn^{++}. Cd^{++}, Hg^{++}, and Cu^{++} are also effective in increasing the observed accumulation under these conditions. As in the case of the substrate-supported reaction (2), only narrow concentration ranges of each of the latter three cations are effective, and the optimal concentration varies considerably with different preparations of mitochondria. A preliminary experiment with concentrations of cation between 10^{-5} M and 2×10^{-4} M is usually sufficient to establish the effective concentration. In each case, however, the increased accumulation of Mg^{++} was completely sensitive to oligomycin and dependent on the addition of ATP. A number of other cations (Mn^{++}, Ni^{++}, Sn^{++}, Ca^{++} Pb^{++}, and Co^{++}) did not activate Mg^{++} accumulation under these conditions and in these concentrations. The values shown in Table I for Mn^{++} and Ni^{++} are typical results. These studies indicate that certain heavy metal ions, notably Zn^{++} and Cd^{++}, possess the property of increasing the energy-linked accumulation of Mg^{++} by heart mitochondria when energy is supplied either as an oxidizable substrate (2) or as ATP (Table I).

While the behavior of the cations studied on the accumulation of Mg^{++} supported by ATP was quite analogous to their effect on the substrate-supported reaction, the effects of CMS on the ATP-supported reaction differed strikingly from those on the substrate-dependent reaction. In the case of the substrate reaction, CMS inhibited the uptake of Mg^{++} and antagonized both the accumulation of Mg^{++} and the additional respiration caused by Zn^{++} (2). The data of Table I show that CMS activates the reaction 2- to 3-fold when ATP is the source of energy for Mg^{++} uptake in mitochondria treated with rotenone to suppress the oxidation of endogenous substrates. The additional uptake depends on ATP and is inhibited by oligomycin. The effect of CMS is not duplicated by *N*-ethylmaleimide and is much more difficult to observe with HMB (Table I). HMB is usually effective only at much lower concentrations than CMS. Both *N*-ethylmaleimide and HMB inhibit the substrate-dependent accumulation of Mg^{++} (2), indicating that these reagents, while they do not duplicate the effects of CMS, do interact with the components of the mitochondrial energy transport system.

TABLE I

Effect of cations and —SH group reagents on ATP-supported accumulation of Mg^{++} by isolated heart mitochondria

Heart mitochondria (5 mg of protein) were incubated for 3 min at 38° in 3 ml of a medium of sucrose (0.25 M), $MgCl_2$ (10 mM), Tris phosphate (1 mM, pH 7.0), ATP (disodium salt neutralized with Tris, 3 mM), Tris-*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (6 mM, pH 7.0), and rotenone (7 μM). The suspensions were then cooled rapidly in ice water and centrifuged for 5 min at 17,000 rpm. The resulting pellets were rinsed quickly with cold 0.25 M sucrose and extracted with perchloric acid. The resulting extracts were then analyzed for Mg^{++} by atomic absorption spectroscopy (2).

Additions	Mitochondria Mg^{++}	
	No addition	Oligomycin (1 μg/mg)
	mμmoles/mg	
Experiment 1		
None	108	80
None, ATP omitted	60	
Zn^{++} (150 μM)	230	
CMS (67 μM)	217	85
CMS + Zn^{++}	558	100
CMS + Zn^{++} (ATP omitted)	70	
Cd^{++} (80 μM)	317	90
N-ethylmaleimide (60 μM)	90	
HMB (23 μM)	117	
HMB + Zn^{++}	317	
Experiment 2		
None	75	
Zn^{++} (150 μM)	192	67
Cd^{++} (160 μM)	190	70
Hg^{++} (20 μM)	158	50
Experiment 3		
None	70	
CMS	213	69
Cu^{++} (50 μM)	190	66
Ni^{++} (33 μM)	80	
Mn^{++} (33 μM)	70	

When Zn^{++} is added in the presence of CMS, the accumulation of Mg^{++} supported by ATP is increased much more than with either reagent separately. The amount of Mg^{++} accumulated under these conditions compares favorably with the amount accumulated in the presence of substrate at comparable temperature and time of incubation (*cf.* Fig. 3 for a quantitative comparison of the Mg^{++} accumulated by these two pathways.) The reaction remains completely sensitive to oligomycin and dependent on ATP (Table I). Similar increases are seen when Zn^{++} is added in addition to HMB. The synergistic enhancement of Mg^{++} accumulation in rotenone-treated mitochondria by CMS and Zn^{++} is shown more clearly in Fig. 1. In this experiment even high levels of Zn^{++} (200 μM) had little effect on the accumulation in the absence of CMS. CMS alone increased the observed accumulation from about 50 to 180 mμmoles of Mg^{++} per mg of protein, while the combination of Zn^{++} and CMS caused an accumulation of 500 mμmoles of Mg^{++} per mg. The observed ATPase activity of these mitochondria increased with the increase in accumulated Mg^{++} (Fig. 1*B*). This observation is compatible with the concept that increased energy is required

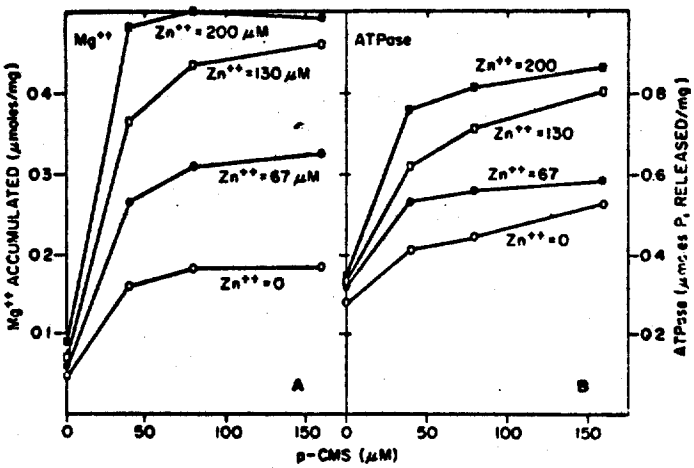

FIG. 1. *A*, the effect of increasing concentrations of CMS and Zn^{++} on the ATP-dependent uptake of Mg^{++} by heart mitochondria. The experiments were carried out as described in the legend for Table I with the exception that the concentration of Tris-*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (pH 7.2) was raised to 13 μM and phosphate was omitted. *B*, the effect of increasing concentrations of CMS and Zn^{++} on the ATPase activity of heart mitochondria. The preparation of mitochondria and the medium employed were identical with that of *A*. The suspensions were incubated for 3 min at 38° with shaking, and the reactions were stopped by the addition of perchloric acid. The increase in inorganic phosphate was then determined by the method of Lindberg and Ernster (14).

TABLE II

Uptake of Zn^{++} by heart mitochondria in presence of ATP

Mitochondria were incubated and extracted as described in the legend for Table I. Mg^{++} and Zn^{++} were then estimated by atomic absorption spectroscopy. A Zn^{++} concentration of 67 μM is equivalent to 40 mμmoles per mg of protein.

Additions	Mitochondrial cations	
	Mg^{++}	Zn^{++}
	mμmoles/mg	
No Zn^{++} added	106	2
Zn^{++} (67 μM)	100	22
Zn^{++} + CMS (67 μM)	340	17
Zn^{++} + CMS + oligomycin	115	21

to support the additional Mg^{++} accumulation which occurs in the presence of Zn^{++} + CMS. The increased ATPase, like the additional accumulation of Mg^{++}, is completely sensitive to oligomycin.

The data of Table II establish that over half of the available Zn^{++} can be recovered in the mitochondrial pellet following these incubations. Therefore, the low uptake of Mg^{++} in the presence of Zn^{++} alone cannot be explained solely by a lack of Zn^{++} available to the mitochondrion. As has been reported previously (2), the uptake of Zn^{++} by the mitochondrion does not appear to require energy.

Properties of Mg^{++} Accumulation Reaction in Presence of CMS and Zn^{++}.—In the absence of CMS and Zn^{++}, the combination of high Mg^{++} concentrations (17 mM), high temperature of incubation (38°), and longer times of incubation (10 min) were found necessary to show the accumulation of Mg^{++} supported by ATP with any consistency (15). The markedly higher levels of Mg^{++} accumulated in the presence of Zn^{++} and CMS can readily be shown in short (3 min and less) incubations at 30° and with much lower concentrations of Mg^{++}. Optimal accumulation is still observed when the concentration of Mg^{++} is between 10 and 17 mM. The addition of P_i is not essential for vigorous accumulation of Mg^{++} (Fig. 1), but the addition of 1 to 3 mM P_i does increase the uptake to some extent. Tris-*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (16) was found to provide effective pH control with no discernible effect on Mg^{++} accumulation.

Examination of the rate of the ATPase reaction associated with the additional Mg^{++} accumulation revealed no lag period. The reaction proceeded linearly over a period of 3 min at 38° (Fig. 2) both in the presence of Zn^{++} and CMS and in the absence of additions. Therefore, estimates of ATPase activity obtained after 3-min incubations (such as that reported in Fig. 1) appear to reflect the rate of the reaction as well as the total ATP hydrolyzed.

As reported above, both the accumulation of Mg^{++} and the ATPase activity which accompanies it in the presence of Zn^{++} and CMS are sensitive to oligomycin. Titration of these activities with oligomycin established that the levels of oligomycin which inhibit these activities were nearly identical with the amount of antibiotic necessary to inhibit the dinitrophenol induced ATPase activity under similar conditions (0.15 μg of oligomycin per mg of protein produced from 45 to 55% inhibition of all three reactions).

The accumulation of Mg^{++} in the presence of ATP, Zn^{++}, and CMS is relatively insensitive to dinitrophenol. The data of Fig. 3 show that more than 10^{-4} M dinitrophenol is necessary to abolish the uptake of Mg^{++} under these conditions. For comparison, the dinitrophenol sensitivity of the accumulation of Mg^{++} in the presence of Zn^{++} with the ascorbate-TMPD substrate system is also shown. This reaction is sensitive to considerably lower concentrations of dinitrophenol than is the

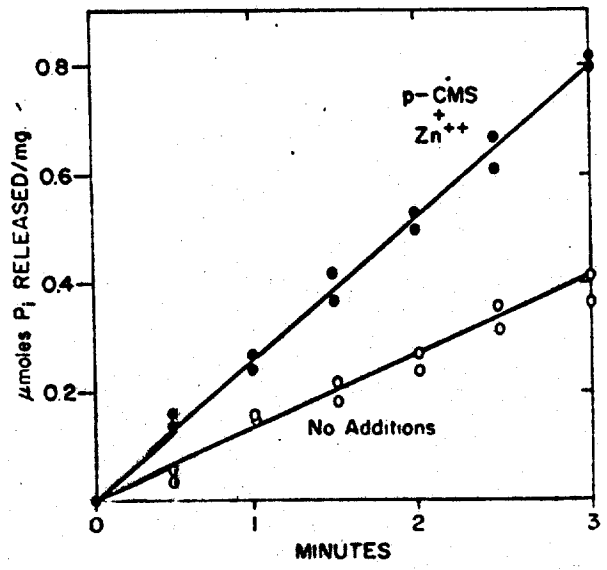

FIG. 2. ATPase activity as a function of time at 38°. When indicated, Zn^{++} and CMS were added to a concentration of 67 μM each. The incubation conditions were those given in the legend for Table I. The reaction was started by the addition of mitochondria which had been equilibrated with rotenone. Samples (0.2 ml) were withdrawn and analyzed for P_i at the indicated times. Two separate determinations of each reaction are plotted

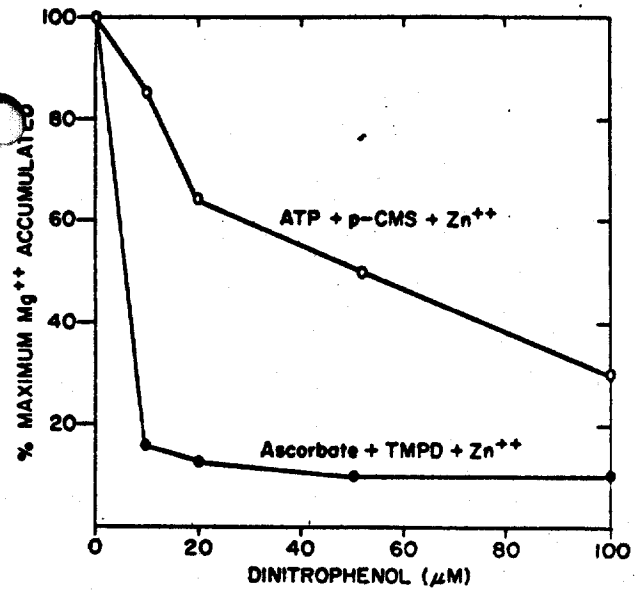

Fig. 3. The effect of dinitrophenol on the accumulation of Mg^{++} by heart mitochondria. The response of two different reactions is shown: the accumulation of Mg^{++} supported by ATP in the presence of CMS and Zn^{++}, and the accumulation supported by ascorbate and TMPD in the presence of Zn^{++}. The reaction conditions were identical with those described in the legend for Table I. The amount of Mg^{++} accumulated by the substrate-supported reaction in the absence of dinitrophenol was 500 mμmoles per mg; the ATP-supported reaction in the absence of dinitrophenol resulted in the accumulation of 340 mμmoles per mg. The substrate-supported reaction medium contained Tris-ascorbate (5 mM), Tris phosphate (3 mM), TMPD (0.1 mM), sucrose, and Mg^{++}, and was supplemented with Zn^{++} acetate (67 μM).

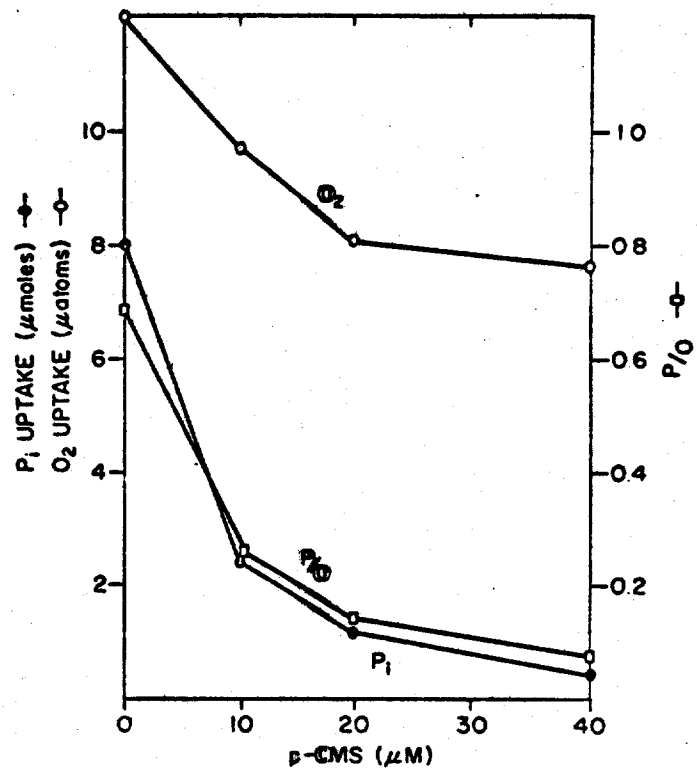

Fig. 4. Effect of CMS on oxidative phosphorylation with ascorbate-TMPD as substrate. Oxygen uptake was estimated by the polarographic procedure previously described (2), and inorganic phosphate uptake by the method of Lindberg and Ernster (14).

ATP-supported reaction in the presence of Zn^{++} and CMS. A similar discrepancy was also noted when the sensitivity of the accumulation of Mg^{++} to *m*-chlorocarbonylcyanide phenylhyrazone (17) was investigated. The ATP- supported reaction was much less sensitive to low concentrations of *m*-chlorocarbonylcyanide phenylhydrazone than was the substrate-supported reaction (50% inhibition levels were 10^{-6} M for the ATP-dependent reaction and 10^{-7} M for the substrate-dependent reaction).

Effect of CMS on Mitochondrial Energy Transfer Reactions—The concentrations of CMS used in these studies strongly inhibit oxidative phosphorylation (Fig. 4) when ascorbate and TMPD are the substrates. It has little, if any, effect on State 4 respiration with ascorbate-TMPD (2), but abolishes State 3 respiration under these conditions. The mercurial does not inhibit dinitrophenol-activated respiration (2, 18), but abolishes the additional respiration induced by Zn^{++} in the presence of Mg^{++} (2). Several early studies have also established that HMB (and therefore presumably CMS) inhibits the dinitrophenol-induced ATPase of intact mitochondria (19, 20). The data presented in Fig. 5 confirm the sensitivity of this reaction to CMS in our preparations. Zn^{++} has little effect on dinitrophenol-ATPase, but addition of Zn^{++} as well as CMS to the reaction flask results in high ATPase activity which is independent of the concentration of dinitrophenol present (Fig. 5). This observation is compatible with the relatively low sensitivity to dinitrophenol of the ATP-dependent accumulation of Mg^{++} which occurs under these conditions. In other words, the combination of Zn^{++} and CMS induces Mg^{++} uptake and the

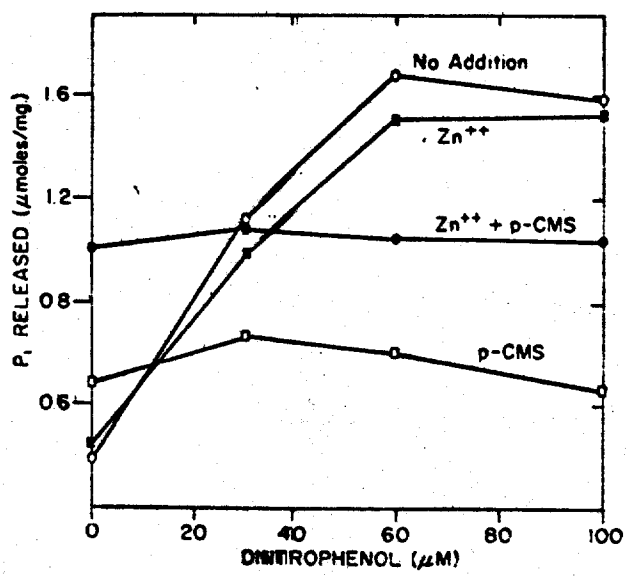

Fig. 5. Effect of CMS and Zn^{++} on the dinitrophenol-induced ATPase of heart mitochondria. Mitochondria (5 mg of protein) were incubated for 3 min at 38° in a medium of sucrose (0.25 M), Tris-*N'*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (15 mM, pH 7.2), Tris-Na_2-ATP (3.3 mM), $MgCl_2$ (10 mM), and the indicated concentration of dinitrophenol. Where indicated, CMS was added to a concentration of 80 μM and Zn^{++} to a concentration of 130 μM. The reactions were stopped by the addition of $HClO_4$, and the increase in P_i was estimated by the method of Lindberg and Ernster (14).

TABLE III

Change in pathway of energy flow for accumulation of Mg^{++} induced by CMS

Mitochondria (5 mg of protein) were equilibrated with rotenone (1 mμmole per mg) prior to incubation (3 min at 38°) in 3 ml of a medium consisting of sucrose (0.25 M), $MgCl_2$ (10 mM), Tris-*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (15 mM, pH 7.2), Tris-ATP (3.3 mM), Tris phosphate (1 mM), Tris-ascorbate (5 mM), and TMPD (0.1 mM). Zn^{++} was present at a concentration of 130 μM and, where indicated, CMS at a concentration of 80 μM. The dinitrophenol concentration was 30 μM and the oligomycin concentration was 0.3 μg per mg of protein. The mitochondria were isolated by centrifugation and extracted with perchloric acid. The Mg^{++} content of the extracts was estimated by atomic absorption spectroscopy.

Possible energy sources	Additions	Mg^{++} accumulated		
		No addition	Plus DNP	Plus oligomycin
		mμmoles/mg		
ATP, ascorbate-TMPD....	Zn^{++}	200	58	200
ATP, ascorbate-TMPD....	Zn^{++} + CMS	260	320	125

ATPase activity which accompanies the uptake, whether dinitrophenol (at low concentrations) is present or not. Both the Mg^{++} accumulation and the ATPase observed in the presence of dinitrophenol are sensitive to oligomycin.

Effect of CMS on Accumulation of Mg^{++} in Presence of Both Substrate and ATP—The accumulation of Mg^{++} in the presence of both substrate (ascorbate-TMPD) and ATP is somewhat lower than that observed with substrate alone. It has been suggested that this decreased accumulation is the result of competition between Mg^{++} uptake and phosphorylation of the ADP derived from ATP (15). This system, however, provides two potential sources of energy to support ion transport, ATP hydrolysis, and respiration. The study summarized in Table III shows that in the presence of substrate and ATP the accumulation of Mg^{++} induced by Zn^{++} is sensitive to dinitrophenol, but insensitive to oligomycin. The reaction therefore appears to use very little of the available ATP as a source of energy for ion uptake. If CMS is also added under these conditions, there is a slight increase in the observed uptake of Mg^{++} and a remarkable change in inhibitor sensitivity. In the presence of CMS, the reaction is now sensitive to oligomycin and insensitive to dinitrophenol. Addition of the mercurial, therefore, results in a shift from a reaction supported primarily by substrate to a reaction supported by the hydrolysis of ATP. Control experiments have established that increasing the concentration of P_i and ATP and increasing the pH to 7.4 do not result in oligomycin-insensitive precipitation of Mg^{++} salts under those conditions. The pH of the strongly buffered incubation medium did not rise above pH 7.2 during any of the reported experiments.

DISCUSSION

These studies have established that CMS activates the ATP-dependent accumulation of Mg^{++} by isolated heart mitochondria, and that under these conditions a marked increase in the ATPase of the mitochondrion is also observed. Both the uptake of Mg^{++} and the ATPase remain sensitive to oligomycin. Zn^{++}

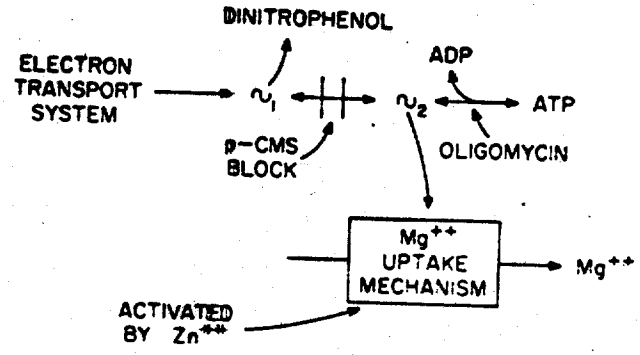

FIG. 6. Scheme showing suggested points of action of CMS and Zn^{++} on the energy-linked accumulation of Mg^{++} by heart mitochondria.

ion, a potent activator of the substrate-supported accumulation of Mg^{++} (1, 2), is relatively ineffective on the ATP-dependent reaction. In combination with CMS, however, Zn^{++} causes a marked increase in the ATP-supported accumulation of Mg^{++}. Previous studies have established that the effect of Zn^{++} on the substrate-supported reaction is antagonized effectively by CMS (2). These observations indicate, therefore, that CMS and Zn^{++} affect the uptake of Mg^{++} by the mitochondrion in quite different ways. The scheme shown in Fig. 6 advances a possible explanation for observed behavior of these reagents.

Mechanism of Zn^{++} Action—The following considerations suggest that Zn^{++} (and perhaps Cd^{++} and other heavy metal cations) activates the accumulation of Mg^{++} by direct action on the mechanism for ion uptake, as distinguished from possible effects on the energy transfer system. (*a*) Under the appropriate conditions, Zn^{++} increases the accumulation of Mg^{++} both in the presence of substrate (2) and in the presence of ATP. (*b*) Zn^{++} appears to alter the permeability of the mitochondrion to Mg^{++} and K^+ ions.[2] (*c*) Zn^{++} decreases P:O ratios only slightly and induces little ATPase activity except in the presence of CMS. These considerations indicate little change in the pathway of energy utilization by mitochondria in the presence of Zn^{++}. The changes in permeability and the general increase in the energy-linked Mg^{++} uptake, regardless of the energy source, strongly indicate that Zn^{++} has its primary effect on the portion of the mitochondrial membrane directly responsible for Mg^{++} (and K^+) uptake. In this respect, it appears quite analogous to valinomycin (7, 10), gramicidin (7, 11, 12), and parathyroid hormone (5, 6). While the mechanism by which mitochondria accumulate ions is uncertain, there appears to be general agreement that the antibiotics and parathyroid hormone all affect mitochondrial permeability. These effects may be sufficient to account for the observed increases in mitochondrial ion uptake (6, 12), but additional factors may be involved (9).

Mechanism of CMS Action—In contrast to the postulated action of Zn^{++} on the ion transport mechanism of the mitochondrion, the action of CMS appears to be localized in the energy transfer system. The following points favor the suggestion that CMS interferes with energy transfer between the dinitrophenol-sensitive site and the intermediate (or condition) which supports Mg^{++} accumulation (Fig. 6).

1. CMS inhibits oxidative phosphorylation and State 3 respiration with ascorbate-TMPD as substrate (Fig. 4).

[2] G. P. Brierley, in preparation.

2. The mercurial inhibits the accumulation of Mg^{++} which is supported by substrate oxidation (2) but activates accumulation of this ion in the presence of ATP (Table I and Fig. 1).

3. CMS inhibits the extra oxidation seen when Zn^{++} activates the uptake of Mg^{++} in the presence of ascorbate-TMPD (2), and dinitrophenol relieves this inhibition. This observation parallels that of Fonyo and Bessman (18) who noted that HMB inhibits respiration dependent on ADP, Ca^{++}, and arsenate, but not that stimulated by dinitrophenol.

4. Previous studies have established that HMB inhibits dinitrophenol-induced ATPase (19, 20) and the present study has confirmed that CMS also inhibits this activity (Fig. 5).

5. CMS activates the ATPase which parallels the ATP-supported accumulation of Mg^{++} (Fig. 1).

6. In combination with Zn^{++}, the ATP-dependent accumulation of Mg^{++} and the parallel ATPase are increased to a much greater degree than in the absence of Zn^{++} (Fig. 1). Both of these reactions, Mg^{++} uptake and ATPase, are unaffected by low concentrations of dinitrophenol but sensitive to oligomycin (Figs. 3 and 5 and Table I).

7. Addition of CMS causes the accumulation of Mg^{++} in the presence of both substrate and ATP to switch from a predominately substrate-supported reaction to a predominately ATP-supported reaction (Table III).

The present study, in conjunction with the report of Fonyo and Bessman (18), would appear to establish that HMB and CMS should be classed as inhibitors of mitochondrial energy transfer (21). One could then infer that a thiol group is involved in the transfer of energy at the indicated point (Fig. 6).

Interruption of the transfer of energy from ~1 to ~2 in this scheme would explain the observed effects of the mercurial on respiration, on the substrate-dependent uptake of Mg^{++}, and on dinitrophenol-induced ATPase. It would also explain the relative insensitivity to dinitrophenol of the ATP-supported accumulation of Mg^{++} and the ATPase which accompanies this reaction. Activation of the ATP-dependent uptake of Mg^{++} could result from a block at the point indicated if it is assumed that even in the rotenone-treated mitochondria some residual endogenous respiration generates enough ~1 to rephosphorylate a portion of the ADP formed. It should be recalled that phosphorylation of ADP competes very successfully with Mg^{++} accumulation in heart mitochondria (15). If CMS effectively removed this "back pressure" on the ATP-supported accumulation of Mg^{++}, the net effect would be an apparent activation of ion accumulation and ATPase. Our present data cannot eliminate this possibility, although it should be pointed out that addition of concentrations of rotenone, cyanide, or antimycin, which result in vanishingly small endogenous respiration, does not duplicate the effect of CMS on the ATP-supported reaction.

In addition, we have recently found that CMS activates mitochondrial ATPase strongly in the absence of added cations (other than Tris) and activates the ATPase of electron transport particles.[3] These results suggest that an actual activation of the enzymes responsible for mitochondrial ATPase as well as inhibition of energy transfer may be involved under the conditions of our experiments. Studies of the activation of the ATPase activity of myosin (22, 23) and the ATPase of submitochondrial particles under conditions which would be unlikely to result in Mg^{++} accumulation (24) provide ample precedent for such an activation. The fact that in our experiments the increased ATPase activity is closely associated with a massive, energy-linked accumulation of Mg^{++} suggests that the increased energy available from the additional ATP hydrolysis can be efficiently coupled to ion movement.

The failure of *N*-ethylmaleimide to duplicate the activation of ATPase and Mg^{++} accumulation seen with CMS and the relative ineffectiveness of HMB under these conditions suggest that the strongly anionic character of CMS as well as its reaction with thiols may be important to the activation process.

In our opinion, the results of this study are much more compatible with the more classical models of oxidative phosphorylation involving a sequential transfer of an energized state through intermediates of unknown structure to ADP (*cf.* Reference 25) than with the chemiosmotic mechanism proposed by Mitchell (26). The existence of an energy-linked ion accumulation which is insensitive to dinitrophenol would appear to be at odds with the mode of action of uncouplers proposed by Mitchell (26), *i.e.* that uncouplers prevent the establishment of a H^+ gradient by increasing the permeability of the membrane to H^+. We realize, however, that the dinitrophenol-insensitive accumulation of Mg^{++} reported in the present study represents a rather artificial system, since the measurement is made in the presence of relatively high concentrations of ATP, Zn^{++}, Mg^{++}, CMS, and dinitrophenol. Taken at face value, however, the experiments reported here indicate that the mitochondrion is capable of concentrating ions by an energy-linked reaction which is not sensitive to dinitrophenol, provided that CMS is present.

[3] G. P. Brierley, C. T. Settlemire, and W. E. Jacobus, in preparation.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 242, No. 18, Issue of October 10, pp. 4324-4328, 1967
Printed in U.S.A.

Ion Transport by Heart Mitochondria

IX. INDUCTION OF THE ENERGY-LINKED UPTAKE OF K^+ BY ZINC ION*

Received for publication, April 3, 1967

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SUMMARY

The accumulation of K^+ by isolated heart mitochondria suspended in isotonic sucrose is markedly stimulated by the addition of Zn^{++}. The accumulation requires a source of energy, the presence of a permeant anion such as acetate, and the presence of either inorganic phosphate or arsenate. The reaction is inhibited and reversed by uncouplers of oxidative phosphorylation and by anaerobiosis. It is also inhibited by Mg^{++} and Na^+ ions and by ethylenediaminetetraacetate and other Zn^{++} chelators. The Zn^{++}-dependent accumulation is accompanied by increased respiration and extensive reversible swelling of the mitochondria. The extent of the observed swelling and the time course of swelling are dependent on the concentration of K^+ and of Zn^{++}. In the absence of added K^+, Zn^{++} induces a rapid energy-linked expulsion of the endogenous K^+ of the mitochondrion. These studies indicate that the addition of Zn^{++} under carefully defined experimental conditions is sufficient to induce the transport of K^+ by heart mitochondria. This transport of K^+ closely resembles that reported by other investigators in the presence of valinomycin, gramicidin, and parathyroid hormone.

Zn^{++} and certain other heavy metal ions markedly increase the energy-linked accumulation of Mg^{++} by isolated heart mitochondria under the appropriate experimental conditions (1–4). The relationship between the increased ion accumulation in the presence of Zn^{++} and other examples of induced ion uptake is not yet clear. The effects of valinomycin (5–8) and gramicidin (5, 8, 9) appear to be limited to increased transport of monovalent cations while parathyroid hormone (10, 11) increases both K^+ and Mg^{++} transport. In the present communication, studies are presented which indicate that Zn^{++} shares with parathyroid hormone the ability to induce K^+ transport as well as Mg^{++} transport in heart mitochondria.

* This research was supported in part by Public Health Service Grant HE09364, by Program Project Grant 09884 from the National Heart Institute, and by a grant in aid from the American Heart Association. A preliminary report of this study has been presented (1). For the previous study in this series see reference 2.

‡ Established Investigator of the American Heart Association.

MATERIALS AND METHODS

Beef heart mitochondria were prepared by the Nagarse procedure as previously described (4). The uptake and release of K^+ was followed with a Beckman model 39047 electrode essentially as described by Pressman (5). Electrode response was calibrated by titration with known concentrations of K^+. Oxygen consumption was measured with either the Beckman oxygen electrode as previously described (2) or the YSI 5331 electrode. Mitochondrial swelling and shrinking was monitored by recording the changes in absorbance at 540 mμ with the use of a Beckman DK-2 spectrophotometer or at 546 mμ with an Eppendorf photometer. The cuvette of the Eppendorf was stirred with a magnetic stirrer; no stirring was used with the DK-2. The two measuring systems give qualitatively identical results. In a number of experiments three parameters (absorbance, oxygen uptake, and K^+ concentration) were recorded simultaneously with an apparatus consisting of a standard YSI electrode chamber mounted on the Eppendorf photometer and equipped with a small side arm to accept the K^+ and reference electrodes. Exact experimental conditions and the composition of the suspending medium are detailed with the individual experiments reported.

RESULTS

Heart mitochondria respiring with ascorbate and *N*,*N*,*N'*,*N'*-tetramethylphenylenediamine as substrate and suspended in 0.25 M sucrose buffered with Tris-acetate and Tris-phosphate show little tendency to swell or to accumulate K^+ in short term experiments at 25°. The addition of low concentrations of Zn^{++} under these conditions results in a rapid and extensive uptake of K^+ by the mitochondria, extensive reversible swelling, and activation of respiration. The series of experiments shown in Fig. 1 documents this response and shows some of the requirements of the system. Fig. 1*A* shows the response of the K^+ electrode, optical density trace, and oxygen electrode in a control experiment in which no Zn^{++} was added. It also shows the response to the addition of 67 μM Zn^{++} as the acetate salt

With this addition to a suspension containing 2 mM K^+ a total of about 190 mμmoles of K^+ per mg of protein enters the mitochondrial phase at a rate of about 210 mμmoles per min per mg. Respiration is increased from a rate of 0.24 to a rate of 0.32 μatom of O_2 per min per mg of protein, and the optical density of the suspension decreases by nearly 0.3 optical density unit. At the anaerobic point both the K^+ uptake and the swelling reverse, and a rapid loss of K^+ to the medium and contraction of the mitochondria occur. The uptake of K^+ and the simultaneous changes in volume and respiration are prevented by the addition of dinitrophenol and other uncouplers, EDTA and other Zn^{++} chelators, or cyanide before the addition of Zn^{++}. The traces for K^+ and swelling obtained under these conditions are virtually identical with the control traces shown in Fig. 1*A*. Zn^{++} added after the cuvette has gone anaerobic also produces no response. Oligomycin does not inhibit the reaction. The uptake of K^+ and the swelling are rapidly reversed by the addition of an uncoupler after the accumulation has begun and before the anaerobic point (Fig. 1*B*). Substitution of Cl^- for acetate in the suspending medium results in much less accumulation of K^+ and less swelling. Omission of P_i from the medium prevents the uptake of K^+, the swelling, and the activation of respiration (Fig. 1*C*). Addition of P_i (or arsenate) after the Zn^{++} results in stimulation of K^+ accumulation and the expected response with the other parameters. It should be noted that Zn^{++} produces a noticeable effect on the K^+ electrode in the absence of P_i. Control experiments in the absence of mitochondria with the complete suspending medium show negligible effects of Zn^{++} on the electrode when P_i is present in the medium.

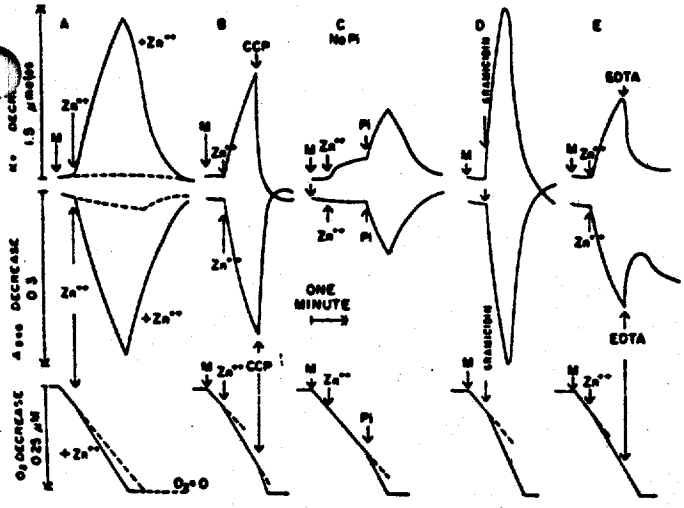

FIG. 1. The effect of Zn^{++} on mitochondrial K^+ content, volume, and rate of oxidation. The measurements were carried out simultaneously in the apparatus described in the text with the use of a Beckman cation-sensitive electrode for K^+ concentration, a Clark electrode for O_2, and the change in optical density at 546 mμ as the measure of swelling. Mitochondria (7.5 mg of protein) were added to 9 ml of the following medium: sucrose (0.25 M), Tris-acetate (20 mM, pH 7.0), Tris-phosphate (2 mM), Tris-ascorbate (5 mM), *N,N,N',N'* tetramethylphenylenediamine (0.1 mM), and rotenone (7 μM). The suspension was stirred at 25°; with the exception of *D*, the uptake of K^+ was initiated by the addition of Zn^{++}-acetate (100 μM). In *D*, the inducer was gramicidin (0.3 μM). The *dotted traces* in *A* show the results of a control incubation in which the addition of Zn^{++} was omitted. *B* shows the effect of the addition of the uncoupler *m*-chlorocarbonylcyanide phenylhydrazone (*CCP*, 10 μM) after the accumulation has proceeded for 1 min. In *C*, Tris-phosphate was omitted from the original suspending medium and added to the same final concentration at the indicated point. *E* shows the effect of the addition of Tris-EDTA (200 μM) 1 min after the Zn^{++}.

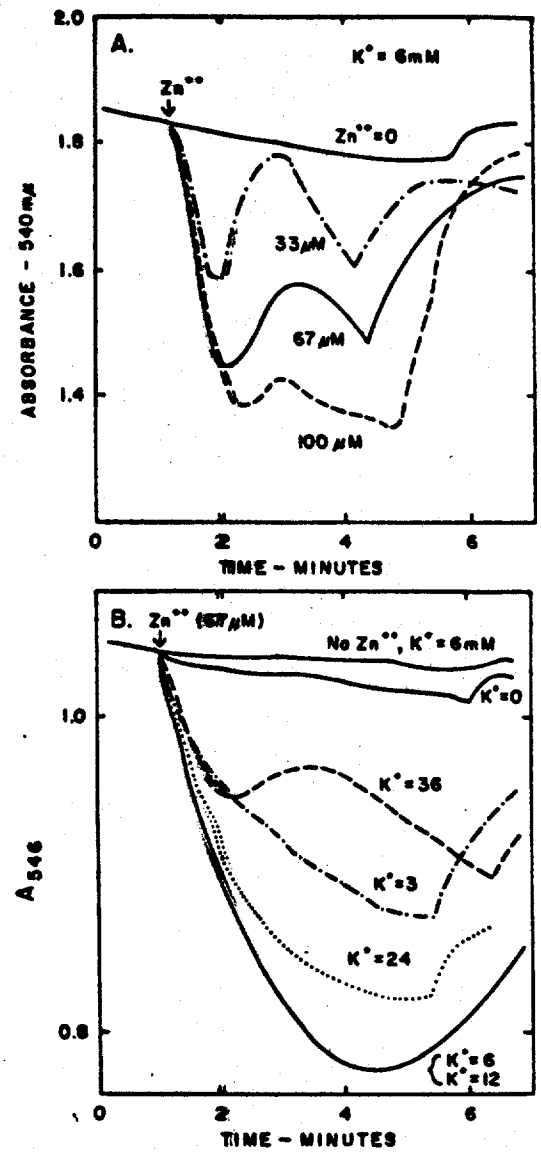

FIG. 2. The effect of varying the concentration of Zn^{++} and of K^+ on the swelling of beef heart mitochondria. In *A*, swelling was monitored at 540 mμ in a 1-cm cuvette by using a Beckman DK-2 spectrophotometer. Zinc acetate was added to the indicated concentration after a 1-min incubation at 25°. The suspending medium consisted of the following: sucrose (0.25 M), Tris-acetate (20 mM, pH 7.0), Tris-phosphate (2 mM), potassium acetate (6 mM), Tris-ascorbate (5 mM), *N,N,N',N'*-tetramethylphenylenediamine (0.1 mM), and 1.2 mg of mitochondrial protein in total volume of 3.0 ml. In *B*, swelling was followed at 546 mμ with an Eppendorf photometer equipped with a circular cuvette 0.75 inch in diameter. The contents were stirred with a magnetic stirrer. The K^+ content of the medium described above was varied as indicated, and swelling was initiated by the addition of zinc acetate (67 μM). The total volume of the incubation mixture was 6 ml; 5 mg of mitochondrial protein were present.

The response of heart mitochondria to addition of Zn^{++} closely resembles the reported response to valinomycin and gramicidin in media containing K^+. Fig. 1*D* shows the result of the addition of 0.3 μM gramicidin to a suspension of mitochondria under the conditions of the experiments described

above. The uptake of K^+ and the swelling both proceed at a much greater rate than that observed in the presence of Zn^{++}, but the total amount of uptake and extent of swelling are roughly the same.

The activation of K^+ uptake by Zn^{++} can be reversed to a large extent by the addition of EDTA after the accumulation has begun. Fig. 1*E* shows the effect of the addition of 200 μM EDTA 1 min after the accumulation of K^+ had been induced by the addition of 100 μM Zn^{++}. Another series of experiments (12) has shown that this treatment rapidly removes bound Zn^{++} from the mitochondrion as well as Zn^{++} in solution. Zn^{++} removal results in a rapid extrusion of K^+ and contraction of the mitochondria although this contraction is followed rapidly by an irreversible swelling phase with no indication of K^+ uptake with the K^+ electrode (Fig. 1*E*). EDTA addition before the addition of Zn^{++} does not result in swelling under these conditions.

Titration of the reaction with increasing concentrations of Zn^{++} revealed that the optimal response occurred at between 67 and 100 μM Zn^{++}. The study shown in Fig. 2*A* establishes that these two concentrations of Zn^{++} resulted in identical rates of swelling but that the extent was greater at the higher concentration. Concentrations of Zn^{++} over 100 μM gave similar responses to that shown for 100 μM. The traces reported in Fig. 2*A* also show a decided tendency to rebound, *i.e.* undergo a cycle of swelling, contraction, and swelling again. The second inflection point corresponds to the anaerobic point. This incipient oscillation is not obtained in all preparations of mitochondria under these conditions (6 mM K^+). However, when preparations which show no tendency to rebound are titrated with K^+ (Fig. 2*B*), oscillations become apparent at higher concentrations of K^+. Fig. 2*B* also establishes that the optimal concentration of K^+ for the accumulation reaction is between 6 and

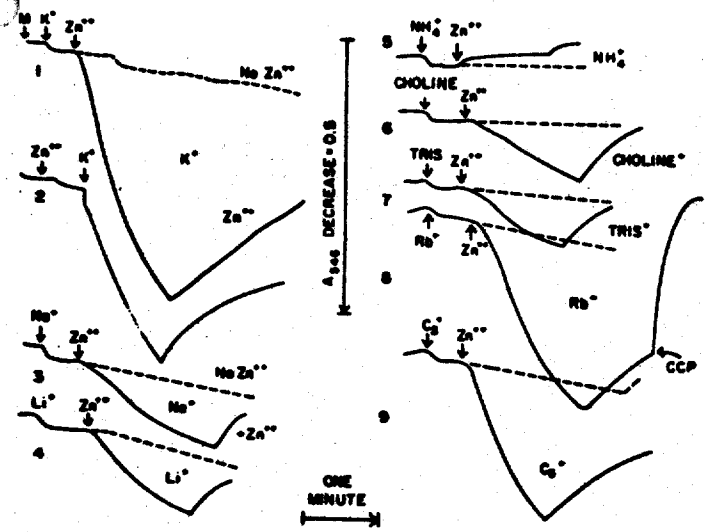

FIG. 3. The swelling of mitochondria in the presence of Zn^{++} and various monovalent cations. In each case the *dotted trace* represents a control incubation in which Zn^{++} was omitted; the *solid trace* is the response to the addition of Zn^{++} (100 μM). In each case the incubation medium consisted of sucrose (0.25 M), Tris-acetate (20 mM, pH 7.0), Tris-phosphate (2 mM), Tris-ascorbate (5 mM), *N,N,N',N'*-tetramethylphenylenediamine (0.1 mM), rotenone (2 μM), and mitochondria (5 mg of protein). The total volume was 6 ml. At the indicated point the monovalent cation was added as the chloride salt to a final concentration of 6 mM. *Runs 1* and *2* show the response to K^+ addition before and after Zn^{++}; *run 3* shows the response to Na^+; *run 4* to Li^+; *run 5* to NH_4^+; *run 6* to choline; and *run 7* to additional Tris. *Run 8* gives the response to Zn^{++} in the presence of Rb^+ and the reversal by *m*-chlorocarbonylcyanidephenylhydrazone (*CCP*); *run 9* shows the response in the presence of Cs^+.

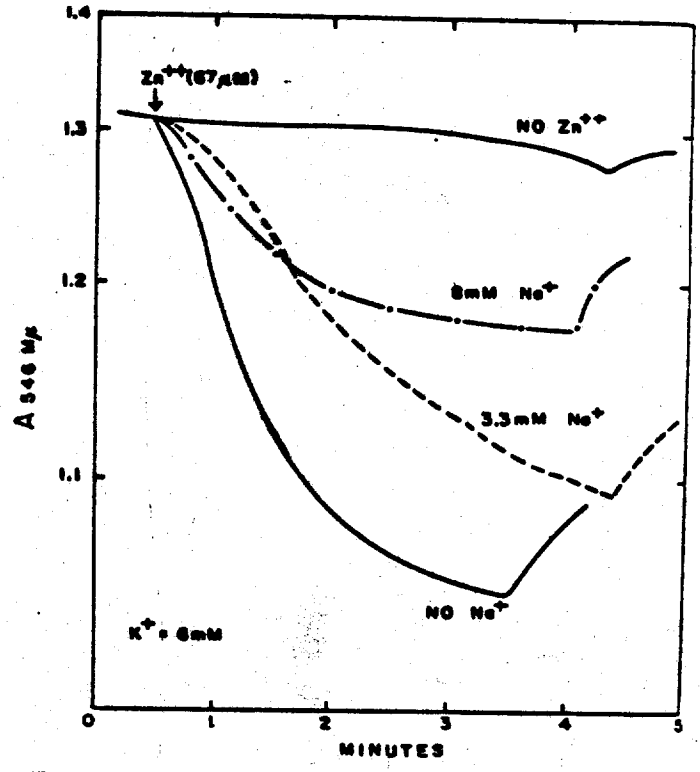

FIG. 4. Inhibition of Zn^{++}-induced, K^+-dependent swelling by Na^+. Swelling was monitored at 546 mμ with the Eppendorf photometer. The medium described in the legend for Fig. 1 was employed with the addition of 2.5 mg of mitochondrial protein to 6 ml of the medium. The K^+ concentration was 6 mM; Zn^{++}, 67 μM.

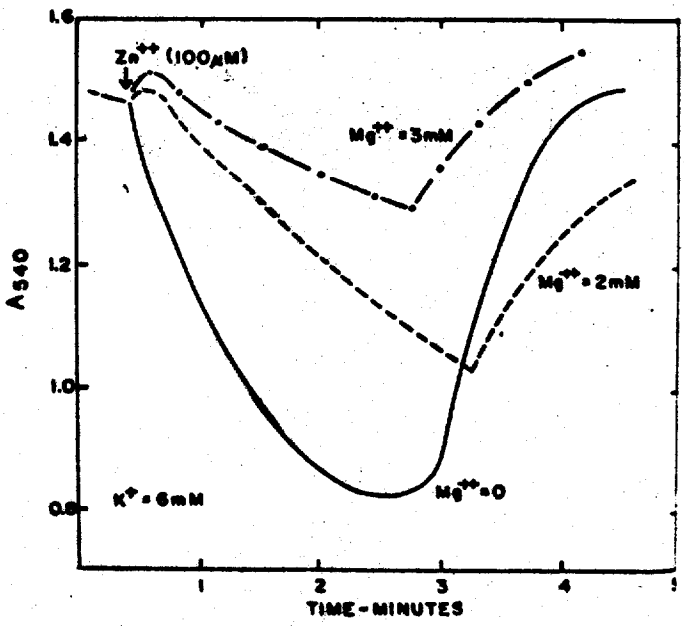

FIG. 5. Inhibition of Zn^{++}-induced, K^+-dependent swelling by Mg^{++}. Swelling was monitored at 540 mμ on the DK-2 spectrophotometer with 3 ml of the medium described in the legend for Fig. 1. The concentration of K^+ was 6 μM; that of Zn^{++}, 100 μM.

12 mM. Studies with the K^+ electrode (not shown) verify that the rate and extent of K^+ uptake are much greater from 6 mM K^+ solutions than from solutions containing 2 mM K^+ such as those reported in Fig. 1. In the experiment presented in Fig. 1 of Reference 1, over 500 mμmoles of K^+ were taken up at a rate of 550 mμmoles of K^+ per mg of protein per min.

The specificity of the induction of monovalent ion transport by Zn^{++} was also investigated. Cd^{++} substitutes rather well for Zn^{++} in the role of inducer, and some slight response was obtained with Cu^{++}. Other divalent ions tested did not induce K^+ uptake.

The specificity for the monovalent ion is shown in Fig. 3. This study establishes that mitochondria swell to the extent of about 0.5 optical density unit in the presence of 6 mM K^+ when 100 μM Zn^{++} is added. Addition of K^+ after the Zn^{++} is only slightly less effective (*trace 2*). Substitution of 6 mM Na^+, Li^+, $choline^+$, or Tris for K^+ results in a slight Zn^{++}-dependent swelling which reverses on anaerobiosis. Substitution of 6 mM NH_4^+ results in a slight contraction under these conditions (*trace 5*) while Rb^+ and Cs^+ are only slightly less effective than K^+ in supporting the Zn^{++}-dependent swelling.

The Zn^{++}-dependent uptake of K^+ and the associate swelling are inhibited by Na^+ (Fig. 4) and more strongly by Mg^{++} (Fig. 5).

The addition of Zn^{++} to suspensions of mitochondria in the absence of added K^+ results in a rapid release of endogenous K^+. The study shown in Fig. 6 establishes that the release of K^+ is accompanied by activation of respiration requires a source of energy (in this case provided by ascorbate-*N*,*N*,*N'*,*N'*-tetramethylphenylenediamine respiration as shown in Fig. 6*B*), and requires the presence of P_i (Fig. 6*C*). Shrinkage of the mitochondria is frequently observed to parallel the release of endogenous K^+ (Fig. 6*A*). The amplitude of this change is small, and a secondary swelling usually follows as shown in Fig. 6*A*. The secondary swelling reverses when the system

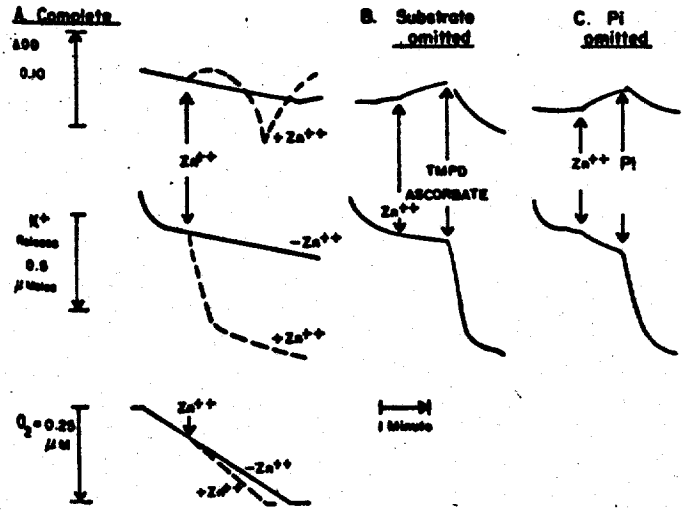

FIG. 6. Energy-linked release of K^+ following addition of Zn^{++}. K^+ appearance in the medium was monitored with the K^+ electrode. Absorbance at 546 mμ and oxygen utilization was measured simultaneously in 9 ml of the following medium described in the legend for Fig. 3 but with the omission of added K^+. The reaction was started by addition of 7.5 mg of mitochondrial protein. In *A* the response of the complete system to the addition of Zn^{++}-acetate (67 μM) is shown. In *B* the substrates were omitted initially and added at the indicated point. *C* shows the result of omitting P_i initially and the addition of P_i after the Zn^{++}.

becomes anaerobic. Since Tris is the only cation added under these conditions, it appears possible that the swelling trace may reflect movements of this cation (*cf.* Fig. 3 also).

DISCUSSION

The results of this study establish that Zn^{++} (and Cd^{++}) induces a massive energy-linked uptake of K^+ under conditions in which respiration is maintained. The response of the mitochondrion to the addition of Zn^{++} closely resembles that previously reported to the addition of the toxic antibiotics valinomycin (5-8) and gramicidin (5, 8, 9) and to parathyroid hormone (10, 11). Each of these reagents causes the rapid uptake of K^+ which is closely associated with swelling and contraction cycles, and each activates respiration. In each case the reaction requires respiration and is prevented or reversed by anaerobiosis and uncouplers of phosphorylation. There is also some indication that Zn^{++}, like valinomycin (13, 14) and certain other antibiotics (15) can produce oscillations in the K^+ content and the volume of the mitochondrion under the proper conditions. In the absence of external K^+, Zn^{++} induces an energy-linked extrusion of the endogenous K^+ of the mitochondrion. Under these conditions, valinomycin (6) and certain histones (16) have been reported to produce a similar effect. The ability of Zn^{++} to increase the energy-linked uptake of Mg^{++} as well as K^+ is a property which has been reported for parathyroid hormone (10) whereas the effects of the antibiotics appear limited to the monovalent cations. The ability of the membrane to discriminate between Na^+ and K^+ in the presence of Zn^{++} supports the argument that the effects of the heavy metal are rather specific and do not result merely in a generalized increase in permeability to ions.

A major difference in the requirements for the induction of K^+ transport by Zn^{++} as compared to other inducers appears to be the necessity for added P_i or arsenate. Acceleration of K^+ transport by P_i in the presence of valinomycin has been reported (6), but the requirement appears much more absolute in the case of Zn^{++} induction. Our most recent experiments indicate that phosphate may be involved in the uptake of Zn^{++} by the mitochondria membrane.[a]

In the case of the activation of Mg^{++} accumulation by Zn^{++} it is possible to advance the rather trivial explanation that the very insoluble heavy metal phosphate permits more effective precipitation of Mg^{++}-phosphate and therefore that the activation of ion accumulation does not occur at the level of the membrane (4). Since the accumulation of K^+ does not appear to involve precipitation of insoluble salts, the present study would appear to establish that Zn^{++} is effective in stimulating ion accumulation at the level of the membrane.

Harris, Cockrell, and Pressman (6) have observed that under some circumstances the effect of valinomycin is seen only in an increased turnover of bound K^+ as indicated by tracer studies. Scott and Gamble (17) have previously reported that a number of heavy metal ions including Zn^{++} and Cd^{++} cause increased exchange but a net loss of bound K^+ from mitochondria. Since it is well established (18) that these ions inhibit respiration with physiological substrates, it appears that the observations of Scott and Gamble (17) could be explained by the combination of increased K^+ uptake induced by the heavy metal in conjunction with secondary degenerative changes which account for the net loss.

[a] G. P. Brierley and V. A. Knight, unpublished observations.

The number of available reagents which affect the flux of ions across the mitochondrial membrane is increasing rapidly as the phenomena of induced ion transport receives more attention. It cannot be assumed that all of these reagents are reactive through a common mechanism, but the use of Zn^{++} as a probe into the mechanism of ion transport appears to offer certain experimental advantages. From a chemical standpoint it represents the simplest inducer yet reported. It is available as an isotope for labeling studies and can be readily estimated chemically. In addition it can be quantitatively removed from the membrane by chelation with EDTA (12). Further studies on the binding of Zn^{++} to the mitochondrion and its relation to the mechanism responsible for the uptake of K^+ and Mg^{++} are currently in progress in our laboratory.

Acknowledgments—It is a pleasure to acknowledge the assistance of Drs. R. N. Bhattacharyya, J. G. Walker, and G. R. Hunter in various phases of this study, and the expert technical assistance of Miss Betty Shearman.

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J. Animal Sci. 18(2): 836-842 (1959)

ZINC TOXICITY IN THE WEANLING PIG

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IN 1934, Todd *et al.* reported the first evidence of a need for zinc in the diet of animals. They found that the addition of zinc salts to the diet of rats improved the rate of growth. Studies by Sutton *et al.* (1937) showed that the zinc tolerance of the rat was between 0.5 and 1.0% of the diet. Further studies with the rat reported by Smith *et al.* (1946) indicated that feeding zinc carbonate at 1.0% of the diet produced a severe anemia in 3 to 5 weeks, and in another study 0.7% zinc carbonate produced a marked anemia within 4 weeks.

Grimmett *et al.* (1937) and Sampson *et al.* (1942) have both described zinc toxicity in the young pig produced by feeding 0.1% zinc in the form of zinc lactate in a diet consisting largely of milk. However, Sampson *et al.* (1942) failed to produce zinc toxicity in the growing pig during the latter stages of development.

The occurrence of parakeratosis has prompted considerable experimental investigation of the required amount of zinc in the diet of the pig (Stevenson *et al.*, 1956; Lewis *et al.*, 1956; Hoekstra *et al.*, 1956; Lewis *et al.*, 1957; Luecke *et al.*, 1957). It appears that both calcium and zinc are involved in the etiology of this nutritional disorder. At any rate zinc salts are being routinely added to practical swine rations.

The purpose of this study was to ascertain the level of zinc tolerance and to characterize zinc toxicity in the weanling pig. The interaction of high dietary levels of zinc and calcium, and the action of high dietary levels of zinc on the blood clotting time were also investigated.

Experimental

Three experiments were conducted with weanling pigs. A description of the diets used is given in table 1.

Thirty-six weanling pigs of various breeds were used in Experiment 1. Outcome groups of six pigs each were formed on the basis of breeding, weight and general condition. The pigs were allotted at random to the zinc treatments from the outcome groups and self-fed *ad libitum* in treatment groups for 42 days in concrete pens. The zinc treatments imposed are given in table 2.

In Experiment 2, 40 weanling pigs of various breeds were used. Outcome groups of five pigs each were formed on the basis of breeding, weight and general condition. The pigs were allotted at random to the zinc treatments from the outcome groups which, in turn, were allotted at random to the calcium treatments. The pigs were self-fed *ad libitum* in individual metal pens with wire mesh floors for 35 days. Calcium was added as 3% dicalcium phosphate which replaced an equal amount of yellow corn. The zinc treatments imposed and the calcium levels are given in table 3.

Sixteen weanling pigs of various breeds were used in Experiment 3. Outcome pairs were formed using the same criteria as in the two previous experiments. The pigs were allotted at random to the zinc treatments from the outcome pairs. Feeding methods and management practices were the same as in Experiment 1. The zinc treatments imposed are given in table 4.

All supplemental zinc was supplied as reagent grade zinc carbonate. Clotting time was measured by the method described by Howell (Wintrobe, 1946). Zinc determinations were performed by the method described in A.O.A.C.

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[2] The authors wish to acknowledge Commercial Solvents Corporation, Terra Haute, Indiana, for funds which made this study possible. We also wish to express appreciation to the staff of the College of Veterinary Medicine at the University of Illinois for assistance in this study.

TABLE 1. PERCENTAGE COMPOSITION OF DIETS FED

Experiment no.	1	2 and 3[a]
Yellow corn, ground	77.2	77.2
Soybean oil meal, solvent	20.3	20.3
Vitamin A & D oil (3000A–600D)	0.5	0.5
Trace-mineralized salt	0.5	....
Iodized salt	....	0.5
Ground limestone	1.0	1.0
Dicalcium phosphate	0.5	0.5
Chlortetracycline, 25 mg./lb.	+	+
Vitamin mixture #37[b]	+	+
Mineral mixture #1[c]	—	+

[a] Contained 46.9 ppm zinc, dry matter basis.
[b] Vitamin additions per lb. of diet were: Thiamine HCl, 0.50 mg.; riboflavin, 1.40 mg.; niacin, 5.0 mg.; calcium pantothenate, 4.5 mg.; pyridoxine HCl, 0.60 mg.; folic acid, 0.35 mg.; inositol, 90.0 mg.; p-amino-benzoic acid, 10.0 mg.; biotin, 0.03 mg.; vitamin B_{12}, 5.0 mg.
[c] $MgCO_3$, 623 mg.; $FeSO_4$, 182 mg.; $MnSO_4 \cdot H_2O$, 54.48 mg.; $CoCl_2 \cdot 6H_2O$, 18.16 mg.; $CuSO_4$, 18.16 mg.; NaF, 3.6 mg.; KI, 1.8 mg.

TABLE 2. PERFORMANCE OF WEANLING PIGS FED ZINC CARBONATE. EXPERIMENT 1

Lot number	1	2	3	4	5	6
Zn added, %	0	0.05	0.10	0.20	0.40	0.80
No. of pigs	6	6	6	6	6	6
Mortality	0	0	0	2	3	1
Av. time on test, days	42	42	42	36.3	33.2	29.5
Av. initial wt., lb.	39	38.5	40.3	38	39.2	36.2
Av. daily gain, lb.	1.56	1.62	1.56	1.24	0.77	0.26
Av. daily feed, lb.	4.35	4.59	4.61	4.05	3.36	1.44
Gain-feed ratio	0.36	0.35	0.34	0.31	0.23	0.18
Hemoglobin values, av.[a]	12.99	12.50	12.39	10.87	13.54	12.55

[a] Gm. per 100 ml. blood.

TABLE 3. PERFORMANCE OF WEANLING PIGS FED ZINC CARBONATE. EXPERIMENT 2.

Zn added, %	No. of pigs	Mortality	Av. time on test, days	Av. initial wt., lb.	Av. daily gain, lb.	Av. daily feed, lb.	Gain-feed ratio
Lot 1–0.0%							
Control	4	0	35	26.75	1.46	4.04	.36
Control+1% Ca.	4	0	35	26.00	1.29	3.35	.39
Av.	4	0	35	26.38	1.38	3.70	.375
Lot 2–0.05%							
Control	4	0	35	28.0	1.46	4.09	.36
Control+1% Ca.	4	0	35	26.50	1.16	3.17	.37
Av.	4	0	35	27.25	1.31	3.63	.365
Lot 3–0.10%							
Control	4	0	35	29.50	1.45	3.89	.37
Control+1% Ca.	4	0	35	26.75	1.48	3.83	.39
Av.	4	0	35	28.13	1.46	3.86	.38
Lot 4–0.20%							
Control	4	0	35	28.25	1.42	3.83	.37
Control+1% Ca.	4	0	35	26.00	1.22	3.31	.37
Av.	4	0	35	27.13	1.32	3.57	.37
Lot 5–0.40%							
Control	4	1	32.75	28.0	0.66	2.56	.26
Control +1% Ca.	4	1	26.75	26.75	0.90	2.81	.32
Av.	4	1	29.75	26.88	0.76	2.68	.29

Results and Discussion

The results of Experiment 1 are presented in table 2.

The control group and the groups fed 0.05 and 0.10% zinc responded about the same in average daily gain, average daily feed and gain-feed ratio. There was no evidence that feeding an additional 0.1% zinc exerted a toxic effect. The addition of 0.2% zinc proved to be toxic, since it depressed average daily gain and average daily feed. Also, there was some indication that the gain-feed ratio was depressed. Compared to 0.2% zinc, pigs fed 0.4% zinc exhibited a greater depression in the measures of performance. Pigs fed 0.80% zinc showed body weight loss and a severely depressed feed intake.

TABLE 4. PERFORMANCE OF WEANLING PIGS FED ZINC CARBONATE. EXPERIMENT 3

Lot number	1	2
Zn. added, %	0	0.40
No. of pigs	8	8
Mortality	0	3
Av. time on test, days	42	38.4
Av. initial wt., lb.	35.25	36
Av. daily gain, lb.	1.52	0.92
Av. daily feed, lb.	3.23	2.61
Gain-feed ratio	0.47	0.35

Figure 1. A. Hematoma in left axillary space of a pig fed 0.2% of added zinc. B. Congestion and hemorrhagic condition of the mesenteric tissue. C. Massive hemorrhage in left axillary space.

In the group fed 0.2% zinc, one pig died on the 15th day and another was sacrificed on the 31st day when it showed severe toxic reactions and death appeared imminent. Three pigs died on the next highest level of zinc: deaths occurred on the 15th, 19th, and 31st day of the test. At the 0.8% zinc level, one pig died on the 24th day and the remainder were sacrificed on the 29th or 31st day when they were in an extreme moribund condition.

Pigs fed 0.2% and 0.4% zinc exhibited similar toxic reactions. Typical ante mortem and post mortem gross lesions are shown in figure 1. In general, the pigs exhibited extensive hemorrhage in the axillary spaces. There was a marked gastritis with some ulceration. The intestine contained extensive hemorrhage and there was a marked catarrhal enteritis. The brain was congested and the lateral ventricles contained a large amount of free clotted blood. In addition, the spleen, lymph nodes, and viscera displayed a hemorrhagic condition. Most of the pigs exhibited an arthritic condition

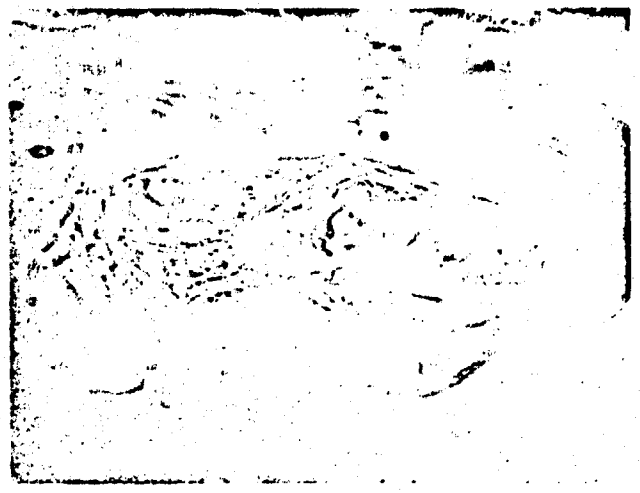

Figure 2. Congestion and swollen joint of pig suffering from zinc toxicosis.

characterized by swollen joints which, on post mortem examination, displayed extensive congestion. Figure 2 shows the syndrome observed.

Pigs receiving 0.8% zinc lost weight the first week of the experiment and started scouring on the third day. The feces were yellow and contained extensive quantities of mucous. Post mortem examination revealed the same general symptoms as described above with marked subcutaneous hemorrhage in the vertical thoracic region. During the post mortem examination it appeared that the blood clotting time was lengthened. Prothrombin time and calcium and phosphorus levels of the blood were studied. Two pigs

TABLE 5. ANALYSES OF VARIANCE OF EXPERIMENTS 1, 2 AND 3

Source of variance	d/f	Mean square: Daily gain	Daily feed	Gain-feed ratio
Exp. 1				
Treatment (T)	4	0.8484**		
Outcome groups (G)	5	0.1310		
T x G	20	0.0919		
Exp. 2				
Between diets (D)	1	0.1210	1.7514	0.0024
Groups within diets (G)	6	0.2826**	2.4327**	0.0038
Zinc levels (Z)	4	0.7158**	1.9192**	0.0230**
Z x D	4	0.0619	0.3658	0.0006
Z x G	24	0.0501	0.2822	0.0027
Exp. 3				
Treatment (T)	1	1.5751**		
Outcome groups (G)	7	0.1127		
T x G	7	0.0717		

** Significant at 1% level.

displayed a prothrombin time of 18 seconds, a somewhat shortened time, while the other three were within the normal range. Average calcium and phosphorus levels were 15.7 and 7.6 mg. %, respectively. These values also appear to be within the normal range according to Dukes (1955). Hemoglobin values (table 2) determined on blood taken the 21st day of the experiment appeared to be within the normal range according to Dukes (1955).

The amount of zinc in liver samples was studied. For five pigs fed 0.8% zinc the liver contained 2.26 mg. of zinc per gram of wet tissue. One pig each at levels of 0.2 and 0.4% zinc showed liver zinc values of 1.60 and 3.10 mg. of zinc per gram of wet tissue, respectively.

The analysis of variance, presented in table 5 shows that the treatments exhibited a statistically significant effect ($P<0.01$), upon the weight gains.

The results of Experiment 2 are presented in table 3. The analyses of variance presented in table 5 shows that the zinc levels exhibited a

statistically significant effect ($P<0.01$) upon average daily gain, average daily feed, and gain-feed ratio. The additional calcium did not exert a significant effect, although it may have alleviated the toxicity produced at the highest zinc intake. The performance of pigs fed 0.2% supplemental zinc or less was satisfactory. There was no mortality or evidence of zinc toxicity. However, pigs fed 0.4% supplemental zinc showed depressed average daily gain, feed intake, and gain-feed ratio. Furthermore, one pig died on the 13th day and another pig was sacrificed on the 24th day of the test. Both exhibited symptoms of zinc toxicosis described previously.

It may appear that the determined maximum zinc tolerance may differ in Experiment 1 and 2. In the initial study 0.2% supplemental zinc depressed performance; however, it failed to influence the results in the succeeding study. Perhaps the difference in daily feed intake between the two tests may explain the variation. At the level of 0.2% supplemental zinc, the total daily zinc intake was greater in Experiment 1 than in Experiment 2.

The results of Experiment 3 appear in table 4. This experiment was conducted to determine the effect of a high dietary level of zinc on the blood clotting time. Again, the performance was depressed when 0.4% supplemental zinc was fed. In addition, three pigs died of zinc toxicosis. The analysis of variance, presented in table 5 shows that the treatment exhibited a statistically significant effect ($P<0.01$) upon the weight gains. However, analysis of variance indicated that the blood clotting time was not affected by the addition of 0.4% supplemental zinc to the control diet.

Smith and Larson (1946) observed a relationship between zinc toxicity and dietary copper in rat studies. They deduced from their findings that supplementary copper partially prevents the anemia that develops on diets containing 0.7 percent zinc without preventing the growth depression. On the other hand, liver extract produced a marked growth improvement without significantly affecting the blood picture. Hence, the level of dietary copper may be an important element in the hematological aspects of zinc toxicity, but it appears unlikely that moderate variations in dietary copper would affect the level of zinc tolerance as indicated by growth.

Summary

Ninety-two weanling pigs were used to determine zinc tolerance and the characteristics of zinc toxicosis. Levels of zinc supplementation, as reagent grade zinc carbonate, up to 0.8% of the diet were fed for 42 days. Corn-soybean oil meal diets were used.

The addition of 0.1% zinc to the diet was the maximum level tolerated. Higher levels produced symptoms of zinc toxicosis. Toxic symptoms were characterized by depressed rate of gain, feed intake and efficiency of gain. In addition, arthritis, extensive hemorrhage in the axillary spaces, gastritis, catarrhal enteritis, congestion of the mesentery, and hemorrhages in the

ventricles of the brain, lymph nodes and spleen were observed. Feeding high levels of zinc frequently caused death within 21 days on treatment. Hemoglobin values determined on the 21st day of the experiment were unaffected by the levels of zinc fed.

Calcium added at 1% of the diet as dicalcium phosphate had no effect upon the level of zinc tolerance or upon the toxic reactions.

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ADDITIVE EFFECT OF CALCIUM AND PHOSPHORUS ON UTILIZATION OF DIETARY ZINC

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THE influence of dietary factors, especially calcium level, on zinc utilization has been the subject of many recent reports. An early report from this laboratory by Ellis (1953) demonstrated that swine parakeratosis was aggravated by high levels of calcium and phosphorus. Since that report, research has disclosed much about the role of zinc in this skin disorder in swine. The report of Stevenson and Earle (1956) and reports of other workers indicate that, in addition to the interrelationship between calcium and zinc, other factors are involved in the etiology of parakeratosis.

The known effects of potassium on skin disorders and the dramatic effects of low potassium levels on hair growth in rats shown in this laboratory (Dolnick *et al.*, 1963) have demonstrated that dietary level of this metal is under some conditions a factor in skin abnormalities in rats. From this background plans were developed to study effects of high and low levels of potassium, calcium, phosphorus and zinc on rat growth and hair development using an experiment of factorial design. As part of the experiment, studies were made of some effects of these variables on zinc metabolism as indicated by retention of zinc in hair, bone, liver and kidney. Observations were also made on effects of treatments on feed efficiency, weights of liver and kidneys, serum inorganic phosphate and serum alkaline phosphatase.

Experimental

Composition of the basal diet and of the basal mineral mixture used in two trials is shown in table 1. The mineral mixture was adapted from salt mixture No. 12 of Jones and Foster (1942), omitting the salts that provide calcium, phosphorus, potassium and zinc. These elements were added separately to the different diets in the form of various salts to provide the amount required to obtain the desired level of each mineral. To illustrate, the amount of salts added in the first trial to obtain the desired mineral levels is shown in table 2.

In making the necessary calculations, mineral content of the diet constituents was considered. Reagent grade salts were used for all compounds except for $CaCO_3$ which was USP grade. Zinc content of the mixed diets was determined by chemical analysis and is given in the last line of table 2. These analyses indicate that the zinc content of the basal diet without the zinc added as basal salt mixture was approximately 13 ppm. This is more than the N.R.C. (1962) requirement level of zinc for rat growth given as 12 ppm of the diet.

It has been shown by Forbes and Yohe (1960) that some of the zinc from isolated soy protein is not utilized by the rat. It was estimated that the soy protein used in these diets provided approximately 5 ppm of zinc. If all this zinc were assumed unavailable, the low zinc diets would still provide from 130 to 150 mcg. of zinc per rat daily, assuming a feed consumption of 10 gm.

Animals and Equipment. Albino male rats, 22 to 28 days of age and from a colony on a breeder-type ration, were used. They were kept in individual cages. All metal parts of equipment available to the animals including feed cups were stainless steel. Tap water, determined by chemical analysis to be practically free of zinc, was available from glass bottles and tubes. Constant temperature rooms were used.

Experimental Design. In each of the two trials 64 rats were allotted into eight outcome groups on the basis of age, weight and genetic origin. Each of the experiments was designed as a 2 x 3 factorial, consisting of eight treatment combinations.

In trial 1 calcium was held constant at 1.2% of the diets. The variables tested were phosphorus at 0.3 and 1.2%, potassium at 0.10 and 0.65% and zinc at 18 and 42 ppm. In the second trial potassium was held constant at 0.65%, the variables were calcium at 1.2 and 0.3%, phosphorus at 1.2 and 0.3%, and zinc at 18 and 42 ppm.

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TABLE 1. COMPOSITION OF BASAL MIXTURES

Ingredients	Percent
Basal ration	
Soybean protein [a]	23.0
Wood pulp [b]	2.0
Soybean oil	5.0
DL-Methionine	0.3
Vitamin mix [c]	3.3
Basal mineral mix	1.3
Dextrinized starch	q.s.[d]
Special mineral mix	q.s.
Total	100.0
Basal mineral mixture	
NaCl	38.150
$MgCo_3$	52.660
$FeSO_4 \cdot 7H_2O$	7.673
KI	0.229
$MnSO_4 \cdot H_2O$	1.145
$CuSO_4 \cdot 5H_2O$	0.138
$CoCl_2 \cdot 6H_2O$	0.005
Total	100.000

[a] ADM C-1 Assay protein; Archer-Daniels-Midland Company, Minneapolis, Minnesota.
[b] Solka-Floc; Brown Company, Berlin, New Hampshire.
[c] Vitamin diet fortification mixture in dextrose; Nutritional Biochemicals Corporation, Cleveland, Ohio.
[d] Amount required.

Blood samples were taken by heart puncture after the animals were anesthetized. The bones, livers, kidneys and hair samples were taken from the rats immediately after sacrifice.

Results

Growth Response and Feed Efficiency. Weight gains for 5 weeks on the diet treatments of both trials 1 and 2 are shown in figure 1. In this chart high mineral levels are denoted by chemical symbols, low levels by a dash. In trial 1 essentially normal weights were obtained when all minerals (calcium, phosphorus, zinc and potassium) were fed at the high levels. When dietary zinc was reduced to the low level (18 ppm), marked reduction in weight gains was evident. Feed efficiency also was significantly reduced. The lower phosphorus and potassium levels also reduced gains, but less so than the low zinc. However, low phosphorus and zinc together failed to reduce weight gains as much as did low zinc alone. Low potassium produced low weight gains in all four treatments, and in these treatment groups low zinc again reduced gains most in the presence of high calcium and high phosphorus.

In the second trial the variables tested were calcium, phosphorus and zinc. Potassium was held constant, since the higher potassium levels in the previous trial did not indicate increased zinc requirements because of high potassium. The first four treatments were similar in both trials, and the results are in good agreement.

The data on weight gains show that either high calcium or high phosphorus apparently increases the dietary requirement for zinc. The data show that, where both high calcium and high phosphorus were fed, there was a substantial difference in weight gains between high and low zinc groups, whereas when animals were on low calcium and low phosphorus, there was no significant difference in growth due to level of zinc (the first two vs. the last two bars, figure 1). In the other groups receiving either high calcium, high phosphorus, or both, differences due to zinc level are evident. Since the differences occur on both balanced and unequal levels of calcium and phosphorus, the depression in gain cannot be ascribed to an unfavorable calcium/phosphorus ratio, as suggested by Forbes and Yohe (1960).

Bone and Hair Zinc and Organ Weights. Figure 2 shows the results of chemical determinations of zinc in ash from left femurs. Composite samples from each of the 16 treatments of both trials were used. High dietary

TABLE 2. MINERAL SUPPLEMENTS TO PROVIDE DESIRED LEVELS OF Ca, P, K AND Zn (Trial 1)

Mineral Supplement	Diet Number							
	17	18	19	20	21	22	23	24
$CaHPO_4 \cdot 2H_2O$, %	3.00	3.00	3.00	3.00	....	....	....	....
$CaCO_3$, %	1.25	1.25	1.25	1.25	3.00	3.00	3.00	3.00
KH_2PO_4, %	2.10	2.10	....	....	....	....	....	....
ZnO mg./kg.[a]	5.85	35.10	5.85	35.10	5.85	35.10	5.85	35.10
$NaH_2PO_4 \cdot H_2O$, %	....	....	2.13	2.13	....	....	0.55	0.55
KCl, %	....	....	0.11	0.11	0.56	0.56	0.11	0.11
K_2HPO_4, %	....	....	....	....	0.70	0.70	....	....
Zn found in diet, ppm	18	40	19	41	16	40	18	45

[a] 5.85 mg./kg. of ZnO is the amount required to provide the zinc level of 4.7 ppm furnished by 4% of the Jones and Foster (1942) salt mixture.

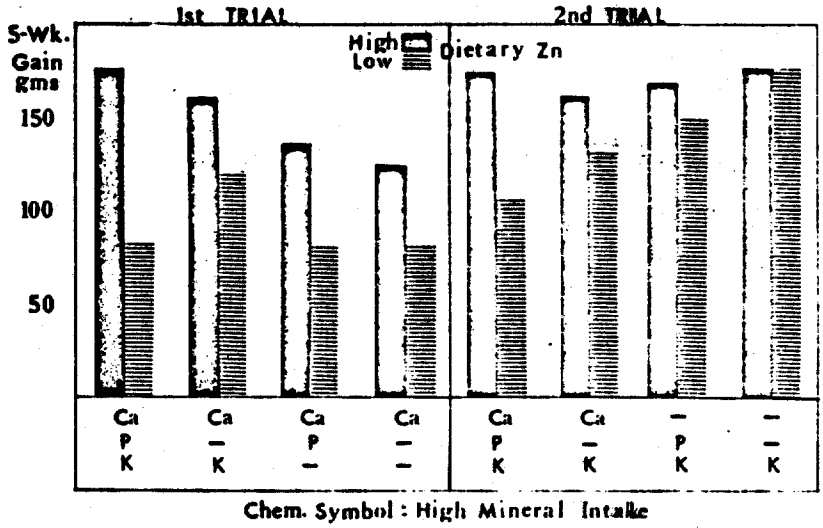

Figure 1. Five-week weight gains of the rats in trials 1 and 2. The sixteen bars illustrate effects of varying the four minerals in the sixteen treatment groups.

zinc (denoted by the solid bars) invariably resulted in higher level of bone zinc when other dietary minerals were the same. Results (as illustrated by the bar chart) suggest that low levels of calcium, phosphorus, or both, tend to produce higher bone zinc.

Retention of zinc in hair was less influenced by other minerals in the diet than the zinc in bone, but followed a similar pattern as to treatment effects. The differences in liver zinc were found to be even less marked, but here again the pattern of treatment effects was similar.

Treatment effects on organ weights from both trials were similar. Liver weights did not vary significantly. Kidney weights expressed as percent of body weight were not affected significantly by zinc level, but averaged higher with high dietary levels of phosphorus and lower with high levels of calcium and potassium.

Hair Coat and Skin Abnormalities. On the

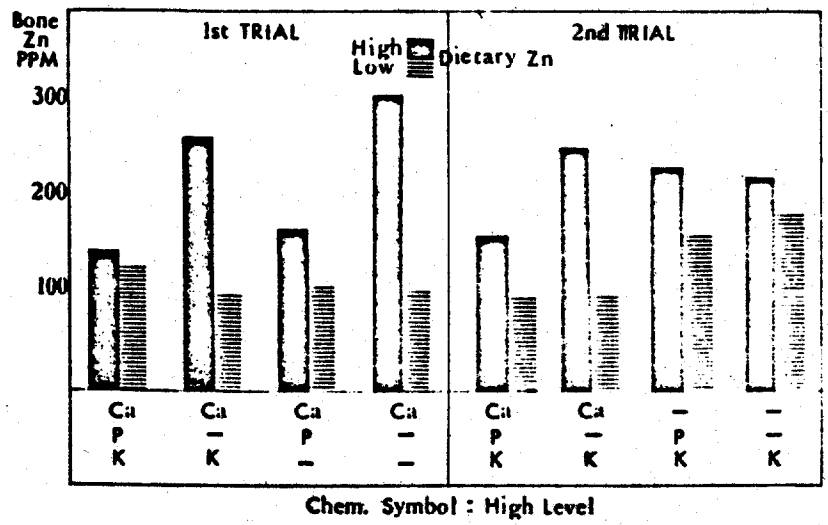

Figure 2. Zinc retention in fat-free dry matter of left femurs in parts per million as determined by chemical analysis. Treatment effects are illustrated for trials 1 and 2.

low potassium diets in trial 1 many of the rats showed the typical symmetrical denuding pattern (figure 3, A) that has been previously observed on this basal diet at variable levels of calcium and phosphorus with low potassium (Robins *et al.*, 1965). Low zinc, high calcium and high phosphorus increased the incidence and severity of this condition in the animals of trial 1 fed low potassium.

A third trial was conducted to test this effect further and to observe the specific action of the conditioned zinc deficiency on hair coat. Using the high-calcium, high-phosphorus, high-potassium diet, in which zinc was further reduced to 8 ppm, hair and skin abnormalities of several types were noted. Figure 3 (B) shows a typical shaggy condition compared with a normal animal receiving the same diet including an additional 20 ppm of zinc. Figure 3 (C) shows symmetrical pattern of hair loss on posterior dorsum and hips. Figure 3 (D) shows a typical denuding of hair and skin lesions. All of these animals except the one shown in figure 3 (A) were receiving the high potassium levels and zinc intake of approximately 80 mcg./day. These abnormalities are assumed to be a result of the conditioned zinc deficiency, because they are typical of zinc deficiency symptoms of rats that have been reported by others (Follis *et al.*, 1942), and they did not occur on diets having the higher level of zinc.

Blood Serum Studies and Statistical Treatment of Data. Alkaline phosphatase determinations made on composite samples from the first trial showed some differences among treatments. Alkaline phosphatase was 15% lower on the low zinc diet. Samples from individual animals were analyzed in the second trial. However, individual variations were large, and analysis of the data showed that treatment effects were not significant.

Results of analysis of the serum inorganic phosphate values from trial 2 are shown in table 3. Others have reported effects of zinc on serum phosphorus, but the data in this table indicate that no significant effects of zinc on serum phosphorus were obtained. However, the main effects on serum phosphorus of calcium and of phosphorus and the calcium x phosphorus interaction were highly significant.

Table 3 also includes the mean squares resulting from analysis of weight gains from trial 2. Much of the data was analyzed in this way, but space limits the presentation of

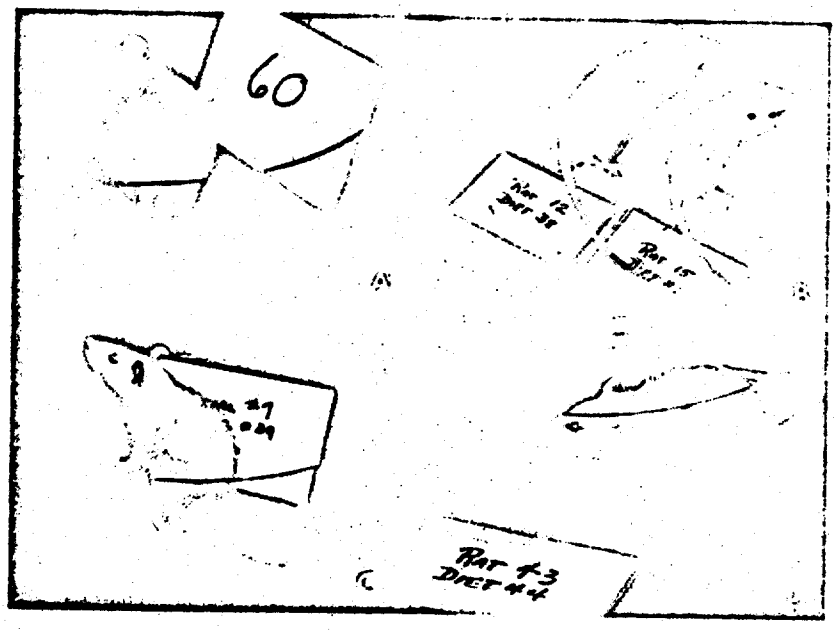

Figure 3. Effects of dietary mineral variation on hair coat.
A. Symmetrical pattern of denuding from low potassium.
B. Effects of high- and low-zinc intake, showing shaggy hair coat from zinc deficiency.
C. Symmetrical pattern of hair loss which occurs frequently in zinc deficiency.
D. Denuding and skin lesions typical of zinc deficiency.

TABLE 3. VARIANCE ANALYSIS OF WEIGHT GAINS AND SERUM INORGANIC PHOSPHORUS (TRIAL 2)

Source of Variation	d.f.	Mean squares: Weight gains	Mean squares: Serum inorganic phosphorus
Total	63	....	....
Blocks	7	175.57	0.49
Treatments	7	4,688.79**	7.63**
Ca	1	9,312.25**	23.94**
P	1	1,764.00*	18.15**
Ca x P	1	420.25	7.76**
Zn	1	12,210.25**	0.50
Ca x Zn	1	5,852.25**	0.77
P x Zn	1	2,756.25**	0.36
Ca x P x Zn	1	506.25	1.94
Error	49	258.46	0.67

* P<.05.
** P<.01.

this material. These data illustrate that a conditioned zinc deficiency can be produced by high levels of either calcium or phosphorus, and that the effect of these minerals is additive and independent. When the treatment effects are separated into individual degrees of freedom, the main effects of calcium, phosphorus and zinc are all highly significant. This is also true of the calcium x zinc and phosphorus x zinc interactions; however, the nonsignificant mean square for the calcium x phosphorus interaction denotes the independent effects of these minerals in the diets on weight gains.

Summary

Factorially designed experiments, each using 64 male albino rats, were used to investigate high and low levels each of four dietary minerals. The minerals and levels studied were zinc (18 and 42 ppm), calcium (0.3 and 1.2%), phosphorus (0.3 and 1.2%) and potassium (0.1 and 0.9%). Data for weight gains show that either high calcium or high phosphorus apparently increases the dietary requirement for zinc. Analysis of the data indicates that high calcium and high phosphorus independently cause a conditioned zinc deficiency and that this effect is additive. The higher levels of zinc produced increased zinc retention in bone, hair and liver at all levels and all combinations of calcium, phosphorus and potassium.

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ORAL ZINC SULPHATE IN LEG ULCERS

SIR,—Dr. Husain (May 31, p. 1069) shows that oral zinc sulphate in the treatment of chronic leg ulcers is well worth giving to patients in hospital. He states, however, that "it is difficult to predict the effect of zinc sulphate on ambulant patients". My experience with oral zinc sulphate as an adjunct to the treatment of chronic leg ulcers in ambulant patients leads me to believe that the same satisfactory results are obtained. For over two years I have prescribed 220 mg. capsules of zinc sulphate t.d.s. to all elderly patients treated for chronic leg ulcers. None have shown any untoward effects from the zinc sulphate and none have complained of nausea even on direct questioning. The ulcers appear to heal much more rapidly than ulcers treated with pressure bandages alone. Ulcers in a few patients who had been heavy drinkers healed remarkably quickly; I attribute this to their extra need of zinc, since patients with liver damage have been shown to be zinc-deficient.[10] Like Dr. Husain, however, I am wary of prolonged therapy until more is known of the long-term effects of added zinc.[11]

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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 113, 617–628 (1966)

Effects of Zinc on Adenosine Triphosphatase Activity and Superprecipitation of Actomyosin from Skeletal Muscle of Rabbit[1]

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Zinc activates the ATPase of actomyosin from skeletal muscle of the rabbit in the absence of other added divalent cations, at low ionic strength. Maximum activation in the presence of 1.0 m*M* ATP, pH 7.4, is induced by a Zn concentration of 0.05–0.08 m*M* (about 10^{-6} *M* free Zn^{++}). Under these conditions, 1.5 moles of Zn are bound per 10^5 gm of actomyosin. The effect of Zn depends on the interaction between actin and myosin, and has no apparent dependence on Ca^{++} even when the concentration of this cation in the assay medium is reduced to values lower than those required for activation of the Mg-stimulated enzyme.

Zinc and magnesium act synergistically if the concentration of either cation is insufficient to activate the ATPase maximally, but, in the presence of 1.0 m*M* ATP-Mg (plus Ca, 0.04 m*M*), Zn has only an inhibitory effect.

Stimulation of the ATPase activity of actomyosin by Zn in the absence of other added divalent cations is accompanied by superprecipitation, and the rate and extent of superprecipitation under these conditions approach those induced by Mg.

Comparison of the properties of the Zn-ATPase with those of the Mg-ATPase shows that the activity of the Zn-ATPase is less susceptible to KCl and pCMB than is the activity of the Mg-ATPase.

The effect of Zn on the ATPase activity of myosin at low ionic strength is similar to that of Mg inasmuch as low concentrations of Zn (<0.04 m*M*) are only slightly stimulatory and higher concentrations are inhibitory.

We suggest that the Zn^{++} and Mg^{++} cations interact with actomyosin at the same active sites of the enzyme, but that Zn is bound more firmly than Mg and holds the structure of the activated enzyme more rigidly.

The role of magnesium and calcium in muscle contraction and relaxation has become increasingly defined, but that of zinc has not been studied in detail, particularly with respect to the chemical basis of the effects of this cation on muscle activity. Such effects on skeletal muscle include potentiation of the tension developed during twitch (1, 2), relaxation of glycerol-extracted muscle fiber bundles (3–5), stimulation or inhibition of the ATPase activity of myosin (6–8), and inhibition of myofibrillar ATPase (3, 7).

The studies on the effects of Zn on the isolated contractile proteins have normally been conducted in the presence of Mg and Ca, and these cations have occluded the specific effects of Zn[2] (9). We therefore studied the effects of this cation, at low ionic strength, on the properties of the ac-

[1] This research was supported by grants from the N.S.F. (G19811 and GB-3088) and the Muscular Dystrophy Associations of America.

[2] After preparation of this manuscript, a recent report on the effect of Zn on myofibrillar ATPase activity in the absence of Ca and Mg came to our attention (32). The results reported corroborate in part our findings with actomyosin.

tomyosin and myosin from skeletal muscle in the absence of other added divalent cations. We also investigated the effects of Zn on the ATPase activity of actomyosin as modified by Ca, Mg, pCMB,[3] ATP, and KCl.

Our results show (a) that Zn substitutes for Mg in activating the ATPase activity and inducing superprecipitation of actomyosin suspensions; (b) that the Mg- and Zn-activated enzyme differs in its response to Ca, pCMB, and KCl; (c) that Zn and Mg at low concentrations act synergistically with respect to their activating effect on the ATPase of actomyosin; and (d) that the ATPase activity of myosin is only slightly influenced by concentrations of Zn which maximally stimulate the ATPase of actomyosin.

EXPERIMENTAL

Preparation of muscle proteins. Actomyosin of rabbit skeletal muscle was prepared from homogenized muscle extracted for 24 hours with Weber-Edsall solution. The procedure was essentially that described by Maruyama and Watanabe (10) except that KCl was used instead of tetramethylammonium, and EDTA was included only occasionally as specified in other sections.

L-Myosin was prepared by the procedure of Portzehl *et al.* (11).

Actin was obtained from acetone-dried powder of muscle (12) and purified according to the method described by Barany *et al.* (13).[4]

Measurement of ATPase activity. The rate of hydrolysis of ATP was determined from the inorganic phosphate liberated during a predetermined period of incubation (1–3 minutes). The amount of ATP hydrolyzed was less than 20% of the original ATP in the assay media. Under these conditions a rectilinear relationship exists between the amount of ATP split and the time of the reaction. The system, buffered at pH 7.4 in 16 m*M* tris, contained 1.2–1.5 mg of protein, and Zn, Ca, Mg, and KCl as indicated in the legends in a total volume of 5.0 ml. The reaction was started by adding 1.0 ml of an ATP solution whose pH had been adjusted previously to pH 7.4 with KOH and was terminated by adding 2.0 ml of 20% trichloroacetic acid. The suspensions were filtered immediately through Whatman No. 41 paper, and samples of the filtrates were analyzed for phosphorus (14). All procedures were carried out at 23°C.

In a series of experiments designed to correlate the ATPase activity with the amount of Zn bound by actomyosin, the protein concentration was about 2 mg per milliliter and the final volume was 8.0 ml. In these experiments the reaction time was reduced to 15 seconds.

The buffers used in the pH-dependence studies consisted of equimolar mixtures of solutions of tris and maleic acid adjusted with KOH to the desired pH. Calculation of the final concentrations of K in the assay media take into consideration the amount of K added with the buffers.

Superprecipitation. The rate and extent of superprecipitation of actomyosin at 23°C were determined from turbidity measurements of the suspensions made with a DU Beckman spectrophotometer at 350 mμ in 1-cm cells (15). A magnetic stirring device was adapted to the spectrophotometer to permit constant mixing of the suspensions. It consists of a small electric motor attached to the bottom of the cell compartment of the spectrophotometer to drive a magnetic bar inside the cell.

Analyses of zinc and magnesium were performed by absorption spectrophotometry (16) with a Perkin - Elmer absorption spectrophotometer, model 303. Preparation of the samples for analysis was as follows: assay media containing the same reagents as described for the measurements of ATPase activity were prepared in 12-ml centrifuge tubes. In this series of experiments the final volume was 8.0 ml and the concentration of protein was 2.0 mg per milliliter. Ten seconds after adding ATP to a final concentration of 1.25 m*M*, the tubes were centrifuged in an International clinical centrifuge at maximum speed for 90 seconds. Five ml of the clear supernatant fluid was collected and added to 1.0 ml of 20% trichloroacetic acid to ascertain that the supernatant fluid was protein-free. Samples of the supernatant fluid-trichloroacetic acid mixture were then analyzed for Zn, Mg, and inorganic phosphate.

The pellets obtained above were rinsed three times with distilled water, resuspended in 8.0 ml of 0.25 *M* sucrose adjusted to pH 7.4 with a few drops of 20 m*M* tris buffer, and centrifuged as before. The supernatant fluids were collected and analyzed for Zn and Mg. We found that the sucrose solution does not remove the bound Mg or Zn and provides a practical method to remove cations trapped with supernatant fluid in the pellets. The cations bound by the actomyosin were determined by atomic absorption spectros-

[3] Abbreviations used: pCMB, *p*-chloromercuribenzoate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetate; tris, tris-(hydroxymethyl)aminomethane.

[4] We are grateful to Dr. M. Barany for donating some of the actin preparations.

copy in trichloroacetic acid extracts (final trichloroacetic acid, 4%) of the pellets obtained before the sucrose washes or after one or two washes with sucrose solutions. The correction for the amount of cations in the second wash fluid trapped in the pellet was negligible when compared to the total amount of cations retained in the pellet.

Protein analyses were performed by the biuret method (17) standardized by Kjeldahl analyses of the nitrogen.

Special treatment of actomyosin and reagents. When it was essential to minimize the contamination of the assay media with Ca and Mg, the actomyosin and ATP were treated with Chelex-100 resin.

Eight ml of a solution of actomyosin (5 mg per milliliter) in 0.5 *M* KCl buffered in 35 m*M* tris was transferred to a 12-ml centrifuge tube, and about 3 gm of wet Chelex-100 resin in the K form was added. The suspension was mixed and allowed to stand for 5 minutes at 0°C. Centrifugation of the actomyosin-resin mixture in an International table centrifuge at maximum speed for 5 minutes separates the resin from the protein solution. The centrifugation procedure was repeated on the supernatant fluid containing the actomyosin.

In some experiments the resin treatment was omitted and 10^{-3} *M* EDTA was added to the actomyosin suspension, which was then washed exhaustively with 0.1 *M* KCl or dialyzed to reduce the concentration of EDTA to less than 10^{-7} *M*.

Treatment of the ATP was as follows: Fifty ml of 40 m*M* ATP, neutralized with 1 *M* KOH, was passed through 20–30 gm of wet Chelex-100 resin, in the K form, packed in a sintered funnel. This treatment decreased the Ca and Mg of the ATP solutions to below the limits of detectability by absorption spectrophotometry (10^{-6}–10^{-7} *M*). The concentration of ATP after this treatment was determined from its extinction coefficient of 259 mμ.

All other compounds were of reagent grade. Distilled water was further deionized by passing it through a Bantam demineralizer.

RESULTS

Effect of zinc on ATPase activity of actomyosin. Zinc activates the ATPase of actomyosin from skeletal muscle of the rabbit in the absence of added Mg or Ca. Figure 1 shows that Zn is at least as effective an activator of the enzyme at low ionic strength as Mg in the presence of 0.04 m*M* Ca. At Mg concentrations higher than 0.01 m*M* the Mg-activated ATPase requires Ca^{++} in concentrations of the order of 10^{-6}–

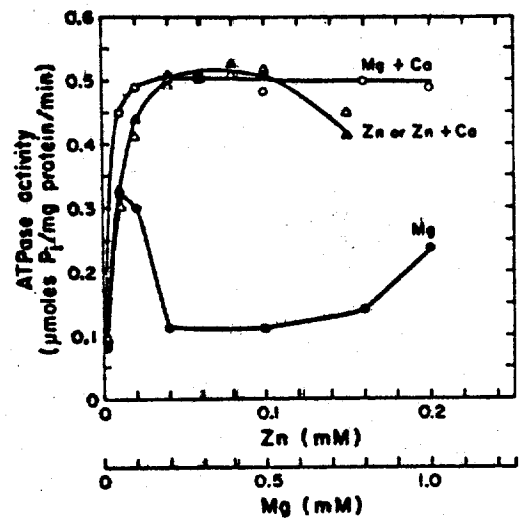

FIG. 1. ATPase activity of actomyosin at various concentrations of Zn or Mg with and without Ca. The assay media contained 47 m*M* KCl, 1.0 m*M* ATP, 0.3 mg of protein per milliliter, Zn or Mg as shown in the abscissa, and was buffered at pH 7.4 with 16 m*M* tris. When added, Ca was present in a concentration of 0.04 m*M*. The actomyosin was washed with 1 m*M* EDTA and the ATP was treated with Chelex-100 resin, as described in the text, to minimize the amount of Mg and Ca contaminating these substances.

10^{-6} *M* (24), but this cation appears to have no significant effect if Zn, in concentrations up to 0.2 m*M*, is the activating cation. Maximum activation by Zn in the presence of 1.0 m*M* ATP and 47 m*M* KCl at pH 7.4 occurs when the concentration of added Zn is 0.06 m*M*, and concentrations of Zn higher than 0.08 m*M* are inhibitory. This is in contrast to the activation of the enzyme by Mg plus Ca which remains constant between 0.1 and 1.0 m*M* Mg (Fig. 1).

Zinc also activates the ATPase in the presence of Mg if the concentration of the latter cation is insufficient to activate the enzyme maximally, but the degree of activation and the concentration of Zn required for maximum activity depend on the concentration of Mg. If the concentration of Mg is optimal for the enzyme, Zn acts as an inhibitor even at low concentrations (Fig. 2). The synergistic effect of Zn and Mg on the ATPase activity is shown in Fig. 2 for two suboptimal concentrations of Mg.

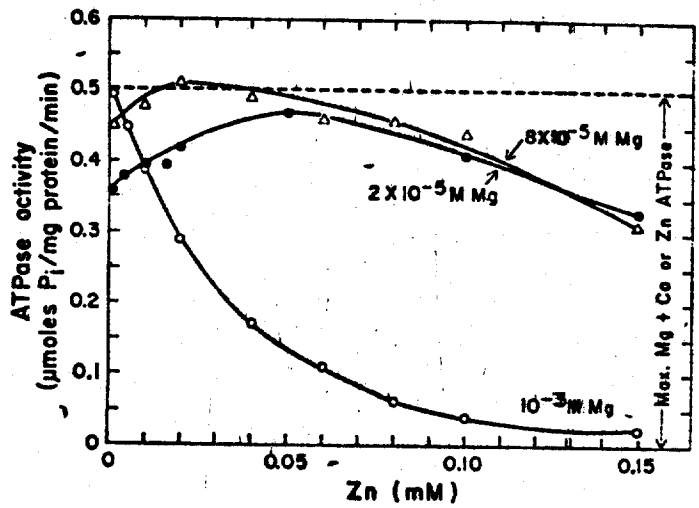

Fig. 2. The effect of Zn on the ATPase activity of actomyosin at various concentrations of Mg (plus Ca, 0.04 m*M*). The assay medium was the same as that described in Fig. 1, except for the concentrations of Zn and Mg which varied as indicated.

0.02 and 0.08 m*M*, which, in the presence of 0.04 m*M* Ca, stimulate the ATPase activity to 75 and 90%, respectively, of the maximum value obtained with optimum concentrations of either Mg, in the presence of Ca, or Zn alone (Fig. 1). The concentration of Zn required for maximum activation varies inversely with the concentration of Mg, and the results in Fig. 2 indicate that the two cations may be less effective as activators when present simultaneously in the assay medium than an optimum concentration of either cation.

We observed that at equimolar concentrations of Mg-ATP (1.0 m*M*), Zn has only an inhibitory effect on the ATPase activity. This latter observation has also been made by other investigators working with myofibrils (3, 7) and has led to the general conclusion that Zn is an inhibitor of the Mg-stimulated ATPase activity of the actomyosin complex. From our results, it appears that Zn can actually substitute for Mg in activating the enzyme, and inhibits it only under special circumstances. It should be noted that Mg also inhibits the actomyosin ATPase under certain conditions (18).

Effect of zinc on the ATPase of reconstituted actomyosin. The ATPase of actomyosin that is activated by Mg is a property imparted by actin on the myosin moiety of the protein complex. Myosin is also an ATPase whose activity is stimulated by Ca and inhibited by Mg. To decide whether the Zn-activated ATPase is a property of the actomyosin or of the myosin, we studied the effect of Zn on the rate of ATP hydrolysis by myosin and by actomyosin reconstituted from myosin and actin. The results of these experiments, summarized in Fig. 3, show that concentrations of Zn up to 0.04 m*M* have only a slight stimulatory effect on the ATPase of myosin and that higher concentrations of Zn are inhibitory. Adding actin to myosin in the ratio of 1:6 by weight increased the ATPase activity from 0.12 μmole P_i per milligram protein per minute, in the absence of added divalent cations, to 0.40 μmole P_i per milligram protein per minute in the presence of 0.04 m*M* Zn. Concentrations of Zn higher than 0.06 m*M* are inhibitory (Fig. 3).

We also tested the effect of cadmium on the enzymic activity of myosin and reconstituted actomyosin. The effects of this cation on the ATPase activity of the two proteins at low ionic strength are qualitatively similar to those induced by Zn, but Cd is only 30–40% as effective an activator of the ATPase of actomyosin (Fig. 3).

The above results make it apparent that Zn and, to some extent, Cd act like Mg

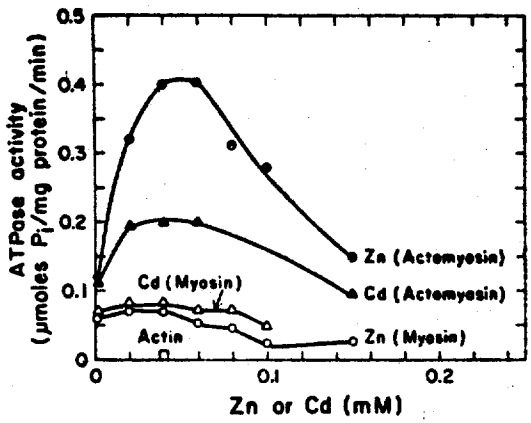

FIG. 3. Activation of the ATPase of myosin and of actomyosin reconstituted from myosin and actin by Zn and Cd. The assay media contained 40 m*M* KCl, 1.0 m*M* ATP, Zn or Cd as indicated in the abscissa, 0.3 mg of myosin/ml, and 0.05 mg of actin/ml when present. The system was buffered at pH 7.4 with 16 m*M* tris.

in increasing the rate of hydrolysis of ATP by actomyosin, but not by either myosin or actin. [Actin has no enzymic activity (Fig. 3).]

In contrast to the results reported here on the effect of Zn and Cd on the ATPase of myosin, there are those of Blum (6), who showed that these cations enhance the Ca-stimulated ATPase activity of myosin in the presence of 5 m*M* Ca at ionic strengths of about 0.3. We confirmed these results in preliminary experiments, but include in our report only those results obtained at low ionic strength and in the absence of added Ca.

Superprecipitation in the presence of zinc. The Mg-stimulated ATPase activity of actomyosin is closely associated with the phenomenon of superprecipitation (19). We therefore tested whether Zn, like Mg, induces superprecipitation of actomyosin at low ionic strength. Figure 4 shows the extent and rates of superprecipitation as measured by the change in optical density of actomyosin suspensions at 550 mμ. The Mg which normally contaminates actomyosin preparations and ATP salts was removed by Chelex-100 resin as described in a previous section. When we took this precaution actomyosin suspensions did not superprecipitate over a period of 5 minutes after adding ATP unless Mg and Ca, or Zn were also added. Magnesium in a concentration of 1.0 m*M*, in the absence of Ca, inhibited the slow superprecipitation which was normally observed after adding 2.0 m*M* ATP in the absence of added divalent cations (control) (Fig. 4) or when only Ca was present. The slow superprecipitation probably reflects contamination of the medium with trace amounts of Mg. Under these conditions Mg activates the enzyme in the absence of Ca, but not at the higher concentrations of Mg (10, 24).

Maximum superprecipitation in the presence of 1.0 m*M* Mg and 0.2 m*M* Ca requires 3–5 seconds after adding ATP (2 m*M*) and 5–10 seconds when Zn (0.06 m*M*) is the activating cation. There is no apparent dependence of superprecipitation on Ca when Zn is the activating cation. On the other hand, Mg induces superprecipitation only if Ca is also present, even though Ca

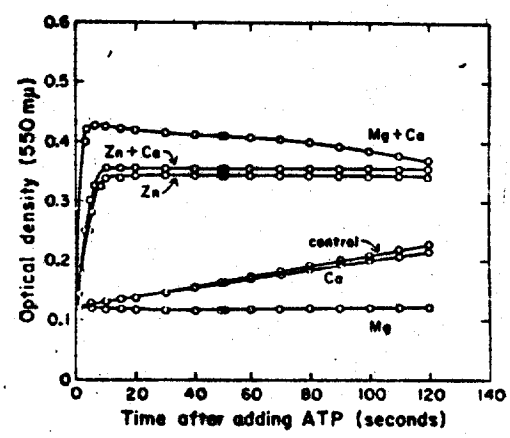

FIG. 4. Superprecipitation of actomyosin measured by changes in turbidity of actomyosin suspensions at 550 mμ. The media contained 0.5 mg of actomyosin/ml, 50 m*M* KCl, 16 m*M* tris at pH 7.4, and 1.0 m*M* Mg or 0.06 m*M* Zn in a final volume of 3.0 ml. Calcium, when added, was present in a concentration of 0.2 m*M*. The reaction was initiated by adding 0.2 ml of a 30-m*M* ATP solution with a microburet to the cell of the spectrophotometer, and the optical density of the suspensions was followed while the suspensions were constantly stirred. Both ATP and the actomyosin were pretreated with Chelex-100 resin to minimize the original concentration of Mg and Ca in the assay media.

alone has no effect on the state of the protein (Fig. 4). The extent of superprecipitation induced by Zn, as measured by changes in optical density and the volume of the pellet of centrifuged actomyosin after superprecipitation, appears to be less than that induced by Mg. Visual observation of the transformation in the actomyosin suspensions that takes place after adding ATP indicates that Zn supports the type of change seen when Mg is present.

Dependence of ATPase activity on KCl concentration. The Mg-stimulated ATPase activity is influenced markedly by the concentration of KCl in the medium (20). Figure 5 shows that the Mg-stimulated ATPase activity of actomyosin decreases rapidly as the KCl concentration of the assay medium is increased gradually from 30 to 130 m*M*. The ATPase activity in the presence of Zn is also dependent on the KCl of the medium, but in a manner distinct from that of the Mg-activated enzyme. Activation by Zn is maximal at a KCl concentration of about 70 m*M*, and the activity drops gradually as the concentration of KCl deviates from this value. At about 50 m*M* KCl, the Zn- and Mg-stimulated ATPase activities are similar under the conditions of the experiments (1.0 m*M* ATP and 1.0 m*M* Mg or 0.06 m*M* Zn at pH 7.4). At a KCl concentration of 130 m*M*, the Mg-stimulated ATPase activity drops to its lowest value, but the Zn-stimulated ATPase activity is still 0.4 μmole P_i per milligram protein per minute. We also observed that at this KCl concentration "clearing" of the actomyosin suspensions occurs with 1.0 m*M* Mg but not with Zn. Both "clearing" and superprecipitation could be easily detected visually, but in a series of experiments carried out at 130 m*M* KCl we also measured the turbidity of the suspensions. The optical density of the assay media containing Mg or Zn was 0.110 at 550 mμ for a protein concentration of 0.4 mg per milliliter before adding ATP. Adding ATP to a final concentration of 1.0 m*M* caused the optical density of the suspensions containing Mg (and Ca, 0.04 m*M*) to drop to an average value of 0.050, whereas the optical densities of the suspensions containing Zn increased to values which averaged 0.350. The decrease caused by KCl in the ATPase activity of the Mg-activated enzyme is shifted to higher KCl concentrations by pCMB (Fig. 5) (21, 22).

Maruyama and Gergely (23) demonstrated that "clearing" under the conditions of our experiments represents dissociation of the actomyosin into actin and myosin, Mg being specifically required for this phenomenon.

p-Chloromercuribenzoate vs. ATPase activity.

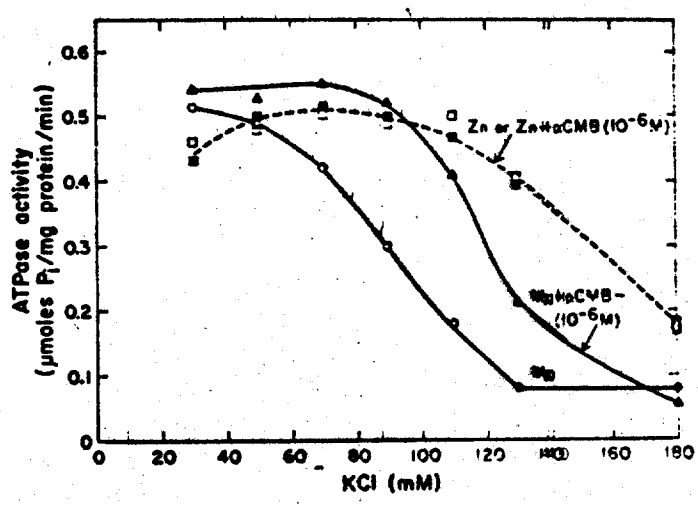

FIG. 5. The effect of KCl on the activity of Mg- and Zn-activated ATPase of actomyosin. The assay media contained 1.0 m*M* ATP, 16 m*M* tris at pH 7.4, 0.3 mg of actomyosin/ml, and 1.0 m*M* Mg (plus Ca, 0.04 m*M*) or 0.06 m*M* Zn. Potassium chloride and pCMB were added as indicated. ■ ■ ■ Zn; □-□-□ Zn+pCMB.

ity. Partial titration of the —SH groups of myosin increases the Ca-stimulated ATPase activity of myosin (6). Enhancement of the Mg-stimulated ATPase activity of actomyosin by pCMB was also reported recently (22). Our results on the effects of pCMB on the ATPase activity of actomyosin at KCl concentrations ranging from 30 to 180 m*M* confirm the latter observation, as was noted above, but we were unable to show a similar effect of pCMB on the Zn-stimulated ATPase (Fig. 5). Concentrations of pCMB higher than 10^{-6} *M* have only an inhibitory effect on the Zn-stimulated ATPase (Fig. 6). Thus, a pCMB concentration of 2×10^{-6} *M*, which produces a 200% increase in the activity of the Mg-stimulated ATPase, inhibits the Zn-stimulated ATPase activity by about 25% (Fig. 6). Since 1.5 mg of actomyosin was present in a total volume of 5.0 ml of the assay medium, it can be calculated that only 0.7 mole of pCMB per 10^5 gm of protein is sufficient to cause the observed effects. This implies that a maximum of only about one-tenth of the total available —SH groups of the actomyosin (7–8 —SH groups per 10^5 gm of protein) is blocked by pCMB when the changes noted above occur.

Activation of ATPase at various concentrations of ATP. The ATPase activity of actomyosin was determined when either 0.06 m*M* Zn or 0.06 m*M* Zn plus 0.5 m*M* Ca was added to the assay media at concentrations of ATP varying from 0.9 to 6.0 m*M*. A similar series of assays was carried out in which 1.0 m*M* Mg was substituted for Zn. A concentration of 0.5 m*M* Ca was used to ascertain that even at the highest concentration of ATP studied, the free Ca^{++} would be of the order of 10^{-5} *M*, since this concentration of free Ca^{++} is necessary for maximum activity of the Mg-activated ATPase of actomyosin systems (24).

The Zn-stimulated ATPase activity is only slightly affected by Ca^{++} at any of the ATP concentrations studied (Fig. 7). A Ca concentration of 0.5 m*M* in the presence of Zn is actually inhibitory for the enzyme at the lowest ATP concentration studied (0.9 m*M*), but lower concentrations of Ca, which at this ATP concentration

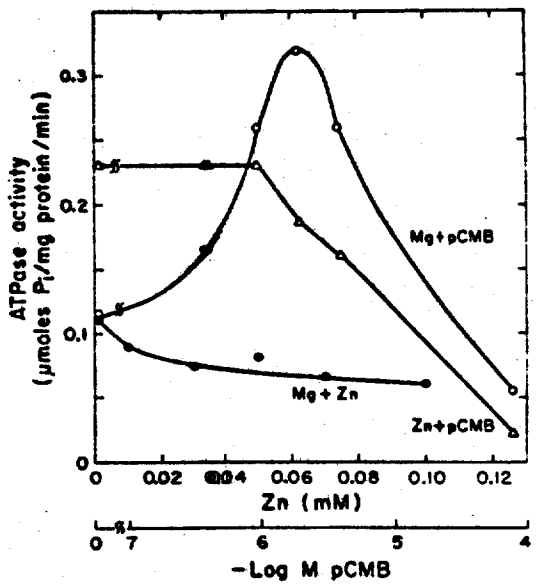

FIG. 6. Effects of varying concentrations of pCMB on the ATPase activity of actomyosin when the activating cation was either 1.0 m*M* Mg (plus Ca, 0.04 m*M*) ○-○, or 0.06 m*M* Zn, △-△. Also the effect of varying concentrations of Zn on the Mg-ATPase activity (Mg, 1.0 m*M*; Ca, 0.04 m*M*), ●-●. The assay media contained 1.0 m*M* ATP, 16 m*M* tris at pH 7.4, 100 m*M* KCl, 0.3 mg of actomyosin/ml, and divalent cations as indicated above.

are sufficient to reactivate the Mg-stimulated enzyme, do not influence the ATPase activity of the Zn-stimulated enzyme (Fig. 1).

The enzyme activity approaches a maximum value of 0.5 μmole P_i per milligram of protein per minute at 2.0 m*M* ATP and remains nearly constant up to ATP concentration of 4.0 m*M*. Higher concentrations of ATP inhibit the ATPase activity (Fig. 7). This inhibition occurs also in the case of the Mg-activated enzyme even when the concentration of free Ca^{++} in the medium is calculated to be at least 10^{-5} *M*, a value higher than that reported to be required for full activation of the ATPase activity of myofibrils in the presence of Mg and 8.0 m*M* ATP (18). Thus, the inhibitory effect of ATP at concentrations above 4.0 m*M* on the Mg- and Zn-stimulated ATPase activity of actomyosin presumably is a peculiarity of actomyosin preparations.

At an ATP concentration of 3.0 m*M*,

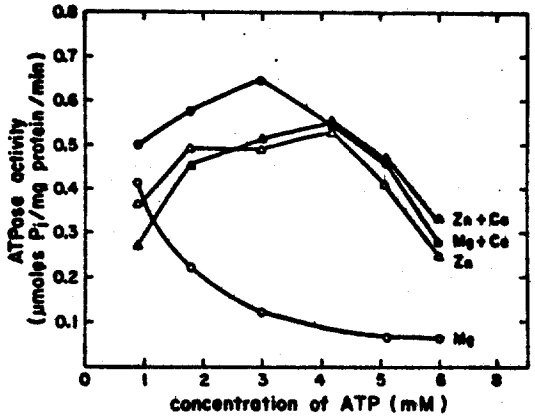

FIG. 7. Stimulation of the ATPase activity of actomyosin by Zn at various ATP concentrations. Results of the activation of the enzyme by Mg are also included for comparison. The assay media contained 16 m*M* tris at pH 7.4, 0.3 mg of actomyosin/ml, and either 0.06 m*M* Zn or 1.0 m*M* Mg. Calcium, when included, was present in a concentration of 0.5 m*M*. The concentration of ATP varied between 0.9 and 6 m*M* and the ionic strength was adjusted to 0.06 with KCl at the various concentrations of ATP.

the Mg- and Zn-ATPase activities (1.0 m*M* Mg or 0.06 m*M* Zn), before adding Ca, are 0.12 μmole P_i per milligram of protein per minute and 0.5 μmole P_i per milligram of protein per minute, respectively. Addition of 0.5 m*M* Ca increases the Mg-stimulated ATPase activity to 0.64 μmole P_i per milligram of protein per minute, but does not have any significant effect on the Zn-stimulated ATPase activity. The response to Ca^{++} in the case of the Mg-activated enzyme suggests that under the conditions of the experiments the medium was in fact depleted of free Ca^{++} when Mg was the activating cation presumably because ATP chelated the contaminating Ca which at lower ATP concentrations sustained the Mg-stimulated ATPase activity at a value of 0.4 μmole of P_i per milligram of protein per minute (Fig. 7). This makes it unlikely that the Zn-stimulated ATPase activity depends on trace amounts of Ca contaminating the assay media. The amount of contaminating Ca^{++} would be even lower at the higher ATP concentrations where we observed only a slight stimulatory effect of Ca on the Zn-activated ATPase.

pH-dependence studies. We studied the dependence of the ATPase activity of actomyosin on pH when Zn was the activating cation. For comparison, experiments were also carried out with Mg, and the results are included in Fig. 8. The ATPase activity of actomyosin is equally low for the two cations at pH values below 5 (0.05 μmole of P_i per milligram of protein per minute) and rises sharply between pH 5 and 6. The rate of rise is somewhat higher in the case of the Zn-activated enzyme so that at pH 6 the activity of the Mg-ATPase is about 0.3 μmole of P_i per milligram of protein per minute, whereas the Zn-ATPase activity is about 0.5 μmole of P_i per milligram of protein per minute. Between pH 6 and 7 the Zn-ATPase activity remains essentially constant at 0.5 μmole of P_i per milligram of protein per minute, whereas the activity of the Mg-ATPase increases gradually up to pH 7.4; at this point the activities of the enzyme stimulated by either cation are equal.

Above pH 7.4 the activity of Mg-stimulated ATPase is higher than that of the Zn-stimulated enzyme. In these alkaline regions competition of the hydroxyl ion for Zn probably becomes a predominant factor in determining the availability of the divalent cation to activate the enzyme.

Binding of zinc by actomyosin. Figure 9 shows the relationship between the concen-

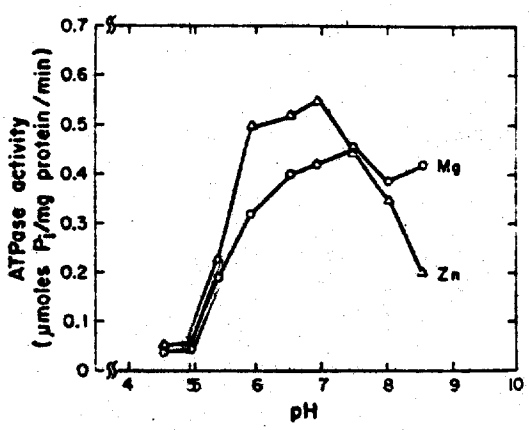

FIG. 8. Dependence on pH of the activity of the Zn- and Mg-ATPase of actomyosin. The assay media contained 10 m*M* tris-maleate buffer adjusted with KOH to the desired pH, 0.3 mg of actomyosin/ml, 50 m*M* KCl, 1.0 m*M* ATP, and either 0.06 m*M* Zn or 1.0 m*M* Mg plus 0.04 m*M* Ca.

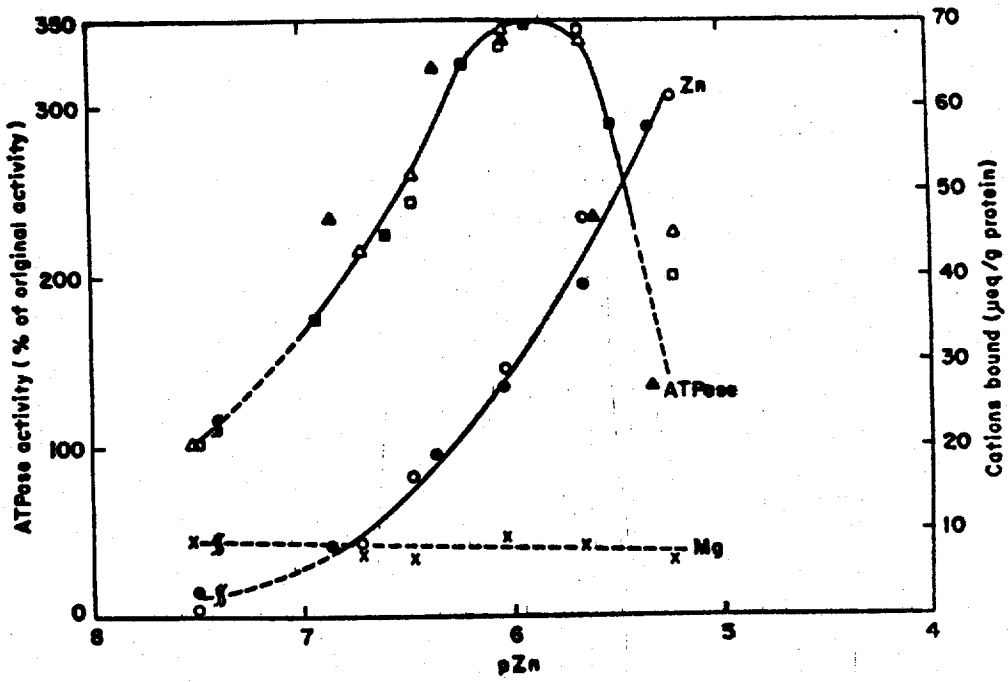

FIG. 9. ATPase activity, and binding of Zn and Mg by actomyosin at various concentrations of free Zn^{++} in the media. The assay media contained 50 m*M* KCl buffered in 16 m*M* tris at pH 7.4, 1.25 m*M* ATP, 2-3 mg of actomyosin/ml, and concentrations of 0–0.25 m*M* of added Zn in a total volume of 8.0 ml. The free Zn^{++} was calculated for pH 7.4 from the total Zn and the average concentration of ATP in the supernatant fluid with a pK_{ATP-Zn} value of 4.76 (30). Triangles and squares represent ATPase activity; circles and crosses represent Zn and Mg bound, respectively.

tration of free Zn^{++} in the assay medium, the amount of Zn bound, and the ATPase activity of actomyosin. When the concentration of free Zn^{++} is about 10^{-6} *M*, the ATPase activity is maximal and 15 μmoles of Zn are bound per gram of actomyosin. This value agrees closely with the amount of Mg bound by actomyosin at optimum activity when Mg is the activating cation.[6] The Zn-stimulated ATPase activity declines rapidly when the amount of Zn bound to actomyosin exceeds 20–23 μmoles per gram of protein.

The actomyosin preparations used in these studies had about 4 μmoles of bound Mg per gram of protein, which could not be removed by washing with 0.1 *M* KCl. This bound Mg was not displaced by Zn at the concentrations used in our studies and presumably is bound very tightly (Fig. 9).

Specificity of Zn for particular binding groups in proteins has not been observed, and sulfhydryl, imidazole, and carboxylic groups all appear to bind Zn to some extent (25–27). Of these groups, only imidazole has its p*K* value in the physiological range, and the dependence of the binding of Zn by myosin on H^+ between pH 6 and 8 suggests that histidine may be involved in the binding of Zn by this protein (7). Hotta and Kojima (7) reported that myosin has 19 Zn binding sites per 10^5 gm. This value is only slightly higher than the 12–16 histidyl residues found per 10^5 gm of myosin or actomyosin (28, 29).

From our results it appears that 1.5 moles of Zn bound per 10^5 gm of actomyosin induce maximum catalytic activity. This indicates that only a small fraction of the total histidyl residues need be associated with the active site of the enzyme if the effect of Zn is in fact mediated through its interaction with the imidazole group. Stracher (28) has recently shown that in the case of myosin only one histidyl residue per

[6] Unpublished observations.

2×10^8 gm of myosin is involved in the catalytic function of the enzyme.

A more detailed study on the binding of Zn by the functional proteins of muscle that would employ the approach of selectively blocking or destroying specific groups in the myosin and actin and observing the effect on the uptake of the cation by these proteins should yield useful information concerning the site(s) in the protein interacting with Zn.

DISCUSSION

Our results show that an enzymatic activity resulting from the interaction of myosin and actin is stimulated by Zn at low concentrations. A similar enzymatic activity of the actomyosin complex requires either low concentrations of Mg (<0.01 m*M*), or Mg and a concentration of Ca^{++} of the order of 10^{-5}–10^{-6} *M* (24). We cannot decide conclusively from our studies whether Zn and Mg interact with the same sites to modify the active center of the actomyosin, or whether the two cations have different loci of interaction with the protein, but cause similar configurational changes around the active center of the enzyme. Zinc and Mg may also interact with ATP to transform the molecule into the proper substrate for the enzyme, i.e., ATP-Mg and ATP-Zn complexes are the true substrates. There is evidence that the ATP-Mg complex is in fact the substrate for actomyosin (18), but we do not yet have similar evidence with respect to the ATP-Zn complex.

Of particular interest are the results indicating that activation of the ATPase and superprecipitation of actomyosin by Zn occur even when the concentration of Ca^{++} in the assay medium is lower than that required for activating the Mg-stimulated enzyme [about 10^{-6} *M* (24) (Figs. 1 and 4)]. The mechanism by which Ca^{++} suppresses the inhibition of the enzyme by Mg is not known, but it appears that the two cations compete for the inhibitory site(s) of the enzyme and that the requirement for Ca^{++} arises only at the higher concentrations of Mg^{++} (<0.01 m*M*) when this cation occupies that site(s) (10, 24). Zinc, at the concentrations investigated, either does not interact with the inhibitory site(s) of actomyosin (i.e., functions like Mg at low concentrations), interacts with the site and has no effect or the effect is simply a Ca-like action. However, since Ca^{++} is very effective in overcoming the inhibitory effect of Mg, we cannot exclude the possibility that remnants of Ca^{++} contaminating the assay medium may have in fact obscured a Ca^{++} requirement of the Zn-activated enzyme. If existent, such requirement must, however, be satisfied by a concentration of Ca^{++} lower than that needed to stimulate the Mg-activated enzyme (Figs. 1, 4, and 7).

Zinc, at concentrations at which it stimulates the ATPase activity of actomyosin maximally, has essentially no effect on the enzymatic activity of myosin, and at higher concentrations inhibits the enzyme (Fig. 3). In this respect Zn is similar to Mg. The effect of the two cations is also similar with respect to their ability to induce superprecipitation, but there exist also several differences between their effects on actomyosin: (a) The Zn-activated ATPase of actomyosin is less susceptible to increase in ionic strength than is the Mg-activated ATPase (Fig. 3); (b) The activity of the Mg-ATPase of actomyosin at ionic strengths between 0.05 and 0.15 is further increased by 10^{-6} *M* pCMB, but this concentration of pCMB has no effect on the ATPase when Zn substitutes for Mg and higher concentrations of pCMB are inhibitory (Figs. 5 and 6); (c) Magnesium, but not Zn, causes "clearing" of actomyosin suspensions under certain conditions.

Zinc binds to —SH groups of proteins (25) and presumably competes with pCMB for the —SH groups of actomyosin, but Zn is distinct from pCMB in its effects on the ATPase activity of this protein; thus, in contrast to Zn, pCMB alone (no divalent cations added) has only an inhibitory effect on the residual ATPase activity,[6] and Zn does not substitute for the mercurial compound in enhancing the ATPase activity of actomyosin when 1.0 m*M* Mg is present (Fig. 6). The inhibition of the Zn-stimulated ATPase activity by low concentrations of pCMB suggests that the sites which, when

[6] Unpublished observations.

blocked by pCMB, enhance the activity of the Mg-activated ATPase (22) (Fig. 6) are occupied by Zn. The added pCMB will then be available to interact with —SH groups of the enzyme that are vital for catalytic function. Edman (5) obtained evidence that Zn and the mercurial compound mersalyl interact with the same functional groups of glycerol-extracted muscle fibers, probably —SH groups. He observed, however, that the relaxing effect of Zn on glycerinated fibers depends on pH within the pH range of 6–8 and concluded that the effect of Zn is also mediated through an interaction with imidazole groups.

The relaxing effect of Zn on glycerinated muscle in the presence of Mg, as studied by Edman (3–5), presumably corresponds to our observation that Zn inhibits the ATPase activity of actomyosin if Mg is also present at concentrations which, in the absence of Zn, activate the enzyme maximally (Fig. 2). Zn alone, in sufficiently high concentrations, also inhibits the ATPase activity of actomyosin (Figs. 1 and 3) and relaxes glycerinated muscle fibers (3). The relaxation is mediated by the concentration of free Zn^{++} which depends on the concentration of ATP since this compound chelates Zn avidly (3, 30). We also observed a dependence of the ATPase activity of actomyosin on the concentration of free Zn^{++} (Fig. 9), but since the concentrations of ATP-Zn complex and the amount of Zn bound to actomyosin also increase with increasing free Zn^{++}, we cannot conclude how the free cation mediates the activation of the enzyme.

Presumably we are dealing with the same form of the active center of the enzyme whether the activating cation is Zn or Mg; it is unlikely that two different configurations of the enzyme act on the substrate. The activity of the Zn-ATPase is less susceptible to KCl and pCMB than the activity of the Mg-ATPase (Fig. 5). This suggests that the structure of the activated enzyme is more firmly established by Zn than by Mg as would be expected if Zn had a higher affinity than Mg for the binding sites of actomyosin involved in determining its catalytic center. As suggested in an earlier section, Zn probably competes with pCMB for the binding sites and is expected to compete also with K^+. The rise in activity of the enzyme between pH 5 and 7, when either Zn or Mg is the activator, suggests that the same ionizable group (or groups) of the enzyme participates in its catalytical center irrespective of which is the activating cation. Various lines of evidence suggest that this group (or groups) may be the imidazole of histidine (5, 7).

Activation by Zn of the ATPase of actomyosin and the ability of this cation to induce superprecipitation suggest that Zn can trigger the physiological function of the protein complex. However, in the presence of physiological concentrations of Mg, Zn has only an inhibitory action on the ATPase activity and presumably on contraction. Furthermore, only a small fraction of the total Zn in muscle (0.1–0.2 mmole per kilogram wet weight) is expected to be free. To know whether Zn normally plays a role in the contraction-relaxation cycle of muscle we need further knowledge of the concentration of free cations in muscle at rest and during contraction and an integrated understanding of the role of each cation.

The potentiation of twitch tension induced by Zn on skeletal muscle is apparently related to prolongation of the active state caused by this cation (1, 2); it appears to originate from interactions of Zn with sites at the surface of the muscle cell and presumably is unrelated to the action of Zn on the contractile proteins (31).

ACKNOWLEDGMENT

The authors are indebted to Doctors A. Sandow and A. Weber for many suggestions and stimulating discussions during the course of this work.

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A comparison of the toxicities of zinc and cadmium sulfates.

A note from Caujolle F., (16 December 1963).

P. H. Chanh, G. Mamy, F. Moulas, and L. T. N. Suong

The immediate toxicity from aqueous solutions of zinc and cadmium sulfates was studied with respect to Swyss mice (21-23 gm), Whystar rats (195-225 gm), rural crossed-race rabbit (2.62 kg ± 0.18 kg) and diverses races of dogs. According to manner of administration, the salts were dissolved in variable concentrations.

1. Mice and rats. Preliminary tests and mathematical analysis provided the definition of the characteristic doses to administer. The toxicity criteria selected were LD_{50} in 24 hours and the activity index. Approximate values were obtained by Kaerber's method; the values kept were refined by the probit method advanced by Bliss & Finney. It begins with graphical study and then makes use of the method of maximum probability allowing LD_{50} confidence levels relative to the 5% threshold.

The definitive experiments applied to 660 mice and 701 rats. The administrations always took place in the form of a single dose enterally ingested by means of the sophageal probe or slow ip. aseptic injection. The concentrations had been determined from the preliminary tests and are listed in table 1; the resort to two different concentrations for each manner of administration was necessitated because of the fan-like dispersion of appreciable magnitude in the case of enteral administration.

	zinc sulfate			cadmium sulfate	
	administered solution conc. mM/100 ml	No. of animals affected	administration.	administered solution conc. mM/100 ml	No. of animals affecte
MICE	20.0	110	esophagus	1.0	160
	3.0	240	ip.	0.1	150
RATS	100	160	esophagus	10	260
	10.0	140	ip.	0.1	150

The results obtained and compiled in table II show the following:

a. Contrary to conventional thought, zinc toxicity is higher for mice than for rats, whereas cadmium toxicity is appreciably the same for both species. Comparison of activity indices is significant.

MICE			RATS	
zinc sulfate	cadmium sulfate	characteristic results mM/kg	zinc sulfate	cadmium sulfate
		ESOPHAGUS ADMINISTRATION TESTS		
		24 hour maturity:		
2½	0.1400	maximum dose never lethal	5.00	0.040
3.77	0.3100	LD_{50} K & B	8.48	0.370
3.88	0.312	JD_{50} Bliss	8.65	0.602
(4.13-3.66)	(0.354-0.275)		(9.37-7.99)	(1.229-0.294)
5.50	0.600	minimum dose always lethal	15.00	1.600
		"Distal" terms:		
2½	0.140	infra-lethal dose	5.00	0.030
3.71	0.275	LD_{50} K & B	7.86	0.234
3.73	0.280	LD_{50} Bliss	7.98	0.200
(3.94-3.52)	(0.314-0.250)		(8.67-7.35)	(0.278-0.145)
5 days	4 days	crisis time	9 days	10 days
		IP. ADMINISTRATION TESTS		
		24-hour terms:		
0.05	0.0090	maximum non-lethal dose	0.65	0.0080
0.59	0.0133	LD_{50} K & B	0.80	0.0102
0.60	0.0129	LD_{50} Bliss	0.81	0.099
(0.69- .53)	(0.0141- 0.0119)		(0.87-0.76)	(0.0108-0.0090)
1.12	0.027	minimum dose always lethal	0.93	0.150
		"distal" terms:		
0.04	0.0090	infra-lethal dose	0.60	0.0080
0.26	0.0122	LD_{50} K & B	0.73	0.0097
0.31	0.0117	LD_{50} Bliss	0.74	0.0095
(0.45-0.21)	(0.0132-0.01014)		(0.80-0.69)	(0.099-0.0090)
14 days	4 days	crisis time	11 days	8 days

TABLE II: Zinc and cadmium sulfates toxicity.

b. Except for massive doses, the activity of both metals even when administered ip. is slow to manifest itself; the divergence between the activity index and LD_{50} for 24 hours is particularly pronounced in the rat when cadmium sulfate is administered through the esophagus.

Complementary tests were performed with rats of 350 ± 30 gm weight chloralized (chloralosé) with solutions (made isotonic by means of sodium chloride) of 20 mM/100 ml of zinc sulfate or 1 mM/100 ml of cadmium sulfate, for intravenous perfusion. Perfusion speed was such that death occurred within 50-70 minutes, the time of death being determined by electro-cardiography. Tests carried out with two series of 20 animals show that the lethal doses are 0.483±0.033 mM/kg and 0.025±0.002 mM/kg of zinc and cadmium sulfates resp.

	MICE		RATS	
Administration	zinc	cadmium	zinc	cadmium
ip.	20.50	39.44	48.91	33.71
esophagus	246.70	94.37	527.59	67.41

TABLE III: Bliss activity indices expressed in mg/kg of metal

2. RABBIT.

Intravenous perfusion of 10 mM% zinc sulfate solutions or of 1 mM% cadmium sulfate solutions rendered isotonic by means of sodium chloride and set for a pH of 7 ± 0.1 by means of diluted soda was performed at a rate causing death in about 60 minutes. The tests were performed on 11 urethanized rabbits and on 18 "vigil" ones.. The results show zinc is ten times less toxic than cadmium (test duration: 50-70 minutes

toxicity, mM/kg	zinc sulfate	cadmium sulfate
"vigil" rabbit	0.357± 0.066	0.036± 0.002
urethanized rabbit	0.331± 0.025	0.035± 0.005

TABLE IV

3. DOG

For the concentrations applied to the rabbits, the perfusions to the chloralized dogs (22 subjects of both races) yielded very distinct results despite the disparity of stock (weights from 6.6 to 26.6 kg); lethal doses in millimoles/kg are 0.330 ± 0.069 for zinc sulfate and 0.037 ± 0.003 for cadmium sulfate. These values show the ten-fold larger toxicity of cadmium.

One result shows that the immediate toxicity of zinc is higher for mice than for rats.

Another result concerning iv. administration shows that cadmium is approximately 10 times more toxic than zinc with respect to dogs and rabbits and 19 times more toxic with respect to rats.

Finally there are toxicity ratios of the same order (12 to 14) for enteral administration in rats and mice but they are appreciably different for ip. ingestion: in the latter case the toxicity ratio is 46 for mice and 82 for rats.

Such variations are representative of the difficulties of compared toxicology.

EFFECT OF ZINC ON OXYGEN UPTAKE, PHAGOCYTOSIS AND DEVELOPMENT OF PLISS' LYMPHOSARCOMA

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Zinc is a biotic element. When intake of this microelement is insufficient, there is diminished growth(mice and rats)[13,15,16,17], parakeratosis in swine[18]. Its entry into a series of metalloenzymes (carboanhydrase,carboxypeptidase,yeast alcohol dehydrogenase, glutamic dehydrogenase etc.)indicates its important significance in vital processes.

Our experimental data show that zinc can heighten oxygen uptake in microdosesincrease phagocytic activity in a series of animals [7,11].

Notice that cancerous tissue selectively incorporates zinc. In dense rat tumors the zinc content is 8times higher than in muscles tissue. In Brown-Pire tumors the zinc content is 3.2 times that of the lungs,6 times that of the liver and 2.9 times that of the kidneys [2,3]; there is always significantly more zinc in cancers than in adjoining tissue.[5,11,12]

As R. Ye. Kavetskiy [4] noted, during the growth of cancers there is a disruption in various types of metabolism,especially, a decrease in the level of uptake and carbohydrate metabolism changes. This illustrates that a problem in the origin of these generalized disruptions of carbohydrate metabolism and oxidative processes and their significance in the development of tumors remains open still.

The capacity of zinc to stimulate the intensity of metabolism and pgagocytosis in the rat was the object of our experiment. We attempted to establish: 1) whether there was a change in oxygen uptake in the rat after tumor growth; 2) if zinc affects oxygen uptake in rat tumors; 3)what effect this element has on phagocytosis in these rats; 4) what effect zinc has on growth and development of tumors and on the viability of the animals.

White rats weighing between 150--200 g were injected subcutaneously with Pliss lymphosarcoma obtained from the Leningrad Institute of Oncology, Academy of Medical Sciences USSR.

Zinc chloride in physiological saline solution was daily injected subcutaneously into the tumor, beginning 5--8 days after transplant when the tumor reached a size corresponding to about 1 g. The daily zinc dosage was 120 μg/kg.

A Miropol'skiy[6] apparatus was used to determine oxygen uptake. The obtained data was converted to 100.0 wt at normal pressure and a temperature of 0°. Phagocytic reaction was judged according to phagocyte number and phagocytic index. Neoplasm growth was observed by measuring the area of the growing tumor according to Ellis'[1] formula.

The raw data was analyzed by variational statistical methods.

OXYGEN UPTAKE: In a group of control rats (15 animals) oxygen uptake measured at 6,13,18, and 28 days from the start of observations, varied between 112--118 ml/hr/100 gms animal wt.

Oxygen uptake in cancerous rats (13 animals) in this period was, after adjustment: 86 ml, 97 ml, 92 ml, 72 ml, that is an average of 32.1% lower than in healthy rats (P=0.01).

EFFECT OF ZINC ON OXYGEN UPTAKE OF RATS WITH LYMPHOSARCOMAS: In these animals(10 rats) given zince at the beginning, as a rule, was increased oxygen uptake (21.8%) in comparison with cancerous rats that did not receive zinc (9 animals). But later there was a gradual decrease of this effect (fig. 1). On the 6th day of zinc injection, when oxygen uptake increase was at a maximum, there was a statistically reliable difference in indicators among the comparative groups (P =0.05).

LEUCOCYTE PHAGOCYTIC ACTIVITY: On the tenth day after transplant, there was observed in rats with sarcomas, increased leucocytic phagocytic activity in comparison with healthy animals (P=0.01). Thus, in animals(healthy) the following indices were observed: phagocytic index --4.92; phagocyte no.--68.3. In the tumorous the control phagocytic index was 7.46, and the phagocytic no.--75; in tumorous rats, given zinc, the phagocytic index was 7.59 and the phagocytic number 66.5.

On the 20th day after transplant the indicators were: cancerous control rats, phagocytic index--0.26 and phagocyte no.--9.2, in experimental animals given zinc, phagocytic index--0.79 and phagocyte no.--19 (P<0.001).

Thus, the determination of leucocyte phagocytic activity showed an increase in animals receiving zinc solution.

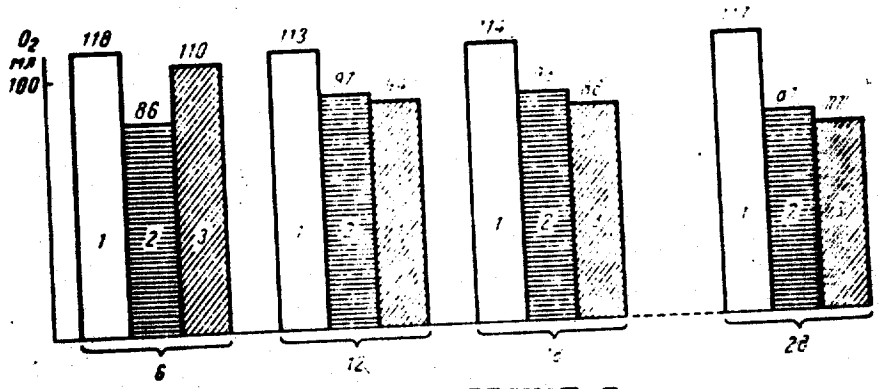
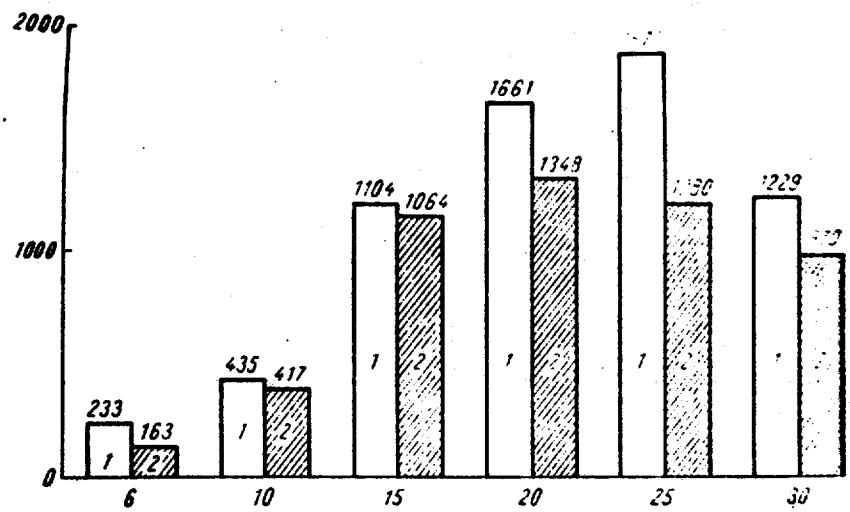


FIGURE I
EFFECT OF ZINC ON OXYGEN UPTAKE BY RATS WITH PLISS LYMPHOSARCOMA

1--healthy; 2--lymphosarcoma; 3--lymphosarcoma=zinc; 4--ordinate axis--in ml/hr/100 g at 0°C and 760 mm Hg st.; abcissa--day of determination

Phagocytic activity in the zinc-fed tumorous rats and in those surviving longest was higher than in healthy control animals. Thus, for example, in the first group indicated, the phagocytic index was 12.24 and the phagocyte no. was 88, in healthy animals the phagocytic index was 4.92 and the phagocyte no. 68.3(P=0.001).

FIGURE II.



EFFECT OF ZINC ON GROWTH OF PLISS LYMPHOSARCOMA IN RATS

1 average dimensions of tumor area in mm^2; 2--same for rats given zinc; Ordinate axis--area in mm^2; abcissa--day of measurement

Thus, by the tenth day in all tumorous rats ,there was noted some increase in phagocytic activity by blood leucocytes. On the 20th day after tumor transplant, it diminished both in control rats and in rats given zinc. However in rats given zinc the activity was somewhat higher than in control animals. Thus zinc stimulates the protective responses of the organism to some degree.

EFFECT OF ZINC ON TUMOR DEVELOPMENT AND RAT VIABILITY: To study tumor growth in both control and experimental rats we calculated the area of the neoplasm within 5--10 days and each 5--10 days thereafter. In the zinc rat group tumor dimensions were smaller on the average than in cancerous control animals (fig. 2) .Thus the average area by the 25th day for the zinc-fed rats(29 animals) was: 1080± 10.8 mm^2 and in the cancerous control animals (11 rats)-- 1870± 9.6 mm^2 (P=0.001).

Giving zinc heightens the viability of the animals. Thus, of 41 control rats ,4 survived (9.76%),in the zinc fed rats,38 of 86 rats survived (44.18%). This difference in viability was reliable (P=0.001). Animals given zince and surviving the first experiment with the lymphosarcoma were observed 6 months and then given a second injection of the same tumor.

In this group, consisting of 30 rats, the tumor grew in only 13 (43.3%),whereas in intact rats the tumor grew in 100%. Of the 13 rats infected and given zinc a second time,5 animals (37.5%) survived.

CONCLUSIONS

1. Rats with Pliss lymphosarcoma have a diminished oxygen uptake in comparison with healthy rats.

2.During daily subcutaneous injection of rats with zinc chloride in 120 μg/kg dose there occured: a)increased oxygen uptake in the first days

after transplant and b) inhibition of tumor growth, c)increased phagocytic activity by blood leucocytes and d) increased animal viability.

THE EFFECT OF ZINC ON OXYGEN CONSUMPTION, PHAGOCYTOSIS AND DEVELOPMENT OF PLISS LYMPHOSARCOMA

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In daily subcutaneous injections of zinc dichloride solution in a dose of 120 mg/Kg) in rats with the transplantable Pliss lymphosarcoma the following phenomena were observed in them in comparison with control tumor-bearing animals greater oxygen consumption during the first 5—10 days of zinc dichloride injections, the increase of the phagocytic activity, reduction of the tumor growth area, higher survival rate among these animals.

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Biochem. J. (1965) 95, 475

The Metal Ion Activation of the Alkaline β-Glycerophosphatase of Rabbit Small Intestine

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(*Received* 14 *July* 1964)

1. A fraction of intestinal epithelial cells from rabbit small intestine that contained nuclei and microvillus membranes served as a source of alkaline-β-glycerophosphatase activity. 2. The greater part of the enzyme activity could be released from the subcellular particles by disintegration of the latter, followed by centrifugation at 40000*g* and butanol extraction of the resulting sediment. 3. Further purification of the enzyme was achieved by diethylaminoethylcellulose chromatography and by gel filtration. 4. Dialysis of the purified enzyme preparations against EDTA gave an essentially inactive enzyme. High activity could be restored by adding $Zn^{2+}+Mg^{2+}$, $Zn^{2+}+Co^{2+}$, $Mg^{2+}+Co^{2+}$ or Co^{2+} alone to these inactive preparations. Neither Zn^{2+} nor Mg^{2+} added singly to the assay system restored more than a small part of the enzyme activity. 5. The optimum Zn^{2+} concentration was about 0·2–1m-equiv./l., whereas Mg^{2+} and Co^{2+} had optimum concentrations about 30–60m-equiv./l. 6. If added in excess of the optimum concentration, Zn^{2+} strongly inhibited the enzyme under all conditions tested. 7. In the presence of an optimum concentration of Co^{2+} (33m-equiv./l.) in tris buffer at the optimum pH (8·8 at 37°), K_m for the β-glycerophosphatase was 0·3mM.

A review of 30 investigations in which alkaline phosphatase has been assayed shows that in only four investigations published since 1940 has a magnesium salt not been included in the assay solution to provide the activating metal ion for the enzyme (excluded from this count are those assays where omission of magnesium salt constituted a 'control' on an assay in which magnesium salt was included). The investigations in which a magnesium salt was deliberately omitted from a routine assay medium stem from the work of Garen & Levinthal (1960) and are discussed below. Recommended assay procedures (Heppel, 1955; Morton, 1955*a*; Volkin, 1955) each involve the deliberate and obligatory addition of Mg^{2+} to the assay system. Only one of these authors (Morton, 1955*a*) mentions other activating bivalent metal ions but states categorically that added Mg^{2+} give maximum activation. J. W. Porteous & B. Clark (unpublished work) found that only 25–75% of the alkaline-β-glycerophosphatase activity of homogenates of epithelial cells of rabbit small intestine could be recovered from the isolated subcellular fractions of these cells when enzyme assays were performed in the presence of added Mg^{2+}. Satisfactory recovery of the activity among the subcellular fractions was achieved (Porteous & Clark, 1965) when the homogenates and the individual fractions were dialysed against water and assayed in the presence of added Co^{2+}. Accordingly the activation by bivalent metal ions of crude and partially purified preparations of the alkaline β-glycerophosphatase of the epithelial cells of rabbit small intestine was investigated. Preliminary accounts of the results have been published (Clark & Porteous, 1963*a*,*b*).

EXPERIMENTAL

Isolation and purification of enzyme preparations. Except where otherwise indicated all manipulations were carried out at 2–4°. Fraction III of homogenates of rabbit-intestine epithelial cells (Porteous & Clark, 1965) was dialysed against 5mM-EDTA, pH 7·4, for 2hr. and then for 16hr. against glass-distilled water adjusted to pH 7·4 with $NaHCO_3$. The non-diffusible material was called fraction IIID and used as a source of crude enzyme.

Enzyme preparations of higher specific activity were prepared from the combined subcellular fractions III and IV (Porteous & Clark, 1965) in the following way. The suspension (approx. 100ml. containing 3–10mg. of protein/ml.) was dialysed for 16hr. against glass-distilled water. The non-diffusible material was called preparation *a*. Preparation *a* was diluted to 200ml. with glass-distilled water,

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homogenized in an Ato-Mix Blender (Measuring and Scientific Instruments Ltd.) at full speed for 2min. and filtered through a small plug of glass wool. The filtrate (preparation *b*) was centrifuged ($2{\cdot}4\times10^6$g-min.) to give a sediment containing approx. 80% of the enzyme activity but only 30% of the protein of preparation *a*. The sediment was taken up in glass-distilled water (20–25ml.) to give preparation *c*. Part of this suspension (20ml.) was extracted with butan-1-ol (8ml.). The solvent was added dropwise with efficient stirring and the mixture heated in a water bath (37°) for 20min. After cooling in an ice bath the mixture was centrifuged at 1500g for 30min. The upper butan-1-ol layer and the interface layer of solid material were discarded. The lower aqueous phase was dialysed for 3hr. against glass-distilled water. The non-diffusible material (preparation *d*) was centrifuged ($1{\cdot}5\times10^6$g-min.) to give a water-clear supernatant (preparation *e*). The sediment was discarded.

DEAE-cellulose column chromatography. Preparation *e* was applied to a DEAE-cellulose column (1·5cm.×10cm.) that had been equilibrated with 0·05M-tris buffer, pH7·1. The column was then washed with 0·05M-tris buffer, pH7·1 (200ml.), before applying a linear concentration gradient of NaCl (0–0·7M) in 0·05M-tris buffer, pH7·1. The enzyme was eluted as a water-clear solution between 0·2M- and 0·4M-NaCl. The contents of the tubes containing enzyme activity were combined to give preparation *A*, which contained 31% of the activity of preparation *e* and showed a 50-fold increase in specific activity (enzyme units/mg. of protein) over preparation *a*. No further activity could be eluted from the DEAE-cellulose columns with NaCl concentrations up to 1·0M. The remainder of the activity could be eluted as a clear solution with 1·0M-NaCl containing 0·1% of sodium deoxycholate or sodium taurocholate. Removal of the deoxycholate or taurocholate by dialysis left a non-diffusible turbid enzyme preparation.

Gel filtration. For this 20g. of Sephadex G-100 (Pharmacia, Uppsala, Sweden) was suspended in 0·05M-tris buffer, pH7·1, and allowed to stand for 24hr. A column (2·2cm. diam.) was then filled with the suspension to a height of 55cm. and 0·05M-tris buffer, pH7·1, allowed to pass through the column for 16hr. Previously concentrated fraction *A* (1·5ml. containing 112 units of enzyme activity) was then applied to the column. Elution was carried out with 0·05M-tris buffer, pH7·1, at a flow rate of 6–12ml./hr. Fractions were collected every 20min. and assayed for enzyme activity. The contents of the tubes containing activity were combined to give preparation *S* (55 enzyme units). The water space external to the Sephadex beads (V_o) was determined by measuring the volume of buffer necessary to elute a diluted sample of indian ink applied to the top of the column.

Enzyme activity measurements. The following method was used for tissue preparations [fractions I–X described by Porteous & Clark (1965) and dialysed preparations of these fractions] and for relatively crude enzyme preparations such as *a*, *b* and *e* described in the present paper (Table 5). The enzyme was preincubated at 37° for 20min. in tris buffer (43 or 66mM) containing (unless otherwise specified) $CoSO_4$ (17mM). Sodium β-glycerophosphate was then added to give a concentration of 17mM and to bring the volume to 2ml. The final pH value was 8·8 at 37°. The reaction was terminated after 20min. by the addition of 50% (v/v) trichloroacetic acid (0·5ml.). The assay tubes were cooled in ice and the contents filtered after 10min. A sample of the filtrate (2ml.) was then analysed for inorganic orthophosphate by the method of King (1932). Control assay tubes from which substrate or enzyme had been omitted were incubated with each assay.

The same method was used for the assay of partially purified preparations (preparations *d*, *e*, *A*–*D* and *S*; Table 5) except that the tris buffer concentration was raised to 170mM and that deproteinization and filtration were found to be unnecessary and were omitted; enzyme activity was terminated by the addition of 2N-H_2SO_4 (1ml.). Ammonium molybdate (0·5ml. of a 5% solution) and 0·1% aminonaphtholsulphonic acid reagent (0·5ml.) were then added in succession. The tubes were left at room temperature for 20min. before measuring the extinction (Unicam SP.3 colorimeter; Unicam Instruments, Cambridge) with a red filter (Ilford no. 204).

Preliminary experiments were carried out to ensure that the liberation of orthophosphate was linear with respect to time of incubation and protein concentration, and that the assay was carried out at the optimum substrate concentration and pH value. The experimentally determined Michaelis constant (preparation *A*; Table 5) for β-glycerophosphate was approx. 0·3mM at the optimum Co^{2+} ion concentration (34m-equiv./l.) and at pH8·8 (37°). It was necessary to preincubate the preparation for 20min. at 37° in the presence of tris buffer and metal salt, but in the absence of β-glycerophosphate, to obtain maximum enzyme activity.

Variations in the composition of the enzyme assay system are described in the Results section and principally involve replacement of the $CoSO_4$ by other specified salts.

Enzyme units. One unit of enzyme activity was defined as that amount of enzyme which released 1μmole of inorganic orthophosphate when incubated for 20min. at 37° and at pH8·8 with sodium β-glycerophosphate (17mM) and $CoSO_4$ (17mM) in tris buffer (43–170mM).

Buffer solutions. Tris buffer consisted of tris base (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) adjusted to the required pH with HCl and diluted to the molarity indicated.

Determination of protein. Protein was determined by the method of Lowry, Roseborough, Farr & Randall, as modified by Miller (1959). Elution of protein from DEAE-cellulose columns was followed by measuring the extinction value of each fraction at 280mμ.

Determination of pH values. The pH measurements were made with a direct-reading pH-meter (model 23A; Electronic Instruments Ltd., Richmond, Surrey).

Reagents. DEAE-cellulose was prepared by the method of Peterson & Sober (1961), with 100–200 mesh Solka Floc (Brown Co., Boston, Mass., U.S.A.) as the starting material. The prepared DEAE-cellulose had pK_a 9·6 and a base content of approx. 0·7m-equiv./g. EDTA, aminonaphtholsulphonic acid and sodium β-glycerophosphate were laboratory reagents of high purity. Other chemicals used were of AnalaR grade. Glass-distilled water was used throughout.

RESULTS

Metal ion activation of crude alkaline β-glycerophosphatase. The effect of several bivalent metal ions on the alkaline-phosphatase activity of fraction IIID [fraction III of a homogenate of rabbit-

intestine epithelial cells (Porteous & Clark, 1965) dialysed against EDTA and water; see the Experimental section] is shown in Table 1.

Each metal salt was added to the enzyme assay system to give a final concentration of 17mM-salt (cation concentration of 34m-equiv./l.). At this concentration, Co^{2+} had the greatest effect on the activity of the enzyme; Ca^{2+}, Ba^{2+} and Fe^{2+} were each more effective than Mg^{2+}. The effects of different concentrations of added Co^{2+}, Ca^{2+}, Fe^{2+} or Mg^{2+} on enzyme activity are shown in Fig. 1.

At all concentrations of added cations (from 0·6 to 66m-equiv./l.) the enzyme activity was greatest in the presence of added Co^{2+}. Lower activities were obtained with Ca^{2+} and Fe^{2+}, and added Mg^{2+}

Table 1. *Metal ion activation of alkaline β-glycerophosphatase in fraction IIID*

The preparation of fraction IIID and the enzyme assay procedure are described in the Experimental section. The assay system consisted of fraction IIID (0·1ml. containing 0·[illegible]mg. of protein), tris buffer, pH 9·3 (43mM), sodium β-glycerophosphate (17mM) and one of the metal salts indicated (17mM) in a final volume of 3ml. The relative activity of 100 corresponded to the liberation of 0·8μmole of inorganic orthophosphate in the assay system after incubation at 37° for 20min. The specific activity of the enzyme in the presence of Co^{2+} (34m-equiv./l.) was 1·3units/mg. of protein. —, Not measured.

Metal salt added	Relative enzyme activity	
	Expt. 1	Expt. 2
None	15	8
Magnesium acetate	38	31
$CaCl_2$	73	75
$BaCl_2$	46	46
$Ni(NO_3)_2$	12	—
$CoSO_4$	100	100
$MgCl_2$	35	—
Zinc acetate	31	—
$CuSO_4$	19	—
$FeSO_4$	73	72

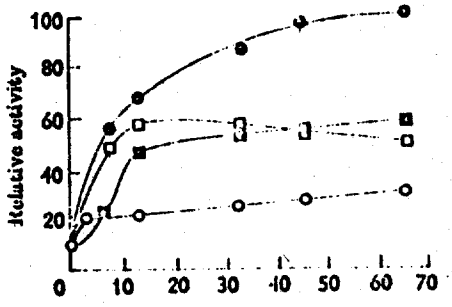

Fig. 1. Metal ion activation of the alkaline β-glycerophosphatase of fraction IIID (Table 5). The assay system was the same as that given in Table 1 except that the concentrations of $CoSO_4$ (●), $CaCl_2$ (□), $FeSO_4$ (□) and magnesium acetate (○) were varied as indicated. The relative activity of 100 for this enzyme preparation is defined in Table 1.

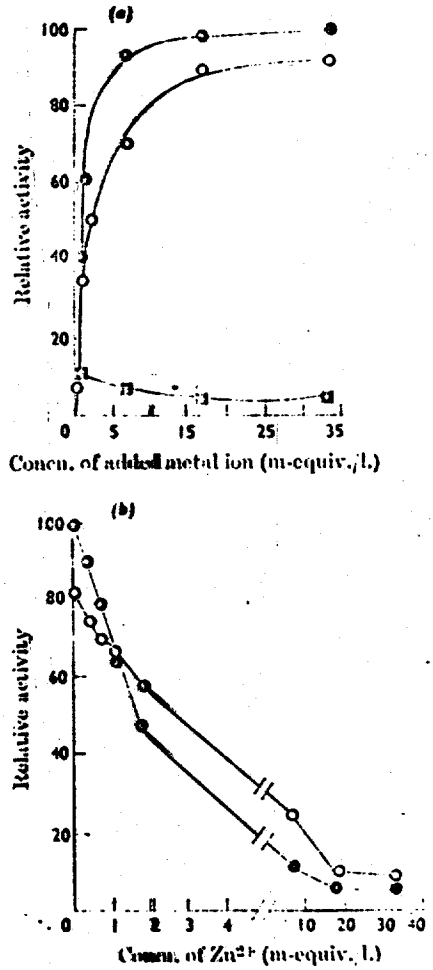

Fig. 2. Metal ion activation of the alkaline β-glycerophosphatase of preparation A (Table 5). Alkaline-β-glycerophosphatase activity was determined as described in the Experimental section except that 17mM-$CoSO_4$ was replaced by the salts indicated. The relative activity of 100 corresponded to the release of 0·93μmole of inorganic orthophosphate in the assay system. In the presence of added Co^{2+} (34m-equiv./l.) the specific activity of the enzyme was 57units/mg. of protein (cf. Table 1). Metal ion concentrations were varied as follows. (a): ●, Increasing $MgSO_4$ concentration; ○, increasing $CoSO_4$ concentration; □, increasing $ZnSO_4$ concentration. (b): ●, $ZnSO_4$ concentration varied in the presence of a constant concentration of $MgSO_4$ (17mM); ○, $ZnSO_4$ concentration varied in the presence of a constant concentration of $CoSO_4$ (17mM).

gave the lowest activities at all concentrations tested.

Metal ion activation of preparation A. Both Co^{2+} and Mg^{2+} activated this preparation but Zn^{2+}, added at concentrations up to 33 m-equiv./l., caused little activation of the enzyme (Fig. 2*a*). The enzyme activity in the presence of added Mg^{2+} rose to a maximum at a concentration of 33 m-equiv./l. The same activity was obtained with a Mg^{2+} concentration of 66 m-equiv./l. This maximum activity was assigned an arbitrary value of 100 and the activity of preparation *A* in the presence of various other metal ions expressed relative to this value (Figs. 2, 3 and 4, and Tables 2 and 3). Alkaline β-glycerophosphatase activity in the presence of added Co^{2+} reached a maximum value of 93, again at a concentration of 33 m-equiv./l. Enzyme activity in the presence of optimum Co^{2+} or Mg^{2+} (33 m-equiv./l.) was markedly decreased by further additions of Zn^{2+} (Fig. 2*b*).

Dialysis of portions of preparation *A* against various concentrations of EDTA at pH 7·4 gave preparations with enzyme activities shown in Fig. 3. After dialysis against 0·5 mM-EDTA, the addition of Co^{2+} to the previously determined optimum concentration gave an activity of 70, whereas Mg^{2+} in place of Co^{2+} gave an activity of only 5. Dialysis of preparation *A* against glass-distilled water for up to 3 days caused only a 10% loss of activity when assayed with Mg^{2+} and no loss of activity when assayed with Co^{2+}.

Preparation *B* was obtained by dialysing a large portion of preparation *A* against 0·5 mM-EDTA pH 7·4, for 4–16 hr. Preparation *B* was then assayed in the presence of added Co^{2+} and Mg^{2+} (Fig. 4). Co^{2+} (33 m-equiv./l.) gave an activity of 92; Mg^{2+} alone (0·7–250 m-equiv./l.) did not restore the activity of preparation *B*, but the further addition of Zn^{2+} (2 m-equiv./l.) restored the activity to 75% of its original value (Expt. 1 in Table 2). The alternative further addition of Cu^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+} (all at 2 m-equiv./l.) had little or no effect on the activity of preparation *B* in the presence of Mg^{2+} (66 m-equiv./l.). The change in activity with change in Zn^{2+} concentration in the presence of a fixed concentration of Mg^{2+} (66 m-equiv./l.) is shown in Table 2 (Expt. 2); almost complete (99%) activation was obtained by the addition of Zn^{2+} at 1 m-equiv./l. Higher concentrations of Zn^{2+} reversed this activation. Since a small amount of EDTA was present in preparation *B* (approx. 0·1 mM in the final assay) the apparent optimum Zn^{2+} concentration was probably higher than it would have been in the complete absence of EDTA. When added alone Zn^{2+} caused only a small activation of preparation *B* (Expt. 2 in Table 2).

Portions of preparation *B* were dialysed at 4° for 16 hr. against glass-distilled water to remove traces of EDTA and then against either 5 mM-zinc sulphate or 5 mM-cobalt sulphate at 4° for 8 hr. Any metal ions not tightly bound to the protein were removed by subsequent dialysis against glass-distilled water at 4° for 16 hr. The portion of preparation *B* treated

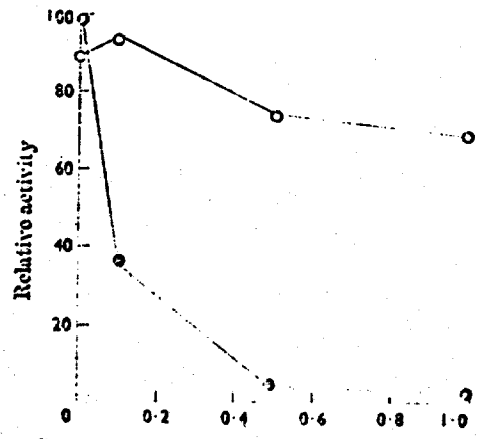

Fig. 3. Effect of dialysis (at 4° for 16 hr. against various concentrations of EDTA, pH 7·4) on the alkaline-β-glycerophosphatase activity of preparation *A* (Table 5). The activity of dialysed preparations was assayed: ○, in the presence of added $MgSO_4$ (17 mM); ●, in the presence of added $CoSO_4$ (17 mM). The assay system was that described in the text except for the variations in the added metal ions. The relative enzyme activity of 100 is defined in Fig. 2.

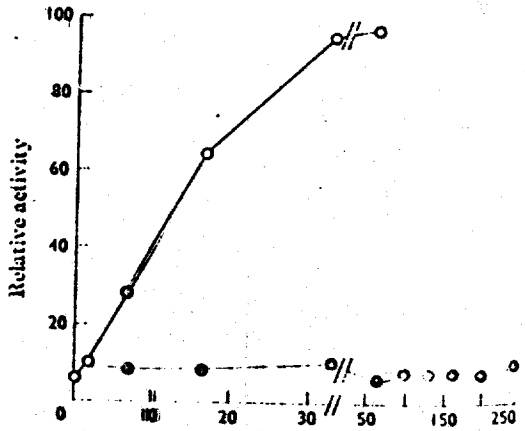

Fig. 4. Metal ion activation of the alkaline β-glycerophosphatase of preparation *B* (Table 5). The relative enzyme activity of 100 is defined in Fig. 2. The assay system was that described in the Experimental section except for the variations in metal ion concentration indicated in the Figure. ●, $MgSO_4$ added to the assay system; ○, $CoSO_4$ added to the assay system.

Table 2. *Metal ion activation of the alkaline β-glycerophosphatase of preparation* B (*Table* 5)

The assay system for Expts. 1 and 2 was that given in the Experimental section except that $CoSO_4$ was replaced by $MgSO_4$, $ZnSO_4$, $CuSO_4$, $CaCl_2$, $MnSO_4$ or $FeSO_4$ as indicated. The relative enzyme activity of 100 is defined in Fig. 2.

Expt. no.	Addition(s) (m-equiv./l.)	Relative activity
1	Mg^{2+} (66)	1
	Mg^{2+} (66) + Zn^{2+} (2)	75
	Mg^{2+} (66) + Cu^{2+} (2)	2
	Mg^{2+} (66) + Ca^{2+} (2)	1
	Mg^{2+} (66) + Mn^{2+} (2)	1
	Mg^{2+} (66) + Fe^{2+} (2)	1
2	Mg^{2+} (66)	5
	Mg^{2+} (66) + Zn^{2+} (0·2)	40
	Mg^{2+} (66) + Zn^{2+} (0·4)	96
	Mg^{2+} (66) + Zn^{2+} (1·0)	99
	Mg^{2+} (66) + Zn^{2+} (2·0)	81
	Mg^{2+} (66) + Zn^{2+} (4·0)	46
	Mg^{2+} (66) + Zn^{2+} (8·0)	18
	None	4
	Zn^{2+} (1)	16
	Zn^{2+} (2)	13
	Zn^{2+} (4)	28
	Zn^{2+} (10)	25

Table 3. *Metal ion activation of the alkaline β-glycerophosphatase of preparations* A, B, C *and* D (*Table* 5)

The assay system was that given in the Experimental section except that the added metal ions were those shown in the Table. The relative enzyme activity of 100 is defined in Fig. 2.

Metal ion(s) added (m-equiv./l.) Preparation...	Relative activity *A*	*B*	*C*	*D*
None	23	3	13	22
Mg^{2+} (33)	100	14	138	177
Mg^{2+} (33) + Zn^{2+} (1)	68	92	80	105
Co^{2+} (33)	77	130	108	148

with zinc sulphate was called preparation *C*, and that treated with cobalt sulphate preparation *D*.

With preparations *A*, *C* and *D* (but not with preparation *B*) Mg^{2+} added alone gave high activity (Table 3). The concomitant addition of Zn^{2+} decreased the activity of preparations *A*, *C* and *D*, but increased the activity of preparation *B*; both preparations *C* and *D* had higher activities in the presence of added Mg^{2+} or Co^{2+} than did the original preparation *A*. Preparation *D* with added Mg^{2+} gave the highest activity of all (177). When added alone Co^{2+} gave a high activity with each of the four preparations.

Sephadex G-100 filtration. A portion of preparation *A* was further purified by passage through Sephadex G-100. The V_o for this Sephadex column was approx. 55ml. The enzyme activity first appeared in the eluate when 56ml. of buffer had passed down the column. A further 20ml. of buffer was required to elute the remaining activity as a narrow symmetrical peak. The enzyme thus appeared to be completely excluded from Sephadex G-100, suggesting a molecular weight greater than 100000. The β-glycerophosphatase activity of the Sephadex G-100 eluate (preparation *S*) was taken as 100, with Mg^{2+} added to a concentration of 66m-equiv./l. This preparation was dialysed against 5mM-EDTA, pH 7·4, for 4hr. and then against glass-distilled water for 16hr.

The dialysed preparation *S* was assayed for activity in the presence of various concentrations of added Mg^{2+}, Co^{2+} and Zn^{2+}, as shown in Table 4. The addition of Mg^{2+} alone (66m-equiv./l.) to the

Table 4. *Metal ion activation of the alkaline β-glycerophosphatase of preparation* S (*Table* 5) *before and after dialysis against EDTA*

The assay system was that described in the Experimental section except for variations in the kind and quantity of added metal ion as indicated in the Table. Each appropriate assay vessel received 0·1ml. of enzyme preparation containing approx. 1μg. of protein. The relative activity of 100 corresponded to the liberation of 0·2μmole of inorganic orthophosphate in the assay system. The specific activity of the enzyme in the presence of Co^{2+} (33·3m-equiv./l.) was approx. 120units/mg. of protein (cf. Table 1 and Fig. 2).

Preparation	Addition(s) (m-equiv./l.)	Relative activity
S (undialysed)	Mg^{2+} (66)	100
S (EDTA-treated)	None	5
	Mg^{2+} (66)	10
	Mg^{2+} (66) + Zn^{2+} (0·07)	65
	Mg^{2+} (66) + Zn^{2+} (0·17)	92
	Mg^{2+} (66) + Zn^{2+} (0·33)	89
	Mg^{2+} (66) + Zn^{2+} (1·7)	85
	Mg^{2+} (66) + Zn^{2+} (3·3)	78
	Mg^{2+} (66) + Zn^{2+} (6·6)	58
	Mg^{2+} (66) + Zn^{2+} (16·6)	37
	Mg^{2+} (66) + Zn^{2+} (33·3)	12
	Zn^{2+} (0·17)	17
	Zn^{2+} (0·7)	16
	Zn^{2+} (3·3)	13
	Zn^{2+} (16·6)	7
	Zn^{2+} (33·3)	9
	Mg^{2+} (66) + Co^{2+} (0·7)	44
	Mg^{2+} (66) + Co^{2+} (1·7)	54
	Mg^{2+} (66) + Co^{2+} (3·3)	56
	Mg^{2+} (66) + Co^{2+} (6·6)	77
	Mg^{2+} (66) + Co^{2+} (16·6)	82
	Mg^{2+} (66) + Co^{2+} (33·3)	84
	Co^{2+} (1·7)	21
	Co^{2+} (6·6)	30
	Co^{2+} (33·3)	39

EDTA-treated preparation *S* gave an activity of 10, but the further addition of Zn^{2+} ions (0·17m-equiv./l.) gave 92% of the original activity. Higher Zn^{2+} concentrations inhibited the enzyme. Added Zn^{2+} alone did not markedly activate the enzyme. When added alone to the EDTA-treated preparation *S* Co^{2+} (33m-equiv./l.) gave an activity of 59. Increased concentrations of Co^{2+} in the presence of a fixed concentration (66m-equiv./l.) of Mg^{2+} gave greater increases in activity than did the addition of Co^{2+} alone. The activity was restored to 84 by the concomitant addition of Co^{2+} (33m-equiv./l.) and Mg^{2+} (66m-equiv./l.).

DISCUSSION

Our results indicate that addition of cobalt salt to crude cell homogenates and to isolated subcellular fractions that had been dialysed against EDTA or against water (Fig. 1 and Table 1; see also Porteous & Clark, 1965), or to various partially purified enzyme preparations (Figs. 2*a*, 3 and 4, and Tables 3 and 4), invariably gave high activity though not necessarily the highest activity obtainable. Partially purified enzyme preparation *A* that had not been dialysed against EDTA (Table 5) gave higher activity with added Mg^{2+} than it did with added Co^{2+} (Table 3 and Fig. 2*a*); preparation *B* (Table 5), on the other hand, was not activated by added Mg^{2+} but was activated by added Co^{2+} (Figs. 3 and 4) or by concomitant addition of Mg^{2+} and Zn^{2+} (Tables 2 and 3), provided that Zn^{2+} was added in low concentration.

We interpret these results to mean that partially purified preparation *A* (Table 5) contained the alkaline β-glycerophosphatase in the form of a metallo-enzyme or metal-enzyme complex (Vallee, 1955) that required the addition of a bivalent metal ion (Mg^{2+} or Co^{2+}) before high activity could be elicited. Treatment of preparation *A* with EDTA (Table 5) then removed any endogenous metal ion affecting the activity of the enzyme and gave essentially inactive preparations (Fig. 4 and Tables 2 and 3) to which full activity could be restored only by adding any pair of the metal ions Co^{2+}, Mg^{2+} and Zn^{2+} (provided that Zn^{2+}, if added, was present in low concentration) or by adding Co^{2+} alone (Figs. 3 and 4, and Tables 2 and 3). This interpretation is supported by the results of experiments in which attempts were made to remove residual EDTA from dialysed enzyme preparations and to equilibrate the presumably metal-free enzyme with Zn^{2+} or Co^{2+} (preparations *C* and *D* in Table 3) and by the results obtained (Table 4) before and after dialysis of preparation *S* (Table 5) against EDTA and water. The results obtained with crude enzyme preparations (Table 1 and Fig. 1) appear to be consistent with this interpretation of the requirement for bivalent metal ions for full activity of the enzyme under the conditions of assay employed.

Table 5. *Summary of the origin of various alkaline β-glycerophosphatase preparations from fractions III and IV of a homogenate of intestinal epithelial cells (Porteous & Clark, 1965)*

Full details of the preparations are given in the Experimental and Results sections.

Starting material	Treatment	Preparation
Fraction III	Dialysed against EDTA, then against water	III D
Fractions III+IV	Dialysed against water	*a*
a	Disintegrated and filtered	*b*
b	Centrifuged; retained sediment	*c*
c	Butanol extracted; aqueous phase dialysed against water; non-diffusible material retained	*d*
d	Centrifuged; supernatant retained	*e*
e	Eluted from DEAE-cellulose	*A*
A	Dialysed against EDTA	*B*
B	Dialysed against water, then against $ZnSO_4$ and once more against water	*C*
B	Dialysed against water, then against $CoSO_4$ and once more against water	*D*
A	Gelfiltration through Sephadex G-100	*S*
S	Dialysed against EDTA then against water	Dialysed *S*

Since other alkaline phosphatases have been shown to be zinc metallo-enzymes (Mathies, 1958; Trubowitz, Feldman, Morganstern & Hunt, 1961; Engström, 1961*a*,*b*; Plocke, Levinthal & Vallee, 1962) it is possible that the rabbit-intestinal alkaline phosphatase also contains zinc as an integral part of its structure; direct evidence for the presence of enzyme-bound zinc is lacking. Most of our results are consistent with this possibility, but it is clear (Tables 3 and 4) that added Zn^{2+} alone confers only low activity on a presumably metal-free enzyme preparation and is, further, a powerful inhibitor of the Co^{2+}-Mg^{2+}-, Co^{2+}-, Zn^{2+}-Mg^{2+}- and Zn^{2+}-Co^{2+}-activated enzyme (Fig. 2 and Tables 2, 3 and 4). It is also clear that the presumably metal-free enzyme cannot be activated by added Mg^{2+} alone (Tables 2, 3 and 4, and Figs. 3 and 4) under the conditions of assay used. That high enzyme activity could be obtained by adding Co^{2+} alone to presumably metal-free enzyme preparations [Figs. 1, 3 and 4, and Tables 1 and 3 (preparations *B* and *D*)] implies that this metal ion can fulfil the functions otherwise fulfilled jointly by a pair of bivalent metal ions (e.g. Mg^{2+} and Zn^{2+}) in the assay system employed. The

concentration of Co^{2+} required to elicit maximum phosphatase activity from the presumably metal-free enzyme was about 50m-equiv./l. in the absence of other added bivalent metal ions (Fig. 4) or in the presence of added Mg^{2+} (Table 4), whereas Zn^{2+}, at least in the presence of Mg^{2+}, was required at a concentration of 0·1–1·0m-equiv./l. (Tables 2 and 4). In the presence of endogenous metal ions (Fig. 2*a*) the optimum concentration of added Mg^{2+} (or Co^{2+}) was about 25m-equiv./l.

Porteous & Clark (1965) prepared a cell homogenate in a sucrose–EDTA medium, isolated subcellular fractions from the homogenate and dialysed each preparation against water. When alkaline-β-glycerophosphatase activity was assayed in the presence of added Mg^{2+} large discrepancies were observed (unpublished work) between the activity of the homogenate and the sum of the activities of the isolated subcellular fractions; the discrepancy was eliminated when assays were carried out in the presence of added Co^{2+} rather than added Mg^{2+}. Endogenous activating metal ions might have been lost in the form of metal ion–EDTA chelates during dialysis of the various preparations, but the discrepancy alluded to cannot be accounted for by a proportional decrease in the activity of each preparation assayed in the presence of added Mg^{2+}. The large amount of adventitious protein in the homogenate was differentially distributed among the subcellular fractions (Porteous & Clark, 1965). It is necessary to assume that endogenous activating metal ions were also differentially distributed during isolation of the subcellular fractions (or differentially lost during dialysis) in such a way that one or more of the fractions containing alkaline phosphatase retained none or a disproportionately small amount of the endogenous activating metal ions retained in the homogenate; it must also be assumed that activating metal ions retained in the dialysed homogenate and subcellular fractions were freely available to the enzyme under assay conditions. In the light of the preceding discussion incomplete recovery of activity among the isolated subcellular fractions would then be obtained when assays were performed in the presence of added Mg^{2+} alone, whereas assays performed in the presence of added Co^{2+} alone would give complete recovery of activity in the homogenate and in the isolated subcellular fractions.

Formation of a non-diffusible chelate with a high stability constant for the activating metal ion(s) should also inactivate the enzyme. It follows that β-glycerophosphatase might be activated or inhibited by adding, or by forming *in situ*, suitable ligands (including other proteins) to chelate with inhibitory or activating metal ions. A change in enzyme activity might then also depend on the period of incubation.

Other results on the activation of alkaline phosphatase. The present results are similar in many respects to those obtained by Cloetens (1941*a*,*b*) with a purified kidney β-glycerophosphatase that was inactivated by dialysis against cyanide and reactivated by added magnesium–zinc, magnesium–cobalt or magnesium mercuric salt mixtures (and to a considerably smaller extent by added cobalt salt alone). The detailed results were interpreted to mean that the enzyme possessed two metal ion-binding sites, each of which had to be occupied by appropriate metal ions to achieve full enzyme activity. Thoai, Roche & Roger (1947) claimed that an inactive and purified dog-intestinal alkaline phosphatase was activated (after preincubation with alanine) by adding only one of the following: Mg^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+} and Mn^{2+}. Thoai *et al.* (1947) and Roche & Thoai (1950) suggested that the single true metal ion activator of Cloetens' enzyme was made unavailable to the enzyme because a non-diffusible protein–cyanide–metal ion complex was formed; they further suggested that a second concomitant metal ion was required solely to displace the true metal ion activator of the enzyme from this complex. It might be argued that a protein–EDTA–metal ion complex was similarly formed; this interpretation of the mode of activation of a β-glycerophosphatase by more than one metal ion seems to be incompatible with the present results (see particularly those shown in Tables 2, 3 and 4).

Most claims that alkaline phosphatases were activated by only one (added) metal ion (usually Mg^{2+}) were made before it was realized that the enzyme from several sources contained zinc as an integral part of its structure (Mathies, 1958; Trubowitz *et al.* 1961; Engström, 1961*a*,*b*; Plocke *et al.* 1962; Plocke & Vallee, 1962; but see also Roche, Bouchilloux & Roger, 1948), and that this indigenous metal was required to activate the enzyme. Any requirement for a second activating metal ion may then depend on the conditions of assay employed. Thus Hove, Elvehjem & Hart (1940), working with crude enzymes, showed a dependence of metal ion activation on the kind of phosphate ester substrate employed; Morton (1955*b*) discussed the effect of extraneous amino acids on metal ion activation of alkaline phosphatases; Garen & Levinthal (1960) showed that purified *Escherichia coli* alkaline nitrophenyl phosphatase was fully active in the presence of Zn^{2+} and M-tris, pH 8·0, but was fully active in 0·01M-tris, pH 8·0, only if Mg^{2+} was also present (see also Plocke *et al.* 1962); Plocke & Vallee (1962) demonstrated that metal ion activation or inhibition of *E. coli* alkaline nitrophenyl phosphatase was partly dependent on the stability constant of the metal ion complex of the buffer components employed in the assay system.

The metal ions shown to activate various alkaline

phosphatases may fulfil one or more of the following roles: (*a*) bonding of the inactive enzyme to an organic activator (Hofstee, 1955) or displacement of an inhibiting metal ion from an organic activator; (*b*) stabilization of the enzyme (Gomori, 1952; Garen & Levinthal, 1960; Levinthal, Signer & Fetherolf, 1962); (*c*) occupation of a specific site in the structure of the enzyme (Mathies, 1958; Trubowitz *et al.* 1961; Engström, 1961*a*,*b*; Plocke *et al.* 1962; Plocke & Vallee, 1962; but see also Roche, Bouchilloux & Roger, 1948); (*d*) displacement of an inhibiting metal ion from the enzyme; (*e*) formation of a chelate or salt with the phosphate ester to give the true substrate (Mathies, 1958); (*f*) formation of a chelate with organic inhibitors (including some substrates), so indirectly activating the enzyme (Gomori, 1952), or displacement of a true activating metal ion from a concomitant inhibitor (Thoai *et al.* 1947; Roche & Thoai, 1950).

Situations (*a*), (*b*), (*c*) and (*d*) appear to be independent of the substrate. Situation (*a*) as originally proposed involved a low-molecular-weight activator that was specific for alkaline phosphatase (see also Abul-Fadl & King, 1949; Alvarez & Lora-Tomayo, 1958). Morton (1955*b*) came to the conclusion that there was no substantial evidence for the existence of such specific organic activators of alkaline phosphatase. Situation (*a*) could possibly be rewritten as: (I) promotion by a metal ion of the reversible dissociation of the active enzyme into inactive or partially active molecules (Reithel, 1963). Such metal ion-dependent reversible associations could conceivably account for several observations that the enzyme consists of several fractions (Gomori, 1952; Mathies, 1958; Engström, 1961*a*; Moss, Campbell, Anagnostou-Kakaras & King, 1961; Grossberg, Harris & Schlamowitz, 1961; Moss & King, 1962). Situations (*b*), (*c*) and (*d*) might be subsumed as: (II) promotion by a metal ion of a conformation of the protein that is necessary for enzyme activity. Situations (*e*) and (*f*) could be summarized as: (III) formation of chelates of metal ions with various molecules so as to permit or promote catalytic hydrolysis of phosphate esters, one or more of the chelates being involved in the mechanism of ester hydrolysis. Situations (I), (II) and (III) are not, of course, mutually exclusive.

The simplest interpretation of the results obtained in the present investigation would be that the rabbit-intestinal β-glycerophosphatase contains a bivalent metal (possibly zinc) as an integral part of its structure, and that a second bivalent metal ion (Mg^{2+} or Co^{2+}) is required to activate the enzyme under the conditions of assay employed; further, if the indigenous metal is removed from the enzyme then full activity may be restored by adding Co^{2+} alone (or by adding appropriate concentrations of any pair of the metal ions Zn^{2+}, Mg^{2+} and Co^{2+}) to the assay system used in the present work. If this interpretation is valid it seems reasonable to suppose that Zn^{2+} or Co^{2+} can fulfil the roles described in (I) and (II) and that Mg^{2+} or Co^{2+} can fulfil the role described in (III).

The Medical Research Council provided generous financial assistance for this and related investigations. We thank Professor W. O. Kermack for useful discussions. The technical assistance of Miss Joan McGill and of Miss Helen Brough at different stages of the work is gratefully acknowledged.

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ORAL ZINC SULPHATE IN LEG ULCERS

SIR,—Dr. Husain (May 31, p. 1069) is uncertain about the effectiveness of oral zinc-sulphate treatment in ambulant patients and the possible ill-effects of prolonged treatment. I have reported successful treatment of elderly patients with bedsores [9]; they received oral zinc sulphate, 220 mg. thrice or twice daily, and they all remained or became ambulant during treatment. In one case treatment continued for 106 days, and, since that report, zinc sulphate has been used "routinely" in this unit for appropriate cases. No ill-effects have been seen in the patients who have been treated. Abbott et al.[10] have given a preliminary report of a double-blind controlled trial of this preparation but their final results have not yet been published. Zinc sulphate seems to be a useful addition to the usual regimen for wound healing, and appears to be safe in the dose mentioned above.

Medo-Chemicals Ltd. was able to supply capsules.

Stracathro Hospital,
Brechin, Angus.

CHRIS. COHEN.

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Unusual Case of Poisoning by Zinc Sulphate

The following case seems worth recording because of the unusual sequelae. In the literature at my disposal I cannot find any description of a similar sequence of clinical events.

CASE HISTORY

An Indian woman aged 35 was admitted into the General Hospital, Johore Bahru, Malaya, at 9.30 a.m. on March 4, 1946. About one and a half hours previously she had taken a dose of "salts" for constipation, and this was followed in a few minutes by vomiting and purging. The substance swallowed was mistaken for Epsom salt, and approximately 1 oz. (28 g.) was taken. The bottle with the remaining noxious contents was brought to hospital, and the white crystals proved on analysis to be zinc sulphate. The vomitus contained the same substance.

On admission the patient was acutely collapsed and very restless. There was no abnormality of the lips, tongue, or buccal mucosa. Treatment with demulcents, morphine, and nikethamide produced marked improvement within twelve hours. On March 5 her general condition was satisfactory, and vomiting had ceased. There had been no haematemesis. She complained of abdominal pain of a colicky nature, but examination revealed no abnormality. The temperature was normal, there was a persistent tachycardia of 90 to 100 a minute, and examination of her urine showed no abnormality, chemically or microscopically. On the 6th progress was well maintained, and there was no blood in the stool. Next day the improvement continued, but she vomited two *Ascaris lumbricoides*. At about 8.30 a.m. on the 8th I was informed that the patient had suddenly collapsed. On examination she was found to be semi-comatose with a marked ketosis. Blood was sent for estimation of the sugar content, and a catheter specimen of urine for examination. Insulin therapy was immediately instituted, 60 units of the soluble product, of doubtful potency, being given intramuscularly, as the intravenous route was impossible owing to collapse. Nevertheless, the patient's condition continued to deteriorate and she died at 7.30 p.m. The blood sugar was 380 mg. per 100 ml., and the urine was loaded with albumin, sugar, and ketones.

Necropsy was performed at 2.30 p.m. on March 9; the report was as follows: "The body was that of a fat woman of average build. Rigor mortis was absent. The peritoneal cavity contained about 4 oz. (114 ml.) of blood-stained fluid; there was no evidence of fat necrosis. Liver: size normal; rather fatty. Gall-bladder: size normal; contained six faceted pigment stones. Spleen: normal. Kidneys: both intensely congested and haemorrhagic. Suprarenals: congested. Pancreas: size normal; generally congested; the tail was very intensely haemorrhagic. Stomach: very congested; mucous membrane eroded and, in places, desquamated; no perforation. Bowel: congested only; no perforation. Bladder: normal; contained about 2 oz. (57 ml.) of turbid urine which, on examination, showed copious albumin, sugar, ketones, and blood—microscopically there were numerous erythrocytes and epithelial cells. Pleural cavities: normal. Lungs: normal. Pericardial sac: normal. Heart: somewhat flabby; otherwise normal." Samples of pancreas, kidney, liver, and suprarenals could not be examined histologically, as facilities did not exist.

COMMENTARY

This seems to be a clear case of poisoning by zinc sulphate. Treatment of the original collapse was successful, and progress was well maintained until the fifth day, when hyperglycaemic coma developed. Unfortunately the circumstances precluded daily examination of the urine, otherwise pancreatic insufficiency and renal damage might have been noted earlier. Nevertheless, twelve hours before the onset of coma no premonitory symptoms or clinical signs of hyperglycaemia were observed. It is regrettable that histological examination of the tissues mentioned could not be carried out, but in view of the gross pathological findings at necropsy I feel that this case should be regarded as one of haemorrhagic pancreatitis and widespread renal damage as a result of poisoning by zinc sulphate, with hyperglycaemic coma and death supervening on the fifth day.

My thanks are due to Major S. N. Mittra, I.A.M.C., who carried out the post-mortem examination.

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Effect of Excess Dietary Zinc on Iron and Copper in the Rat

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Zinc toxicity in the rat is manifested by a hypochromic, microcytic anemia (Sutton and Nelson, '37; Smith and Larson, '46), poor growth (Smith and Larson, '46), reduction in liver catalase and in cytochrome oxidase (Van Reen, '53), depression of heart cytochrome oxidase (Duncan, Gray and Daniel, '53), a decrease in intestinal alkaline phosphatase (Sadasivan, '52) and an increase in liver and kidney alkaline phosphatase (Sadasivan, '52; Van Reen, '53). Since it was found in the above studies that copper supplementation would prevent the anemia and raise the liver catalase and liver and heart cytochrome oxidase to normal or greater than normal values, it has been generally accepted that zinc toxicity precipitates a copper deficiency.

Davis ('58) reported that by increasing the level of zinc in the diet a depression of the copper content in the liver occurs, but only when the copper levels in the diet approach those of borderline or normal of about 5 to 10 ppm. Duncan, Gray and Daniel ('53) reported that the copper content of the hearts of rats was 40% of the normal value when these animals were fed a diet containing 1.0% of zinc and which supplied 0.06 mg of copper per day for a period of 5 weeks. Dick ('54) found that 20 mg of zinc in the daily ration containing 30 mg of copper did not limit copper storage in the liver of sheep, but a significant limitation was found with 100 mg of zinc. Grant-Frost and Underwood ('58) recently reported that feeding a diet containing 0.5% of zinc and 2.1 ppm of copper to growing rats for 6 weeks reduced copper retention. They stated that zinc not only profoundly reduced copper concentration in the blood and tissues but probably also antagonized absorbed copper at the cellular level. They suggested, however, that a deficiency of some other dietary essential affecting hematopoiesis may be occurring as a consequence of the high zinc intake. From work on zinc toxicity of the rice moth, Sivarama Sasty and Sarma ('58) stated that the antagonistic effect of zinc and copper was a reflection of an interference on iron metabolism by zinc which may be a common phenomenon in zinc toxicosis. The present study was carried out to determine what effect a high level of zinc in the diet has on tissue iron and copper that might explain the mechanism of zinc toxicity.

EXPERIMENTAL

Weanling piebald rats of the Long-Evans strain, ranging in age from 21 to 24 days, were used. Males only were used for the time study and an equal number of males and females on each treatment in the other experiments. The rats were individually housed in wire cages with screen bottoms and received feed and distilled water ad libitum. The composition of the basal diet was as follows: casein, 20%; sucrose, 63%; non-nutritive cellulose,[2] 3%; corn oil, 10%; salt mixture, U. S. P. XIV, 4%; 900 I.U. vitamin A, 90 I.U. vitamin D, and 7.5 mg of α-tocopherol per 100 gm of the diet. Other vitamins were provided in the following quantities in milligrams per 100 gm of the diet: thiamine·HCl, 0.5; Ca pantothenate, 5.0; p-aminobenzoic acid, 5.0; riboflavin, 1.0; niacin, 5.0; choline chloride, 100.0; vitamin B_{12}, 0.002; inositol, 10.0; biotin, 0.03; folic acid, 0.03; and menadione, 0.75. The basal and high-zinc diets contained 4 to 5 ppm of copper.

The design of experiments 1, 2, 3 and 4 and the total number of animals on each experiment are shown in table 1. For the time study (table 3), 36 and 18 rats in trials 1 and 2, respectively, were maintained on the following diets: basal and 0.4% of zinc. The supplementary copper or iron was added directly to the dietary mixture. The animals were sacrificed after 3, 4 and 8 weeks on the experimental regimen for experiments 1, 2, and 3 and 4, respectively. In the time study, three rats from each diet were sacrificed after the following periods of time on the diets: weekly for 6 weeks for trial 1, and 1, 3 and 5 days for trial 2. The rats were stunned by a blow on the head and decapitated. With the exception of the animals in experiment 1, the liver and kidneys were perfused *in situ* by inserting a needle into the vena cava and allowing sodium chloride solution (0.9%) to flow through by gravity force until both organs had a pale fawn color. The liver and kidneys were excised, fat and connective tissue removed, rinsed with distilled water and dried to a constant weight at 100°C. The tibia bone was removed, fixed in formalin, decalcified with formic acid and sodium citrate and stained with hematoxylin and eosin.

The method of Peterson and Bollier ('55) was used for the analysis of copper. The zinc content of the tissues was determined by the dithizone method (Shirley et al., '49). The nitroso-R salt method of Sideris ('42) was used for the determination of iron. Hemoglobin level in the blood was measured by the acid hematin procedure and hematocrit by the conventional method.

The data were analyzed statistically and the multiple range test as proposed

Received for publication October 19, 1959.
[1] Published with the approval of the director as Journal Series Paper no. 74.
[2] Alphacel.

TABLE 1

Effect of excess zinc and added copper or iron on growth, hemoglobin and hematocrit

Diet	Mean weight gain	Hemoglobin	Hematocrit
	gm	gm %	%
	Experiment 1 (24)[1]		
Basal	69 ± 6[2]	12.7 ± 6	33 ± 6
0.6% Zn[3]	32 ± 7[4]	8.7 ± 0.5[4]	24 ± 1[4]
	Experiment 2 (40)		
Basal	87 ± 13		
0.5% Zn	59 ± 10[4]		
0.5% Zn + 0.02% Cu[6]	43 ± 7[4]		
0.5% Zn + 0.02% Fe[7]	62 ± 11[4]		
	Experiment 3 (30)		
Basal	165 ± 40	14.5 ± 0.7	39 ± 2
0.4% Zn	129 ± 35[5]	6.9 ± 1.2[4]	20 ± 5[4]
0.4% Zn + 0.01% Cu	126 ± 24[5]	13.0 ± 0.6	38 ± 2
	Experiment 4 (40)		
Basal	165 ± 34	14.9 ± 1.2	38 ± 3
0.4% Zn	129 ± 31[5]	9.1 ± 2.0[4]	28 ± 6[4]
0.4% Zn + 0.04% Fe[8]	122 ± 24[5]	10.7 ± 1.7[4]	31 ± 4[4]
0.4% Zn + 0.08% Fe	140 ± 25[5]	12.2 ± 1.4[5]	33 ± 3[5]

[1] Total number of animals on experiment. Animals were sacrificed after three, 4, and 8 weeks for experiments 1, 2, and 3 and 4, respectively.
[2] Standard error of mean.
[3] Added as zinc oxide.
[4] Significantly less than basal group, $P < 0.01$.
[5] Significantly less than basal group, $P < 0.05$.
[6] Added as $CuSO_4 \cdot 5H_2O$.
[7] Added as soluble ferric phosphate.
[8] Added as $FeSO_4 \cdot 7H_2O$.

by Duncan ('55) was used to test significance of the means.

RESULTS

Data demonstrating anemia and poor growth caused by three different levels of zinc intake are presented in table 1. In experiment 2, one rat fed the high-zinc diet and one fed the zinc plus iron died in two weeks and are not included in the data. In all experiments, animals fed the high-zinc diets had a poor growth rate ($P < 0.01$), which was not improved by the supplementary copper. Iron supplementation at the 0.08% level appeared to give some improvement of growth; however, this was not statistically significant. The experimental animals fed 0.6 and 0.4% of zinc developed anemia. Rapid blood clotting was experienced with the majority of blood samples obtained from the rats on experiment 2; consequently, insufficient data were obtained to give an accurate picture of the hemoglobin levels. The anemia was characterized by a marked reduction in hemoglobin ($P < 0.01$) and red blood cell volume ($P < 0.05$ for experiment 1 and $P < 0.01$ for experiments 3 and 4). Copper added to the diet containing 0.4% of zinc prevented the anemia. Supplementary iron (0.08%) only partially prevented the anemia.

Rats on the initial experiments did not have a change in the color of haircoat, but those on experiments 3 and 4 developed an achromotrichia. It was characterized by the black hair turning a reddish-brown after three to 4 weeks and gradually to gray. The rats receiving the supplemental copper (experiment 3) did not exhibit achromotrichia, nor was this observed in rats receiving 0.08% of iron.

The data showing the effects of excess dietary zinc and excess zinc supplemented with either iron or copper, on the iron, copper and zinc content of the liver and kidneys and copper in the plasma are presented in table 2. The finding of particular interest in the 4 experiments was the significant ($P < 0.01$) reduction of iron in the liver of rats receiving zinc at a high level. A significant ($P < 0.05$) depression of iron also occurred in the kidneys of rats receiving either 0.5 or 0.6% of zinc. Using liver copper as a criterion, the rats receiving a dietary level of 0.6 or 0.5% of zinc did not show a copper deficiency. However, a marked copper deficiency developed in the rats receiving 0.4% of zinc. This was demonstrated by the lowered ($P < 0.01$) content of copper in the liver and the reduced plasma copper.

The effects of supplementary copper or iron on tissue iron and copper in rats receiving a high level of zinc are of particular interest (table 2). The copper caused a further depression of liver iron. On the other hand, adding iron to the diet of the animals fed the high level of zinc in experiment 2 lessened the severity of the decrease in liver iron and caused kidney iron to be maintained almost at the level found in the rats fed on the basal diet. In experiment 4, the level of iron in the liver of rats receiving 0.08% of iron was essentially the same as for rats fed the basal diet and 0.04% partially overcame the loss. Of particular significance are the data which showed that the added iron (0.08%) prevented completely the loss of liver copper. In experiment 3, the added copper prevented the depression of copper in the liver and plasma.

A marked capacity was found for the rat to absorb zinc and store it in the liver. Neither the copper nor the iron supplementation prevented the buildup of zinc in the liver.

The tibia bone from the rats fed a high level of zinc in experiment 3 showed extensive hyperplasia of the marrow, very few erythrocytes, a considerable increase in the number of myelocytes and megakaryocytes and reticular cell proliferation. In experiment 4, the tibia from rats fed the high-zinc diet showed essentially the same picture. Of interest, however, was the finding that the bone marrow was normal for the rats receiving the high-zinc diets supplemented with either 0.04 or 0.08% of iron.

Since the content of both iron and copper in the liver was reduced in rats receiving 0.4% of zinc for 8 weeks, a time study was designed to determine which element was reduced first. The data ob-

TABLE 2

Effect of excess zinc and added copper or iron on tissue iron, copper and zinc

Diet	Iron[1]		Copper[1]			Zinc[1]	
	Liver	Kidney	Liver	Kidney	Plasma[2]	Liver	Kidney
			Experiment 1 (0.6% Zn)[3]				
Basal (12)[4]	378.4 ± 73.7[a]	229.9 ± 54.6	7.5 ± 1.9	23.8 ± 10.1			
Zn (12)	179.7 ± 51.8[a]	159.3 ± 45.6[a]	8.0 ± 1.8	24.5 ± 9.9			
			Experiment 2 (0.5% Zn)				
Basal (10)	361.9 ± 78.9	105.4 ± 9.5	8.6 ± 1.6	16.9 ± 3.8		290.8 ± 39.2	295.2 ± 83.4
Zn (9)	226.9 ± 60.0[a]	73.9 ± 12.0[a]	9.1 ± 1.7	13.7 ± 3.0		573.7 ± 141.7	801.4 ± 190.4
Zn + Cu (10)	174.6 ± 53.4[a]	95.3 ± 31.0	11.4 ± 3.6	29.2 ± 8.2		421.5 ± 78.7	774.8 ± 157.7
Zn + Fe (9)	269.0 ± 80.0[a]	96.7 ± 25.9	8.1 ± 1.0	11.6 ± 2.4		627.3 ± 83.8	864.4 ± 190.3
			Experiment 3 (0.4% Zn)				
Basal (10)	520.0 ± 186.3		9.8 ± 3.3		81.0	106.7 ± 19.9	
Zn (10)	209.5 ± 40.3[a]		3.9 ± 0.9[a]		21.9	627.6 ± 172.2	
Zn + Cu (10)	144.9 ± 49.8[a]		49.4 ± 19.1		81.0	691.0 ± 234.7	
			Experiment 4 (0.4% Zn)				
Basal (10)	564.0 ± 230.5		8.2 ± 1.8			98.1 ± 9.2	
Zn (10)	123.8 ± 29.9[a]		5.3 ± 1.0[a]			530.4 ± 199.0	
Zn + Fe (10)	310.2 ± 167.6[b]		6.0 ± 1.1[b]			505.6 ± 147.3	
Zn + Fe (10)	488.7 ± 138.9		8.5 ± 2.9			444.1 ± 129.0	

[1] Micrograms per gram of dry weight of tissue.
[2] Micrograms per 100 ml of plasma. Values represent pooled samples.
[3] Animals were sacrificed after three, 4 and 8 weeks for experiments 1, 2, and 3 and 4, respectively.
[4] Figures in parentheses show number of animals assayed.
[a] Standard error of mean.
[a] Significantly less than basal group, $P < 0.01$.
[b] Significantly less than basal group, $P < 0.05$.

TABLE 3

Time effect of excess zinc on liver iron, copper and zinc and hemoglobin and weight gain

Days	Basal[1]					Zinc (0.4%)[1]				
	Mean weight gain	Hemoglobin	Iron[2]	Copper[2]	Zinc[2]	Mean weight gain	Hemoglobin	Iron[2]	Copper[2]	Zinc[2]
	gm	gm %				gm	gm %			
					Trial 1					
7	25	10.7	225.4	12.6	96.9	15	11.0	110.4	11.0	757.7
14	29	12.3	437.6	10.4	105.7	21	10.3	77.6	10.3	515.0
21	24	12.8	449.2	10.0	119.2	14	9.1	61.1	8.0	593.2
28	27	11.6	371.0	10.2	146.5	23	7.5	64.6	6.9	457.6
35	23	14.2	296.5	10.3	136.9	24	8.0	64.5	5.1	560.1
42	17	13.3	335.6	10.6	162.0	4	6.8	72.8	4.5	703.4
					Trial 2					
1	2	10.5	154.8	14.0	135.5	0	11.3	118.5	22.4	349.1
3	13	11.6	154.8	12.8	96.7	7	11.3	78.8	19.3	564.0
5	22	11.6	206.0	13.3	105.3	15	11.1	54.6	14.9	591.6

[1] Values are the average of three rats.
[2] Micrograms per gram of dry weight of tissue.

tained from this study are shown in table 3.

Anemia was evident after 14 days. After 6 weeks the average hemoglobin value of the rats receiving zinc was about one-half that of the controls.

After 7 days on the first trial, the iron content in the liver of rats fed the high-zinc diet had dropped to about 50% of that of the controls. A further loss of iron occurred the second week. Thereafter, the iron remained relatively constant. In the second trial the data show a slight loss of liver iron after one day on the diet and a definite loss after three days. A definite depression of liver copper did not develop until between 21 and 28 days on the high-zinc diet, after which the level continued to drop to the end of the trial.

The buildup of liver zinc in the rats fed the high-zinc diet was very rapid, zinc being absorbed and stored after only one day on the diet. It should be pointed out that the accumulation of zinc in the liver paralleled the loss of liver iron.

DISCUSSION

The finding of particular importance to the understanding of the mechanism of zinc toxicity in the rat was the iron metabolism anomaly, demonstrated by the marked lowering of liver and kidney iron. As shown by the time study, the loss of liver iron was an early manifestation of excess zinc in the diet. The time study also indicated that the loss of iron nearly paralleled the accumulation of zinc in the liver. A reduction of liver copper occurred in rats fed high-zinc diets that did not cause early toxic symptoms but not in those animals receiving 0.6 or 0.5% of zinc for three and 4 weeks, respectively. From the work of Grant-Frost and Underwood ('58), who reported that copper retention was reduced in growing rats receiving 0.5% of zinc for 6 weeks, it is possible that for the present work the rats received the diets of 0.5 or 0.6% zinc for an insufficient length of time. From the time study it was indicated that the loss of liver copper occurred after the loss of liver iron and the development of the anemic condition.

In relation to the production of the anemia, the data indicated that it occurred in rats with a reduced liver iron but with a copper level similar to the controls. Also of significance are the data which demonstrated that additional iron in the diet of rats fed the high-zinc diet apparently prevented the loss of liver copper. From these results it may be stated that (a) excess zinc in the diet causes an early and marked loss of liver iron, (b) the reduction of liver copper occurred after the loss of liver iron and may possibly be the result of the reduction in liver iron,

and (c) zinc toxicity anemia, and presumably the reduced activity of certain iron-containing enzymes, appears to be the result of an iron deficiency rather than copper. The fact that anemia was a relatively late sign of copper deficiency (Gallagher, '57) and that anemia of significant degree was not present in "low copper" rats regardless of an 80% reduction of serum copper and a 40% reduction of liver copper (Dempsey et al., '58) substantiates the present view that zinc toxicity anemia is the result of an iron rather than a copper deficiency.

The action of excess dietary zinc in reducing the amount of iron in the liver is similar to the action of manganese described by Hartman et al., ('55), who noted that feeding high levels of this element decreased the level of iron in the liver of lambs. These workers postulated that manganese interferes with iron absorption rather than with hematopoiesis. Preliminary unpublished work at this laboratory indicated that excess zinc does not interfere with iron absorption. Additional work is needed and is being planned to determine the mechanism as to how excess dietary zinc interferes with the metabolism of iron.

The question arises of why the work of other investigators showed an ameliorating effect of copper on zinc toxicity anemia and decreased activity of some iron-containing enzymes. The results of the present study indicate that the answer lies in the ability of copper to mobilize the iron in the liver even when the iron is at a low level, as demonstrated by the additional loss of iron from the liver of rats fed high-zinc diets supplemented with copper. In this connection, Copp and Greenberg ('46) reported that an intraperitoneal injection of copper to iron-depleted rats fed a diet containing 5.0 ppm of copper resulted in an increased rate of radioactive iron utilization for hemoglobin production. This increased utilization was indicated by a greatly decreased storage of radioactive iron in the liver.

It has been reported that zinc is not normally stored in large amounts in the liver (Underwood, '56) nor absorbed from the intestine in large amounts by the rat (Davis, '58). The results obtained in the present study do not confirm these findings but agree with those obtained with the cat (Scott and Fisher, '38).

As reported by other investigators, symptoms of zinc toxicity exhibited by the rats included poor growth and anemia. The poor growth presumably is caused largely but not entirely by reduced food consumption due to the unpalatability of the diet as suggested by Grant-Frost and Underwood ('58). The increasingly pronounced adverse effect on growth noted in the present study as the zinc content of the diet was increased indicates a corresponding decrease in palatability. The anemic condition was cured by supplementary copper, verifying earlier work. In contrast to the findings of Smith and Larson ('46), the present authors noted that the addition of iron to the high zinc diet appeared to alleviate the anemia somewhat. In the present study, the iron intake was approximately three to 4 times the level used by the above workers, which may account for the difference. In relation to this, whereas the increased amount of bone marrow in the tibia of the rats receiving a high level of zinc was indicative of a compensatory mechanism to alleviate the anemia, the bone marrow was normal in those animals receiving either 0.04 or 0.08% of iron.

Since the achromotrichia occurred in the rats fed the high-zinc diet, with low liver copper, and not in the rats fed the 0.08% of iron in the diet, with corresponding normal liver copper, it is assumed that it was induced by a copper deficiency. The lack of an achromotrichia in the rats receiving either 0.05 or 0.06% of zinc also indicates that they were not copper-deficient. This conclusion stems from the work of Gallagher ('57), who reports that the loss of capacity to produce normally pigmented hair is one of the earliest signs of a copper deficiency.

SUMMARY

A study was made of the effect of excess dietary zinc on iron and copper in tissues of the rat. The results showed that zinc toxicosis results in an accumulation of zinc in the liver with an early and

marked loss of liver iron. The data suggest that the reduction of iron is responsible for the production of the anemic condition and presumably the depression of the activity of some iron-containing enzymes. A lowered liver copper may also occur and the data indicate that it may be the result of the reduced liver iron rather than an effect of the zinc. Copper probably acts in counteracting the anemia and reduced enzyme activity of zinc toxicity by further mobilizing the iron in the liver.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Dennis Sikes, University of Georgia, Athens, for the histological examination and Mesdames Martha Girardeau, Gladys Liles and Mary Walton for technical assistance.

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Reduction of Liver Xanthine Oxidase Activity and Iron Storage Proteins in Rats Fed Excess Zinc[1]

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Previous investigations (Cox and Harris, '60; Magee and Matrone, '60; McCall et al., '61) have demonstrated a reduction of liver iron in rats fed a diet containing excess zinc. Cox and Harris ('60) stated that the decrease of liver iron is an early manifestation of zinc toxicity, whereas a decrease of liver copper is a relatively late effect. Recently, Cox and Hale ('62) found a reduction of liver iron in swine fed excess zinc without a concomitant loss of liver copper.

This investigation was initiated to obtain additional information concerning the anomaly in iron metabolism during zinc toxicosis and, hence, to aid in the explanation of the mechanism. In this study, the activity of xanthine oxidase and the concentration of ferritin and hemosiderin in the liver of rats fed a high level of zinc were determined.

EXPERIMENTAL

The experimental procedure was conducted in the same manner as described in an earlier report from this laboratory (Cox and Harris, '60). Throughout the study, 0.4% of zinc (as zinc oxide) was used in the diet to produce the toxicosis. After the various dietary regimens were completed, the rats were killed and the liver was removed for analyses. For xanthine oxidase determination, the livers were placed immediately in cold, 0.039 M sodium-potassium phosphate buffer (pH 7.4) and after being chilled, were blotted, weighed, and homogenized. Xanthine oxidase activity was determined on the homogenates by a colorimetric assay (Litwack et al., '53). The method of Gabrio et al. ('53) was used for the quantitative fractionation of liver ferritin and hemosiderin. Iron content was measured by the method of Sideris ('42) and molybdenum by the thiocyanate technique as described by Sandell ('59).

RESULTS

Data demonstrating the effect of 0.4% of dietary zinc for 8 weeks, on liver ferritin, hemosiderin, and hemoglobin of male rats are presented in table 1. The concentration of both iron storage proteins was reduced ($P < 0.01$); however, the depletion of ferritin (80.7%) was greater than that of hemosiderin (66.6%). Of particular significance, a higher percentage of the total iron loss in the liver came from the ferritin fraction (77.2%) rather than hemosiderin (19.6%). The data also show that the relative distribution of storage iron between the iron proteins did not remain constant, the percentage of hemosiderin remained essentially the same, whereas that of ferritin decreased. As anticipated, the hemoglobin content in the liver decreased.

Data illustrating the effect of 0.4% of dietary zinc on rat liver xanthine oxidase activity are tabulated in table 2. The excess dietary zinc caused a reduction in activity, which occurred after feeding the diet a relatively short time. The data show that after 14 days, a loss in activity of approximately 50% occurred in the liver of the female rats. However, in the male rats, about 70 and 60% reduction in activity was noted after 4 and 7 days, respectively. Since it was demonstrated that the activity of xanthine oxidase was reduced, it seemed pertinent to ascertain

Received for publication August 6, 1962
[1] Published with the approval of the Director as Journal Series Paper no. 100
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TABLE 1
Excess dietary zinc and ferritin, hemosiderin, and hemoglobin in rat livers

Iron[1] as	Diet: Basal (6)[2]	Diet: 0.4% Zn (6)[2]	Total Fe loss
			%
Total	178.9±29.4[3]	46.5±5.8[4]	
Ferritin	126.7±21.4 (70.8)[5]	24.5±3.8[4] (52.7)	77.2
Hemosiderin	38.9±11.4 (21.7)	13.0±2.9[4] (27.5)	19.6
Hemoglobin	13.4± 4.0	9.0±1.6	3.3

[1] Micrograms per gram of wet weight of tissue. The average net weights of the liver for basal and zinc fed rats were 10.0 and 8.1 gm, respectively.
[2] Figure represents number of male animals fed diet for 8 weeks.
[3] Standard deviation.
[4] Significantly less than basal group, $P < 0.01$.
[5] Figures in parentheses show percentage of total iron as ferritin and hemosiderin.

TABLE 2
Excess dietary zinc and xanthine oxidase activity in rat livers

Days fed diet	Sex	Xanthine oxidase activity[1]: Basal[2]	Xanthine oxidase activity[1]: 0.4% Zn[2]
1	F	9.9	7.8
3	F	13.1	11.0
14	F	12.3	6.4
2	M	11.3	7.0
4	M	8.4	2.5
7	M	9.4	3.6

[1] Micromoles of xanthine disappearance per hour per gram of wet weight of tissue.
[2] Values are the average for two rats.

the status of molybdenum in the liver of rats fed excess zinc. Samples from an earlier investigation, in which rats were fed 0.4% of zinc for 8 weeks, were analyzed for molybdenum. No loss of molybdenum was noted; average liver molybdenum value for rats fed 0.4% of zinc was 1.1 ppm (dry weight basis) and for control rats, 1.2 ppm.

DISCUSSION

Granick ('54) stated that the ferritin fraction of liver, rather than of hemosiderin, is more readily available in the body for various metabolic processes of iron. Shoden et al. ('53) reported that iron is mobilized from both ferritin and hemosiderin, and they further stated that the compounds are functionally indistinguishable. They point out, however, that their study did not permit speculation concerning the intracellular exchange between the two compounds. Recently, Morgan ('61) reported that the reduction of storage iron in iron-depleted rats was accompanied by little change in the relative distribution of iron between ferritin and hemosiderin. In the present investigation, although both iron proteins were significantly reduced in the liver, the depletion of ferritin was greater than hemosiderin. It also was noted that a higher percentage of the total iron loss was the result of the loss of ferritin rather than hemosiderin. And, in contrast to the investigation of Morgan ('61), in which he found that the relative distribution of the total iron between the iron proteins remained essentially the same, our results indicate that hemosiderin remained the same but ferritin decreased. These data indicate, therefore, that the ferritin fraction was more labile than hemosiderin and was the major source of iron loss from the liver. However, as pointed out by Shoden et al. ('53), the role of any possible intracellular exchange between ferritin and hemosiderin could not be determined.

In an earlier investigation (Cox and Harris, '60) on zinc toxicosis in the rat, liver iron was reduced to a minimal value, after which no further depletion occurred. Underwood ('56) stated that iron reduction in tissues reaches a base line below which tissues would not release iron under any conditions of stress. Morgan ('61) noted that the complete removal of storage iron from the liver and spleen of iron-depleted rats was not achieved, but no definite lower limit of concentration could

be demonstrated. From these results and those of the present investigation, one may speculate that either the iron in the storage proteins is in a form in which it can not be further released or some mechanism responsible for the iron release has been destroyed.

The present investigation on the role of xanthine oxidase during zinc toxicosis was prompted by the report (Mazur et al., '58) which showed that xanthine oxidase participated in the *in vivo* liberation of liver ferritin iron to the circulation. It was thought that the action of zinc could possibly be one of increasing the activity of xanthine oxidase and consequently increase the rate of removal of iron from the liver. This apparently is not the case, however, since the activity of xanthine oxidase was reduced; in fact, the reduction was found after feeding the diet a relatively short time. In this respect, it has been shown (Cox and Harris, '60) that the loss of iron from the liver of rats fed excess zinc also occurs after feeding the diet a short time. Relevant to these results was the investigation (Kinney et al., '61) which showed that there were no concomitant changes in liver iron or iron absorption associated with decreased xanthine oxidase activity. Strohmeyer et al.[4] also failed to demonstrate a relationship between liver xanthine oxidase and iron metabolism.

Richert and Westerfeld ('54) reported that rats with an iron deficiency did not exhibit an altered liver xanthine oxidase. It could be concluded, therefore, that the reduced enzyme activity noted in the present investigation was not the result of the loss of liver iron which occurs during zinc toxicosis. On the other hand, a rapid loss of liver iron, such as that found in rats with zinc toxicosis, as compared with the relatively slow rate of iron loss of rats fed an iron-deficient diet may be important. The failure to observe a reduction in the molybdenum content in the liver of rats fed excess zinc suggests that the reduced enzyme activity was not due to a loss of this element. The early reduction of the activity of xanthine oxidase precludes the possibility of an effect of dietary protein intake; low protein intake was shown (Westerfeld and Richert, '50) to reduce liver xanthine oxidase.

SUMMARY

Experiments were made to study the effect of 0.4% of dietary zinc on the activity of xanthine oxidase and the content of ferritin and hemosiderin in the liver of rats.

The feeding of a high level of zinc produced a lowering of xanthine oxidase activity in the liver. The loss occurred after the rats had eaten the diet for a short time. Relevant to this result was the observation that liver molybdenum was not reduced in rats fed excess zinc.

Both iron storage proteins in the liver of rats fed excess zinc were reduced. The percentage loss of ferritin was greater than that of hemosiderin, and ferritin contributed a greater amount to the total iron loss from the liver. These results suggest that, under the conditions of the experiment, ferritin was more labile than hemosiderin.

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J. NUTR. 98(4) 459-466

Excess Dietary Zinc for the Maternal Rat, and Zinc, Iron, Copper, Calcium, and Magnesium Content and Enzyme Activity in Maternal and Fetal Tissues [1,2]

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ABSTRACT Excess dietary zinc (0.4%) was fed to the maternal rat during gestation to study its effect on the content of zinc, copper, iron, calcium and magnesium and the activity of various enzymes in maternal and fetal tissues. Succinic dehydrogenase activity was not altered in maternal and fetal liver and heart during gestation. Cytochrome oxidase activity was reduced in fetal liver, but not heart, and maternal liver and heart at day 16 of fetal age only. Xanthine oxidase activity was undetected in fetal liver and heart at day 22, irrespective of treatment. Activity of xanthine oxidase was lower in maternal heart, but not liver, at day 22. Ceruloplasmin activity was undetected in maternal serum at day 22. Changes in mineral content of fetal liver and body and maternal liver, heart, adrenals, kidneys, spleen, gastrocnemius muscle, thymus, and brain at day 22 of fetal age are as follows. Zinc was increased and copper was reduced in fetal body and liver. Iron was lower in the body, but unchanged in the liver. Total calcium and concentration of calcium were higher in fetal liver, but were lower in the body. Magnesium concentration, but not total, was elevated in liver and body. Total zinc and concentration of zinc were elevated in maternal liver, kidneys, and brain and total zinc was higher in thymus. Zinc content was unaltered in the other tissues. Copper was reduced in the liver only. No change was found in iron content of the tissues. Total calcium and concentration of calcium were higher in heart and brain, but reduced in kidneys. No change in calcium content was noted in the other tissues. Total magnesium and concentration of magnesium were lower in spleen; magnesium concentration was reduced in kidneys. The other tissues had no change in magnesium. The changes found in zinc and calcium in the maternal brain suggest further work in the area of brain metabolism and performance. The relation of reduced serum ceruloplasmin to iron metabolism in the maternal and fetal animal on an excess zinc regimen is discussed.

The effect of excess dietary zinc for the maternal rat during gestation on zinc, iron, and copper content of the fetus has been reported (1). Concomitant with an elevation of zinc content in the fetus were alterations in iron and copper levels, which resembled those reported (2–5) for the weanling and adult rat fed excess zinc.

In addition to altered tissue iron and copper in weanling rats fed excess zinc, changes in calcium, phosphorus, and magnesium have been reported. Sadasivan (6) found that normal deposition of calcium and phosphorus did not occur in bones of young rats. Stewart and Magee (7) confirmed Sadasivan's findings, and showed that the changes were alleviated with dietary supplements of calcium and phosphorus. They also observed a decreased retention of magnesium; however, bone magnesium was not affected.

Activity of various enzymes has been found to be altered in tissues of rats fed excess zinc. Reduction in liver catalase and in cytochrome oxidase (8), a depression in heart cytochrome oxidase (3), a decrease in intestinal alkaline phosphatase (9), an increase in liver and kidney alkaline phosphatase (8, 9), a reduction in liver xanthine oxidase (10), and a lowering of serum ceruloplasmin [3] (11) have been re-

Received for publication February 3, 1969.

[1] College of Human Development Research Publication no. 255.

[2] Supported by Public Health Service Research Grant no. HD-02103 from the National Institute of Child Health and Human Development.

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ported. No data are available on the effect of excess dietary zinc during gestation on the activity of enzymes in maternal and fetal tissues.

The present investigation was designed to study the effect of excess dietary zinc for the maternal rat during gestation on the content of zinc, iron, copper, calcium, and magnesium and the activity of succinic dehydrogenase, cytochrome oxidase, xanthine oxidase, and ceruloplasmin in maternal and fetal tissues.

EXPERIMENTAL PROCEDURE

Nulliparous female rats,[4] ranging in weight from 195 to 245 g, were individually housed in galvanized wire cages and received feed and distilled water ad libitum. Composition of the basal diet was as follows: (in percent) casein, 20; sucrose, 63.3; cellulose,[5] 3; corn oil, 10; salt mixture (with sulfate) (12), 2.7; and vitamin mixture,[6] 1. The excess zinc diet was formulated by the incorporation of 0.4% zinc as zinc oxide into the basal diet. Zinc content of the basal diet was 9 ppm.

Experiment 1. Ten rats were mated as previously described (13). The day sperm were found in the vaginal smear was designated as zero-day fetal age, and rats then were randomly assigned to the basal and 0.4% zinc diet. At fetal age 16 days, all rats were killed.

Experiment 2. Ten rats were subjected to the same regimen as described in experiment 1 except that animals were killed at fetal age 21 days.

Experiment 3. Twelve rats were treated according to the same regimen as described in experiment 1 except that at fetal age 22 days, six basal and six experimental rats were killed.

At the appropriate age of the fetuses, mothers were stunned by a blow on the head, decapitated and exsanguinated. Fetuses were removed via abdominal incision, and were stunned, decapitated and exsanguinated. Fetal livers and hearts were removed and pooled separately according to litter. Fetal livers and hearts and individual maternal liver and heart were chilled in cold, 0.039 M phosphate buffer (pH 7.4) prepared with reagent grade KH_2PO_4 and glass-distilled water. Tissues were removed from the buffer, blotted free of moisture, weighed, and homogenized in a Potter-Elvehjem glass homogenizer with cold phosphate buffer. Homogenates were analyzed for succinic dehydrogenase (EC. 1.3.99.1) (14), cytochrome oxidase (EC. 1.9.3.1) (14), and xanthine oxidase (EC. 1.2.3.2) (15). Sera of mothers were analyzed for ceruloplasmin (16, 17). Hemoglobin level in maternal blood was measured by the acid-hematin technique.

Adrenals, kidneys, spleen, gastrocnemius muscle, thymus, and brain were removed from maternal animals at day 22 of fetal age. These maternal tissues and body[7] of the 22-day-old fetus were dried to a constant weight at 100°. Samples for mineral analyses of maternal liver and heart at day 22 of fetal age and litter-pooled livers of the 22-day-old fetuses were obtained from homogenates used for enzyme analyses, and were dried to a constant weight at 100°. Dried samples were wet-digested with nitric and sulfuric acids and analyzed for zinc, copper, iron, calcium, and magnesium by the atomic absorption[8] technique. Data were analyzed statistically by the t test (18).

RESULTS

Data illustrating the effects of feeding 0.4% Zn to the maternal rat on dry weight and enzyme activity of fetal tissues and enzyme activity and hemoglobin level in maternal tissues are tabulated in table 1.

No change was found in dry weights of the liver and heart of 16-, 21-, and 22-day-old fetuses from mothers fed excess zinc. Dry weight of body was significantly less in the 22-day-old fetuses from these mothers.

Succinic dehydrogenase activity was not altered in the liver of 16-, 21-, and 22-day-old fetuses or in the heart of 21-day-old fetuses from mothers fed 0.4% Zn. Activity of cytochrome oxidase was significantly

[4] CFE strain of Sprague-Dawley rats obtained from Carworth, New City, N. Y.

[5] Alphacel.

[6] Vitamins in cornstarch: (amount per kilogram of diet) vitamin A, 20,000 IU; vitamin D, 2,200 IU; (in milligrams) ascorbic acid, 1017; vitamin E as α-tocopheryl acetate, 485; inositol, 110; choline dihydrogen citrate, 3715; menadione, 49.6; p-aminobenzoic acid, 110; niacin, 99.2; riboflavin, 22; pyridoxine·HCl, 22; thiamine·HCl, 22; Ca pantothenate, 66; biotin, 0.44; folic acid, 1.98; and vitamin B_{12}, 29.7 μg (obtained from General Biochemicals Inc., Chagrin Falls, Ohio).

[7] In this report, the term body is used to mean the whole animal minus the liver and heart.

[8] Perkin-Elmer Model 303 atomic absorption spectrophotometer, Perkin-Elmer Corporation, Norwalk, Conn.

TABLE 1

Effect of zinc-excess regimen on fetal dry weight, enzyme activity in maternal and fetal tissues, and maternal hemoglobin

Diet	Age	Dry wt			Succinic dehydrogenase [1]		Cytochrome oxidase [1]		Xanthine oxidase [2]		Serum ceruloplasmin	Hemoglobin
		Body	Liver	Heart	Liver	Heart	Liver	Heart	Liver	Heart		
		mg	*mg*	*mg*							*IU*	*g/100 ml*
						Fetal						
Basal (10) [3]	16		5.3 ± 1.0 [4]	0.7 ± 0.1	12.2 ± 2.4		447.6 ± 28.7	382.5 ± 20.2				
0.4% Zn (12)	16		6.8 ± 0.7	0.8 ± 0.1	13.4 ± 1.1		308.4 ± 17.3 **	321.3 ± 56.7				
Basal (12)	21		48.6 ± 3.9	4.8 ± 0.8	17.6 ± 4.1	43.8 ± 4.9	352.2 ± 18.8	370.6 ± 22.6				
0.4% Zn (10)	21		50.5 ± 1.7	5.1 ± 0.1	16.3 ± 6.1	35.6 ± 8.4	419.4 ± 8.8	330.6 ± 15.4				
Basal (10)	22	546.3 ± 13.6	83.5 ± 10.2	7.6 ± 0.8	23.9 ± 4.8				0.0	0.0		
0.4% Zn (10)	22	439.4 ± 13.6 **	76.2 ± 9.9	7.9 ± 0.7	23.6 ± 6.9				0.0	0.0		
						Maternal						
Basal (5) [5]	16				31.7 ± 2.3	125.9 ± 16.6	270.4 ± 2.6	461.4 ± 30.2				
0.4% Zn (5)	16				36.3 ± 0.1	69.8 ± 18.1	189.6 ± 8.5 **	330.6 ± 31.0 *				
Basal (5)	21				28.8 ± 6.2	118.0 ± 6.5	196.0 ± 15.8	371.4 ± 49.2				
0.4% Zn (5)	21				27.5 ± 5.0	147.5 ± 7.8	281.2 ± 16.2	291.0 ± 3.4				
Basal (6)	22				55.0 ± 6.0	129.3 ± 12.2			708.1 ± 48.8	440.0 ± 51.6	47.1 ± 2.5	14.4 ± 0.4
0.4% Zn (6)	22				50.0 ± 6.0	111.5 ± 12.2			625.0 ± 96.3	235.0 ± 6.3 **	0.0 **	12.0 ± 0.5 **

[1] Microliters O_2 per milligram dry weight of tissue per hour.
[2] Microliters O_2 per gram dry weight of tissue per hour.
[3] Average number of fetuses per litter.
[4] Mean and SE.
[5] Number of animals per treatment.

* Significantly different from basal value, $P < 0.05$.
** Significantly different from basal value, $P < 0.01$.

TABLE 2

Effect of zinc-excess regimen on zinc, copper, iron, calcium and magnesium in fetal liver and body and maternal tissues at day 22 of fetal age

Diet	Dry wt	Zinc [1]		Copper [1]		Iron [1]		Calcium [1]		Magnesium [1]	
		Total	Conc	Total	Conc	Total	Conc	Total	Conc	Total	C
	g	µg	ppm	µg	ppm	µg	ppm	µg	ppm	µg	p
						Fetal [2]					
						Liver					
Basal		4.3 ± 0.3	54.5 ± 5.9	0.9 ± 0.1	11.0 ± 2.1	23.8 ± 5.8	275.1 ± 44.7	0.4 ± 0.1	5.1 ± 1.2	54.0 ± 11.7	632.0
.4% Zn		20.6 ± 3.2 **	277.1 ± 34.1 **	0.2 ± 0.1 **	2.3 ± 0.7 **	19.8 ± 3.0	265.6 ± 34.2	1.0 ± 0.2 *	13.0 ± 1.3 **	85.1 ± 10.8	1128.9
						Body					
Basal		22.7 ± 0.8	41.6 ± 1.3	5.5 ± 1.3	10.3 ± 1.4	87.8 ± 7.4	161.8 ± 13.9	3615.8 ± 108.5	6832.6 ± 217.1	853.9 ± 22.7	1594.3
.4% Zn		20.7 ± 0.6	48.6 ± 1.6 **	1.6 ± 0.3 **	3.4 ± 0.6 **	17.3 ± 1.2 **	39.4 ± 2.5 **	2706.1 ± 91.5 **	6342.7 ± 252.7 **	833.2 ± 19.2	1943.9
						Maternal [3]					
						Liver					
Basal	3.18 ± 0.23	197.2 ± 13.3	62.5 ± 3.3	29.2 ± 2.5	9.2 ± 0.6	265.1 ± 17.5	84.1 ± 4.6	15.5 ± 1.6	4.9 ± 0.4	158.3 ± 158.1	778.2
.4% Zn	2.77 ± 0.21	1162.6 ± 68.0 **	431.6 ± 36.5 **	14.8 ± 1.9 **	5.5 ± 0.7 **	248.5 ± 28.3	93.2 ± 13.1	16.1 ± 1.6	6.0 ± 0.7	390.0 ± 186.6	770.6
						Heart					
Basal	0.18 ± 0.03	9.3 ± 1.2	53.1 ± 7.7	2.5 ± 0.6	13.8 ± 2.6	25.5 ± 1.9	149.4 ± 18.7	0	0	303.7 ± 19.5	1768.3
.4% Zn	0.18 ± 0.01	8.7 ± 0.6	50.0 ± 5.2	1.1 ± 0.2	6.8 ± 1.0	32.5 ± 2.6	188.3 ± 24.1	1.8 ± 0.4 **	9.9 ± 1.9 **	254.2 ± 16.6	1478.9
						Adrenals					
Basal	0.017 ± 0.001	0.5 ± 0.1	29.8 ± 4.0	0	0	2.7 ± 0.3	151.1 ± 12.7	2.1 ± 0.8	119.8 ± 13.0		
.4% Zn	0.021 ± 0.003	0.8 ± 0.1	40.1 ± 5.2	0	0	2.0 ± 0.3	105.2 ± 23.0	2.3 ± 0.3	119.1 ± 12.2		
						Kidneys					
Basal	0.37 ± 0.01	22.6 ± 1.3	61.2 ± 2.9	5.3 ± 0.2	14.3 ± 0.6	58.2 ± 4.8	157.9 ± 12.7	32.3 ± 2.8	87.1 ± 6.3	685.0 ± 10.0	1861.5
.4% Zn	0.40 ± 0.01	58.3 ± 1.8 **	146.7 ± 3.2 **	5.3 ± 0.3	13.2 ± 0.4	64.0 ± 2.5	162.2 ± 9.8	22.2 ± 1.1 **	55.9 ± 2.9 **	695.8 ± 16.6	1753.3
						Spleen					
Basal	0.09 ± 0.01	4.9 ± 0.4	53.6 ± 2.5	0	0	130.5 ± 4.8	1439.5 ± 69.6	8.0 ± 0.2	89.1 ± 4.8	317.5 ± 8.4	3535.4
.4% Zn	0.12 ± 0.01	7.8 ± 0.7	63.5 ± 8.5	0	0	102.8 ± 13.8	986.9 ± 168.8	7.8 ± 0.4	68.5 ± 11.7	183.0 ± 37.0 **	1475.1
						Gastrocnemius muscle					
Basal	0.41 ± 0.03	15.8 ± 1.0	38.6 ± 0.7	0.8 ± 0.1	1.9 ± 0.1	16.0 ± 0.9	39.3 ± 1.3	12.6 ± 0.4	31.2 ± 1.7	703.3 ± 62.0	1723.4
.4% Zn	0.39 ± 0.03	15.9 ± 1.1	41.4 ± 1.3	0.8 ± 0.1	2.0 ± 0.2	16.5 ± 0.8	45.1 ± 6.0	11.7 ± 0.3	31.3 ± 2.2	739.2 ± 86.5	1907.7
						Thymus					
Basal	0.08 ± 0.01	1.6 ± 0.2	20.2 ± 3.1	0	0	10.2 ± 1.0	134.7 ± 24.0	5.7 ± 0.5	72.4 ± 8.5	66.7 ± 7.5	814.2
.4% Zn	0.07 ± 0.01	2.5 ± 0.4 **	34.7 ± 2.6	0	0	10.4 ± 1.3	152.4 ± 20.7	5.0 ± 0.4	74.4 ± 10.8	60.0 ± 7.2	843.0
						Brain					
Basal	0.32 ± 0.02	11.0 ± 0.4	34.1 ± 1.0	2.9 ± 0.1	8.9 ± 0.4	17.8 ± 0.4	55.6 ± 1.9	18.5 ± 0.8	57.5 ± 3.2	385.0 ± 28.3	1192.2
.4% Zn	0.32 ± 0.01	12.8 ± 0.6 *	39.8 ± 1.6 *	3.0 ± 0.1	9.4 ± 0.4	17.6 ± 0.7	55.0 ± 2.5	28.0 ± 1.7 **	87.1 ± 4.8 **	350.0 ± 8.2	1091.8

[1] Average values of total content and concentration in tissues on dry weight basis and SE.
[2] Average values for a mean of 10 fetuses/litter.
[3] Average values for six animals per treatment.

* Significantly different from basal value, $P < 0.05$.
** Significantly different from basal value, $P < 0.01$.

reduced in the liver of 16-day-old fetuses, but not at other fetal ages, from mothers fed high zinc; heart cytochrome oxidase was unaltered in 16- and 21-day-old fetuses. Xanthine oxidase activity was undetected in liver and heart of 22-day-old fetuses from each treatment.

Activity of succinic dehydrogenase was not changed in liver and heart of maternal rats fed 0.4% Zn during gestation. Cytochrome oxidase activity was significantly reduced in liver and heart at day 16, but not at day 21, of maternal rats fed high zinc. At day 22, the activity of xanthine oxidase was significantly lower in the heart, but not liver, of mothers fed excess zinc. Excess dietary zinc caused a significant reduction in the activity of serum ceruloplasmin of maternal rats at day 22; in fact, activity was undetected. At day 22, hemoglobin was significantly lower in the maternal rat fed 0.4% Zn.

Data showing the effect of 0.4% dietary Zn for the maternal rat on total amount (micrograms) and concentration (parts per million) on a dry weight basis of zinc, copper, iron, calcium, and magnesium in fetal liver and body and maternal tissues at day 22 of fetal age are given in table 2.

Liver of fetuses from mothers fed 0.4% Zn contained significantly more total zinc and concentration of zinc than liver of fetuses from mothers fed the basal diet. A significantly higher concentration of zinc, but not total, was found in the body of fetuses from maternal rats fed excess zinc. Total copper and concentration of copper were significantly reduced in liver and body of fetuses from mothers fed 0.4% Zn. Total iron and concentration of iron were significantly lower in the body, but unchanged in the liver, of fetuses from mothers fed excess zinc. Total calcium and concentration of calcium were significantly higher in the liver, but were significantly lower in the body, of fetuses from 0.4% Zn group compared with those from the basal groups. Magnesium concentration only was significantly elevated in liver and body of fetuses from mothers fed 0.4% Zn.

No significant differences were found between treatments in the dry matter content of maternal tissues. Significant elevations occurred in total zinc and concentration of zinc in liver, kidneys, and brain of maternal animals fed 0.4% Zn. Total zinc was significantly higher in the thymus of these animals. No change was found in the zinc content of other tissues examined. The liver of maternal animals fed excess zinc contained a significantly lower total copper and concentration of copper than the liver of mothers in the basal groups. Copper was not detected in adrenals, spleen, and thymus of animals from each treatment by the technique employed. Copper was not quantitatively altered in other tissues. No significant change was found in the iron content of tissues of mothers fed 0.4% Zn. Total calcium and concentration of calcium were significantly higher in heart and brain, but significantly reduced in kidneys, of maternal rats fed excess zinc. No change was noted in the calcium content of the other tissues from these mothers. Significant reductions were found for total magnesium and concentration of magnesium in the spleen and for magnesium concentration in kidneys of mothers fed the diet containing additional zinc.

DISCUSSION

It has been documented[9] (2–11) that excess dietary zinc for the rat produces an antagonism to iron, copper, calcium, magnesium, and various enzymes. Settlemire and Matrone (19) suggested that zinc affects iron metabolism by a) impairing the incorporation of iron into or release from ferritin, which would influence iron absorption and storage, and b) shortening the life span of erythrocytes, which would cause a faster turnover of iron. Van Campen (20) postulated that zinc alters copper metabolism by impairing its absorption as mediated primarily via the direct effect of zinc either in or on the intestine. Data presented by Stewart and Magee (7) indicate that zinc decreases the normal deposition of calcium in bone and reduces both the absorption and utilization of magnesium. Lowered activity of cytochrome oxidase (3, 8) and ceruloplasmin[10] (11) can be attributed to a zinc-induced copper deficiency since feeding copper restored enzyme activity and reduced copper content was found (8) in hearts with low cytochrome oxidase activity.

[9] See footnote 3.
[10] See footnote 3.

Succinic dehydrogenase activity was not reduced in maternal and fetal liver and heart during gestation, whereas cytochrome oxidase was reduced in activity at day 16 of fetal age, but not at day 21. These findings were not consistent with the view that iron and copper metabolism are altered in the maternal and fetal animal. Reduced copper was found (1) in fetal liver at day 18 of fetal age and in maternal liver and heart at day 16. A similar change in copper status was found at day 22 in this study. Iron was reduced in the fetal body, but not liver, at days 16 and 18 (1), and in the body, but not liver, at day 22 in the present study. Iron in the maternal liver was not altered. As will be discussed later, however, iron in both fetal and maternal liver may not be available for utilization. Beutler and Blaisdell (21) found a diminution of succinic dehydrogenase activity in the heart, but not liver, of the iron-deficient rat. Schultze (22) reported that cytochrome oxidase activity was not reduced in the liver and heart of the iron-deficient rat. A reduced cytochrome oxidase activity was found in the heart (22) and liver (22, 23) of rats fed a copper-deficient diet beginning at weanling age. However, though the activity of cytochrome oxidase was reduced in the liver, no change was detected in the heart of copper-deficient adult rats (24). Of particular relevance to the present study, reduced cytochrome oxidase activity was found in the heart (3), which had reduced copper content, and liver (8) of rats fed excess zinc beginning at weanling age. In both cases, feeding copper caused the activity to return to either normal or greater than normal values. Therefore, from the data relating altered enzyme activity to either excess dietary zinc, iron deficiency, or copper deficiency in the rat and the status of copper and iron in the maternal and fetal animal, findings of reduced succinic dehydrogenase activity and a more consistent diminution in cytochrome oxidase were expected. Other than the fact that the changes in enzyme activity were observed when weanling rats were used, no tenable explanation can be ascribed for the lack of agreement in this study.

The absence of xanthine oxidase in the fetal liver at day 22, which occurred irrespective of dietary treatment, is in accord with earlier studies (25, 26). In contrast to an earlier report (10), xanthine oxidase was not lowered in the liver of the maternal rat fed excess zinc; the discordance may be attributed to age difference in the animals since weanling rats were used in the earlier study. Of relevance to the role of a high zinc regimen to the utilization of iron was the reduction of xanthine oxidase in the heart and the absence of ceruloplasmin in the serum of the maternal rat. An absence of ceruloplasmin has been found[*] (11) in the nonpregnant rat fed excess zinc. Ceruloplasmin has been reported (27) to be involved in promoting iron saturation of transferrin and possibly other iron-containing proteins and enzymes, and consequently the turnover of iron in the body. This suggests, therefore, that the reduced xanthine oxidase in the heart may be attributed to decreased iron utilization as the result of the absence of ceruloplasmin. The fact that ceruloplasmin is scarcely measurable (28) at the time of birth of the rat also explains the reduction of iron in the fetal body, but not liver at day 22, and adds substance to the concept that ceruloplasmin plays an important role in iron turnover in the body. In addition to the suggestion (19) that zinc alters iron metabolism by interfering with its incorporation into or release from ferritin, consideration should be given to the alteration of ceruloplasmin when explaining the action of zinc antagonism to iron metabolism. In this connection, not only is the level of ceruloplasmin affected by impaired copper metabolism but possibly by zinc, since when zinc was added to an in vitro system at the concentration found in serum, the ascorbate oxidase activity of ceruloplasmin was inhibited (29).

Calcium was elevated in the fetal liver, but lowered in the body. In contrast, magnesium concentration, but not total, was higher in the fetal liver and body. For the maternal rat, zinc was elevated in the kidneys, brain, and thymus; calcium was higher in the heart and brain, but lower in the kidneys; and magnesium was lower in spleen and kidneys. An elevation of liver calcium was found (30) in calves fed a high level of zinc. Excess dietary zinc was

[*] See footnote 3.

found to decrease the deposition of calcium in bone (6, 7) and the absorption and utilization of magnesium (7). Though it is evident that zinc interferes with calcium and magnesium metabolism, the specific mechanism is not clear. Hormones are involved in the homeostasis of calcium and magnesium, and since zinc is known (31) to be involved with hormonal balance, further study in this area is warranted.

Of particular interest were the changes found in the content of zinc and calcium in the maternal brain. Although zinc apparently does not readily cross the blood-brain barrier in mice and dogs (32), under the condition of a high zinc regimen, zinc was elevated in the brain of the maternal rat and small but consistent linear increases occurred (33) in the brain of lambs. Intracerebral or subcutaneous injections of zinc for mice caused convulsive seizures, which were prevented by intracerebral injection of calcium (34). Though the authors did not analyze the brain for zinc or calcium, they suggested that the seizures were due to calcium depletion in neuronal membranes. Rather than a decrease, calcium was increased, concomitant with an elevation of zinc, in the brain of the maternal rat fed excess zinc. The lack of reduced brain calcium in this study may be due either to the method of increasing zinc in the brain or to the amount of zinc in the brain. The observation of an increased brain calcium in the maternal rat fed excess zinc indicates further investigation in the areas relative to brain metabolism and performance. In this connection, calcium was reduced in the brain of the maternal rat fed a zinc-deficient diet (35). A study to determine if the same phenomenons occur in the brain of the fetus and postnatal animal would be of particular interest.

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THE CARCINOGENIC INFLUENCE OF ZINC IN DRINKING WATER

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The World Health Organization's regional office for Europe in Geneva stated in 1956 that safe drinking water can contain up to 5 milligrams per liter of zinc. However the announcement emphasized that this was only a provisional recommendation subject to change when the effects of the metal are better known.

For nutritive organic substances there has to exist a certain equilibrium among the various ions, and any change in this equilibrium can cause serious disturbances, known in practice as mineral imbalances.

From the work of E. Halme, H. J. Bagg, and Mihalowski, it is known that zinc can cause teratoma and even cancer. Particularly interesting are the observations of E. Halme who found in the waterlines of Finland a concentration of zinc above the limits held to be carcinogenic. According to L. Johannson and T. Weijola, 35% of all the tests of drinking water in Finland show a concentration of over 8 mg. Zn/l; in certain cases even 50-60 mg. Zn/l. In other advanced countries (U.S.A., Germany, England, and France) the concentration of zinc in drinking water was found, in certain cases, to be even greater. According to my observations, the influence of the process of corrosion, particularly in zinc galvanized waterpipes, is decisive.

Experiment A. - I examined the tap water of different countries (Germany, Italy, France) and of different cities (Bremen, Hamburg, Essen; Rome, Milan,

Naples, Paris, Cannes), and I found a zinc concentration of 8-50 mg/l. I also examined the waterpipes of these various places and found that their internal linings were of galvanized zinc.

Since each man needs about 3.0 liters of water a day, many people, after a few decades, receive through their drinking water the optimal concentration of zinc which, in the long run, can cause tumors.

According to the results obtained from the research of Halme and Mihalowski, we know that today many cases of cancer are actually "metal cancers"; particularly from zinc accumulated daily for decades.

✓ Experiment B. - I repeated the experiments of Halme and Mihalowski on mice resistent to tumors. As drinking water I used mineral water from the state springs of Fachingen (Germany), which had a zinc content of 0.016 mg/l. I then artificially added 10-20 mg. of zinc chloride to this mineral water.

1st Group: The first tumor appeared 6-8 months after the start of the experiments. The results are: seminoma, cancer of the bone marrow, and cancer of the uterus. Of the 100 test mice, 10 got cancer (7 + 3).

2nd Group: 40 mice were used in the test. They received mineral water from Fachingen which had no zinc chloride added. None of the animals developed cancer; I found only necrosis (liver, suprarenal cortex) in two cases.

✓ Experiment C. - I found 75 mice decended from mice susdeptible to tumors and used drinking water containing 10-20 mg. of zinc per liter. The first tumor appeared 5 months after the beginning of the experiment. The symptons were: pulmonary adenoma, cancer of the marrow, uterine cancer, and hemangioma. Of the 75 mice, 9 developed cancer.

I then used 25 mice in a test in which I administered mineral water from Fachingen with no zinc chloride added. Of this group of mice, one developed pulmonary adenoma and one hemangioma.

Regarding this situation, Prof. Halme has made some very important observations: "The results give us some very important perspectives on the frequent assertion that cancer, and in particular pulmonary cancer, has in the last few decades been increasing throughout the technologically advanced nations. It must be remembered that in the more recent generation of mice, there are many cases of pulmonary cancer and that these tumors were caused directly by a concentration of 10-20 mg Zn/l., in the drinking water.

In this case it is very interesting to note that the hematic enzime (carbonic anhydrase) is a zinc-containing substance.

Experiment D. - For this test I took 100 mice and gave them mineral water from Fachingen with 10-20 mg/l of zinc added. The animals were kept in glass cages saturated with cigarette smoke.

The first tumor showed up 7 months after the beginning of the experiment. Four of a group of 50 developed cancer (pulmonary adenoma, pulmonary cancer, cancer of the mammaries, and uterine cancer).

Another 50 mice were put to the test. They drank normal mineral water and were kept in glass cages saturated with cigarette smoke. The first tumor formed after 7 days. I must note that in this group I found no mice with cancer; only one with pulmonary adenoma and one with metaplasia of the epithelium of the bronchial mucous membrane.

I think that in this case, tobacco acts as a co-carcinogenic influence as expressed by Prof. Bauer (Heidelberg).

Experiment E. - In this experiment I caged 100 mice in groups of 20. They received Fachingen mineral water with from 0-100 mg. of zinc chloride per liter.

The experiment was continued for three years, always including the mice born during the experiment, but keeping them under special treatment and observation. In this series, I noted a total of 16 deaths due to carcinoma of the uterus, seminoma, cancer ofthe mammaries, and pulmonary cancer. The other deaths were almost always due to cases of inflammation, from the presence of benign tumors or from other causes..

In the meantime I also observed the mice born during the experiment. These mice received, from birth, drinking water containing zinc chloride. From this group I took 100 mice. I found 30 deaths due to cancer in the following forms; sarcoma, leiomyoma, pulmonary adenoma, tumor of the granular cells and fibrosarcoma. The frequency of the tumors was evenly distributed between the two sexes.

If we compare the two tables, it can be stated that among the mice of generations M1, M2, M3, and M4, (i.e., the offspring), tumors formed with greater frequency than in generations M_0 (the parents).

I found that the most favorable concentration of zinc was that of 5.0 - 20.0 mg. Zn/l.

The frequency of tumors of generation M was in total 16 per cent; while in generations M1, M2, M3, M4, it was 30 per cent.

Comparing the two tables, we find that: a) the majority of tumors occurred in mice which, from birth, had ingested zinc from the drinking water; b) the frequency of tumors in subsequent generations increased, (a fact already observed by Halme); c) the time required for the formation of tumors becomes increasingly shorter.

Her I must point out that the formation of tumors in the mice corresponds to the age of cancer in man if you calculate that one year of life for a mouse is equal to 30 years of a man's life.

In order to explain the different values in the tables for the frequency of tumors of the various generations, I radiographed the animals of the experiment and, like Halme, I made it possible to see the accumulations of zinc in the blood vessels and lymphatic glands as well as in the tumors themselves. Thus I was able to ascertain that the amount of zinc in the subsequent generations increased steadily, and that the toxic effect of zinc became steadily stronger.

Experiment F. - I used 100 mice resistent to tumors giving them regular tap water with a zinc content of 8 mg/l. The mice were kept in cages saturated with cigar smoke. The first tumor appeared after 7 months of the experiment. The results showed cases of pulmonary adenoma, pulmonary carcinoma, seminoma, and mammary carcinoma. I used another ten mice as a check and gave them water with a zinc content of 8 mg/l, as I had done in the experimental group. The cages were aired out twice daily in order to keep the mice in fresh air.

The first tumor formed 8 months after the beginning of the experiment. The result was: pulmonary adenoma, carcinoma of the kidneys, and fibrosarcoma. Having found pulonary disturbances in group a) as well as in group b), I am led to believe that the zinc was the primary cause and the smoke the secondary cause of the tumors; a result which corresponds to the opinion of Halme.

Experiment G. - As with the experimental animals, I also examined the elimination of zinc from humans, both well and ill, and I found that the elimination of zinc, using various diets with various concentrations of

zinc (5-100 mg/l.) in their water, varied from 10 to 80 mg/l.

Figure 1 clearly shows that with mice resistent to tumors, the elimination of zinc increases as the concentration of zinc in their drinking water increases.(solid line). At the same time the animals with tumors show a marked diminution in the elimination of zinc.

By means of radiographs (Figure 2), it can be clearly shown that zinc is accumulating in the bodies of the mice with tumors.

As the tumor develops and enlarges, the elimination of zinc becomes steadily weaker and the test animals do not seem to react to an increase in the zinc concentration in the water. On the other hand, the healthy animals do react with a corresponding increase in the elimination of zinc.

My experiments showed that:

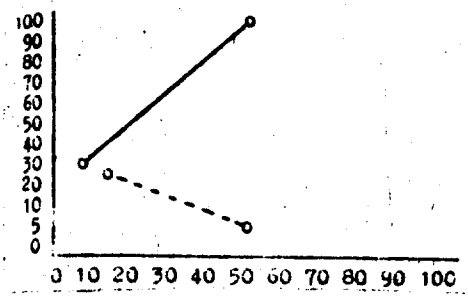
a) the elimination of zinc in healthy persons of various ages is between 11.90 and 12.50 mg. per hundred;

b) the elimination of zinc in patients with internal disorders is between 11.23 and 12.31 mg. per hundred;

c) in the majority of people with cancer, the elimination of zinc is relatively low - between 4.88 and 5.42 mg. per hundred.

It seems generally that a poor elimination of zinc is typical of cancer patients.

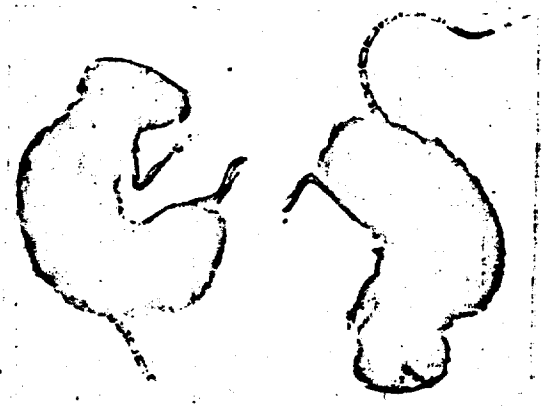
Fig. 1.



——— Mice without tumors

- - - - - Mice with tumors

Fig. 2.



Accumulation of zinc in mice with tumors

Am. J. Physiol. 80(1):31-64 (1927)

AN INVESTIGATION OF THE EFFECT OF LONG-CONTINUED INGESTION OF ZINC, IN THE FORM OF ZINC OXIDE, BY CATS AND DOGS, TOGETHER WITH OBSERVATIONS UPON THE EXCRETION AND THE STORAGE OF ZINC

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Received for publication September 17, 1926

During the last two years, there has been in progress, in the Departments of Physiology and of Ventilation and Illumination of the Harvard School of Public Health, an extensive series of experiments designed to amplify the knowledge already available upon the widespread normal occurrence of zinc in plant and animal tissues and in animal secretions and excretions; to throw new light upon the normal zinc metabolism and upon the significance of zinc to the living organism; and, finally, to obtain information more precise and accurate than now exists in the literature upon the absorption, storage, and excretion of zinc, and upon the physiological and pathological action of zinc and its compounds upon the animal body.

The purpose of the particular experiments reported in this paper was to determine whether or not zinc, administered to animals (cats and dogs) by the gastro-intestinal tract and in the form of a non-corrosive zinc compound (zinc oxide) in fairly large doses, for long periods of time, would occasion acute or chronic damage of any sort, especially to the gastro-intestinal tract itself, to the kidney or to organs in which it might be stored; whether or not zinc taken in this way would be stored and, if so, in what organs; and by what avenues and in what amounts it would be excreted.

REVIEW OF THE LITERATURE. *Zinc absorption.* Zinc compounds taken into the gastro-intestinal tract are, according to Schlossberger (1848), Michaelis (1851), Sacher (1893), Dragendorff (1895), Von Jaksch (1910) and Bernatzik (quoted by Seiffert, 1918), converted in the stomach by the free hydrochloric acid or by free lactic acid, if it be present, into zinc chloride and zinc lactate. Both compounds are, according to the weight of authority, decomposed by the protein substances present, and protein compounds are formed which are either soluble or are in part dissolved by the free acids present. Rosow (1862), investigating the behavior of ingested zinc albuminates, introduced into the stomach of a fasting dog a bag filled with wet zinc albuminate. At the end of three hours, one-half of the albuminate was dissolved and at the end of twelve hours the bag was

empty. This indicates that zinc albuminates ingested or formed in the stomach are dissolved there, but the literature throws no light upon their further digestion, nor upon their chemical form when absorbed, nor their place of absorption. Large amounts of the more inert compounds such as zinc oxide may be only partially changed in the stomach or bowel, and considerable quantities probably pass out in the feces without any changes having occurred (Schlossberger). Indeed, Heller (1847) believed that zinc oxide was physiologically quite inert, that it passed through the gastro-intestinal tract unaffected, and that he could recover it quantitatively in the feces.

Cushny (1924), discussing the general question of the absorption of the heavy metals, wrote: "The form in which the metals are absorbed is quite unknown, but it is not unlikely that they are taken up in insoluble forms by the leucocytes and thus carried into the tissues." This statement is especially interesting to us in view of the fact that the white cell counts in our zinc-fed animals have tended always to be high, though never above the upper limits reported in the literature as occurring in normal animals.

Zinc transportation. The opinion appears to be general that zinc, once it is absorbed, is carried by the blood stream (Michaelis, Delezenne (1919), etc.). Whether the blood plasma or the blood corpuscles act as vehicles remains somewhat uncertain. The only data available on this subject are contained in a paper by Delezenne in which he reported experiments showing that the plasma contained approximately one-tenth only of the total zinc in blood, the rest being present in the cells. Red cells showed only a trace of zinc while leucocytes were very rich in it. This finding ought undoubtedly to be confirmed with the present more exact methods of analysis, especially since the figures given by Delezenne for zinc in mammalian blood, 15 to 25 mgm. per thousand, are decidedly higher than the figures which Lutz (1926) and Fairhall (1926b) in this laboratory have repeatedly obtained for rat, cat and human blood.

The question as to whether or not the lymph stream also transports zinc is another subject for further study. Michaelis investigated the zinc content of the thoracic duct of four horses which had been fed zinc oxide at various times before their death (one and a half to three hours before). In no case was he able to detect zinc in the thoracic duct lymph, although he found zinc in the blood of the portal vein of one of these animals which had twice, previously, taken 22.5 gm. of the preparation. The methods of zinc analysis current in Michaelis' day were not, however, delicate enough to detect very small amounts of the metal.

Zinc storage. Much has been written upon the question of zinc storage, following zinc administration, but, unfortunately, the great majority of investigators (Michaelis, Mazkewitz (quoted by Sacher), Lehmann (1897), Brandl and Scherpe (1899), Jacobi (1899), Seiffert, and Salant, Rieger and

Treuthardt (1918), etc.) report the finding of zinc in various organs and tissues, apparently without knowing that all organs and tissues have a normal zinc content which, once known, obviously becomes a matter of considerable importance before storage of zinc is inferred.

Owing to the absence of normal standards of zinc occurrence in tissues and to the frequent use of poor analytical methods as well, one is justified, in our opinion, in concluding from the literature no more in regard to the storage of zinc in the body in excess of the normal requirements than that the metal is taken up from the blood stream in large part by the liver and is temporarily stored in this organ during the process of gradual excretion. It is possible, but not proved, that zinc may also be stored in smaller amounts in other organs—e.g., the pancreas, thyroid, spleen, bone and kidney—but whether this storage is a transient one or whether some one organ or tissue may store zinc for years as the bone does lead (Minot, 1924) previous work on zinc gives us no convincing evidence.

Zinc excretion. The consensus of opinion (Schlossberger, Michaelis, Mazkewitz (quoted by Sacher), Sacher, Lehmann) in the literature in regard to the excretion of zinc is that absorbed zinc, taken up from the blood and temporarily stored by the liver, is excreted in small amounts by the liver into the bile, but, in the main, is gradually liberated by the liver to the blood stream and is then excreted in minor amounts into the urine by the kidney, but chiefly by the gastro-intestinal tract into the feces. According to Lehmann, the excretion of zinc through the cecum and colon is three to four times as great as through the stomach. Delezenne has reported the finding of zinc in normal pancreatic juice, an interesting fact in view of experimental results which we shall present.

Lewald (quoted by Sacher) brings some evidence to bear that, following the ingestion of zinc oxide, zinc is excreted in milk, but since he presents no figures for normal zinc in milk (Birckner, 1919) his observations are suggestive rather than conclusive. A high zinc content in the skin of animals to which zinc has been administered has been interpreted by Salant, Rieger and Treuthardt as indicating skin excretion of zinc. Recently, Drinker and Fairhall[1] have definitely proved the skin to be a route of normal zinc excretion by demonstrating the presence of zinc in normal sweat.

Studies on the excretion of zinc administered experimentally began as early as 1816 but the early work (Bouchardart, 1816 (quoted by Schlossberger), Heller, Schlossberger, Michaelis, Mazkewitz (quoted by Sacher), Sacher) has little or no significance for us because of the poor methods of zinc analysis used and because of the absence of figures upon normal zinc excretion.

Lehmann, studying the hygienic significance of zinc, fed 1052 mgm.

[1] Unpublished data. Personal communication.

of zinc carbonate (553 mgm. zinc) to a dog in a single dose and recovered in the urine, 8.5 mgm.; in the feces, 540 mgm.—a total recovery of 548.5 mgm. of zinc. In a similar experiment, the same author after the same dose of zinc, recovered 6.9 mgm. in the dog's urine and 400 mgm. in the feces (some lost). A third dog received zinc carbonate by mouth over a period of eleven months. During a period when the daily dose of zinc was 553 mgm., the daily output of zinc in the urine was 4 to 6 mgm. Here again, the normal zinc content of urine and of feces is not taken into account, nor have we any proof that all the zinc in the feces had been absorbed and excreted, though doubtless zinc carbonate is somewhat more soluble in gastric juice than zinc oxide, and, therefore, more likely to be completely absorbed. In spite of these objections, Lehmann's experiments offer qualitative proof at least that the major part of ingested zinc leaves the body in the feces, a minor part in the urine. From the high zinc content of the liver and the bile of his experimental animals, Lehmann inferred zinc excretion in bile, while high zinc figures for the cecum and colon (three to four times as high as in the stomach) led him to regard the large bowel as an important agent in ridding the body of this metal.

Brandl and Scherpe, studying the excretion and storage of zinc, fed zinc malate pills and a solution of the double salt of sodium zinc tartrate to rabbits, guinea pigs and dogs. The amount of zinc recovered in the feces was compared with the amount fed and was shown to constitute a very large fraction of the dose. For example, of 995 mgm. of zinc, given to a dog in the form of a solution of zinc malate mixed with meat, 967 mgm. were recovered in the feces and in the contents of the bowel at autopsy; again, of 420 mgm. of zinc (fed to a dog as the double salt of sodium zinc tartrate) 419 mgm. were recovered in the feces and in the intestinal contents. Although these recovery figures are not quite so good as they appear since the normal zinc content of food and of feces was not taken into account, nevertheless they do indicate, as does the rest of our qualitative evidence, that ingested zinc can be largely recovered in the feces. What proportion of it is absorbed and subsequently excreted, and what proportion passes through the bowel unabsorbed, we are not prepared to venture an opinion.

Following the feeding of zinc oxide to a calf and to a sheep, Lander and Edwards (1912) express the opinion that by far the greater part of the zinc ingested passes out in the feces as zinc sulphide.

Salant, Rieger and Treuthardt administered to animals by mouth, subcutaneously and intravenously zinc malate and zinc acetate. The fate of the zinc when injected intravenously indicated that the gastro-intestinal canal was the chief organ of elimination, since, by the end of two or three days after its administration, one-third to nearly one-half of the zinc given was recovered from feces and from gastro-intestinal contents. The urine, according to these authors, contained but minute quantities of

zinc, amounts which were practically within the limits of experimental error. Although the amounts of the metal present in the skin were small, the evidence, in the opinion of these investigators, was sufficient to warrant the conclusion that zinc may either be stored or eliminated by this organ.

Summarizing briefly the opinions expressed in the literature in regard to the excretion of zinc, we may say that absorbed zinc begins to be excreted promptly after its absorption. A small fraction leaves the body in the urine; the major amount, however, is excreted along the alimentary tract, some of it in the bile, possibly some of it in the pancreatic juice. If the absorbed zinc is greater in amount than the excretory mechanism can handle at once, zinc is temporarily stored, chiefly in the liver, and liberated by this organ as fast as it can conveniently be excreted. A small amount of zinc is excreted in sweat and probably some also in milk.

Local and general action of ingested zinc oxide. The literature contains a vast amount of data upon the results of feeding zinc oxide to man and to animals. In evaluating these data, there is, in our opinion, one all-important question which must be asked in regard to each group of experiments —Was the zinc oxide used chemically pure? This question is, in fact, applicable to all experiments with zinc oxide, regardless of the portal of entry, but we shall consider here only ingestion experiments. The chief contaminations to which zinc oxide is subject are lead, cadmium, arsenic and antimony—substances carried over from the ore in the process of manufacture. Occasionally investigators, working upon the effect of zinc and its compounds upon health, admit lead contamination (Murgia, 1906; Seiffert), and attempt to determine what part zinc plays in causing a given set of symptoms by excluding the disturbances obviously caused by lead, and attributing everything else untoward to zinc. Other writers (Landouzy and Maumené, 1850; Houvler, 1850; D'Amore, Falcone and Maramaldi, 1892) describe rapid and overwhelming intoxications which they attribute to zinc oxide—intoxications so extraordinarily severe and so obviously and totally out of accord with the bulk of experimental evidence, that we are led inevitably to the conclusion that some contaminating substance, probably lead or arsenic, and not zinc oxide itself, occasioned the damage reported.

Experiments reported by D'Amore, Falcone and Maramaldi serve as an excellent illustration of the point which we wish to make. These investigators fed zinc oxide, "the purity of which had been well ascertained," to dogs. The dose at first was 1 gram daily; after three to five days, ½ gram daily. For the first four or five days the animals vomited one-half to one hour after eating; after this the oxide was retained. The dogs at once began to lose weight; they suffered from muscular weakness, which increased to such an extent that they were unable to move. Sensibility

disappeared first in the hind limbs. A remarkable pallor of the mucous membranes developed.

There was change in size and shape of the red cells, a diminution in their number, a tremendous increase in the number of white cells, and a great decrease in the amount of hemoglobin. The urine diminished in amount, and showed amounts of hemoglobin which increased steadily until it became a deep red in color. No red blood cells, however, were present. There was albuminuria (7 to 9 grams per liter of urine), glycosuria (a maximum of 15 to 17 grams of sugar per liter), and the urinary sediment showed hyaline and granular casts.

The dogs died on the tenth to the fifteenth day from the beginning of the experiments. Pathological examination of the organs obtained at autopsy showed, according to the authors, areas of inflammation in the stomach and duodenum (but not of a severity in accord with the importance of the intoxication), together with fatty degenerations of the liver, the pancreas, and the kidney, especially of the epithelium of the tubules. These findings should be borne in mind in order that they may be contrasted with the negative results which we obtained when, using zinc oxide of a high degree of chemical purity, we attempted to repeat these experiments.

With the possibility of contamination firmly in mind, let us now consider the conflicting mass of experiments which have been reported upon the ingestion of zinc oxide by men and by animals. One group of investigators (Heller, Wibmer (quoted by Helpup, 1889), Barbier, Herpin, Home (all three quoted by Sacher), Pera (1911), Lander and Edwards, etc.) has reported negative; another group (Orfila (quoted by Schlossberger), Werneck (1831), van Hasselt (quoted by Helpup), Michaelis, D'Amore and his associates, Hadlich (1908)), positive results following the taking of zinc oxide. Heller in 1817 reported that no symptoms followed the internal administration to man of zinc oxide in sugar solution in doses of 20, 60, and 120 grains a day—a result which led him to conclude that zinc oxide was physiologically inert and, therefore, useless as a medicine. Schlossberger wrote concerning "two people who, suffering from severe nervous troubles, gradually arrived in subsequent use of the metal (zinc oxide) at very strong doses, at which only with one of them sometimes slight vomiting appeared. . . . . The second patient was a student who on account of epileptic fits of many years' standing had taken zinc oxide many weeks."

In a discussion of the medicinal use of zinc oxide, Sacher referred to cases described by Barbier, by Herpin, and by Home in which doses of zinc oxide as large as 1.2 grams, 2.0 grams and 2.5 grams were taken in the course of a day without causing vomiting.

Wibmer (quoted by Helpup) gave a young dog from the middle of September to the middle of October, daily, 10 grains of zinc oxide. Appetite

and stools remained normal. The animal then tolerated to the end of October, 15 grains daily, consuming in six weeks an ounce of the oxide. Autopsy showed everything normal. Lander and Edwards in 1912 gave a calf and a full grown sheep a total of 2220 grains (148 grams) each, in the course of twenty-six days. For fifteen days each animal received 4 grams per day, and then for eleven days, 8 grams per day. No loss of appetite or condition—indeed no symptoms of any sort—were observed.

Pera, arguing in favor of zinc white as a substitute for lead white, cited the absence of toxicity as one of its advantages. He said that zinc oxide has been used as a teniafuge in a dose as large as 30 grams of zinc oxide powder in 60 grams of syrup for the first day and 15 grams the following day—and this without causing the least accident.

In contrast to these negative findings, we have in the literature accounts by a second group of investigators of active symptoms ensuing from the administration of zinc oxide. This group observed in man, burning in the stomach, eructations, griping, increased thirst, loss of appetite, vertigo, hot flashes, and general exhaustion following single doses of 6 to 8 grains of zinc oxide. With 10 grains, Werneck reported, in addition to these symptoms, sharp pains in the diaphragm and between the shoulders, later vomiting and the passage of a thin stool, with exhaustion lasting twenty-four hours.

The following protocol, taken from Michaelis, illustrates the type of result reported by the positive group of experimenters upon the administration of zinc oxide to animals.

A growing cat received in milk with white bread 10 grains of zinc oxide *via humida parata*. Restlessness, vomiting and diarrhea followed. There was much bile in the fecal masses. For three days the animal received on each day half the former dose. On the fourth day it received 6 grains, upon which the number of the stools increased. On the following day, 8 grains. The animal became restless, and devoured greedily what was thrown to it. In the afternoon there was retching and vomiting five times; at the end, bile-stained mucus. In the beginning, the stools were normal, later thin and fluid. On the following day, the animal was dull, apathetic, and ate first in the afternoon some food which had been mixed with 4 grains of zinc oxide. After a half hour the same disturbances as the day before appeared. The cat was very weak and could scarcely stand erect. Dose omitted on the seventh day. On the eighth day, 6 grains, loss of appetite and diarrhea. On the ninth day, perhaps 2 grains, which was soon got rid of; now there appeared a peculiar twitching of the limbs of the animal. It curved itself and stretched continually. Set the cat free and stretching continued. No desire to run away. This symptom developed on the next day into true cramps which attacked especially the hind extremities. The animal then fell on its side and went into violent twitchings, cried out and had tears in its eyes. After two minutes the attack ended and the animal was then free of the twitchings. The two following days, the same dose; no cramps. However, if a mouse was held before this animal, it at once developed vigorous cramps. Fourteen more days the animal received zinc; cramps were observed only once. Then it was killed after it had taken 92 grains (6 + gm.) of zinc oxide.

Autopsy finding: On the cardia two small erosions,—one on the greater curvature, everywhere else the stomach was normal. In the intestine there was much mucus and some reddening, beginning catarrh. Everything else was normal. In the liver 0.024 gm. of zinc oxide was found. Relatively much in the bile. Brain undetermined. Spinal cord showed nothing abnormal. In the urine during the entire time zinc was demonstrable.

The probability that the oxide used by Werneck, Michaelis, D'Amore, etc., contained some contaminating toxic substance is suggested by the conflicting evidence presented by other observers. This probability is converted, in our opinion, into a practical certainty when our own experience is brought into evidence.

EXPERIMENTAL METHODS. *Diet.* Besides the control animals, ten cats and three dogs were used in these investigations. The cats were kept in individual wire cages and were fed once a day canned salmon and milk. We have in the past in this laboratory kept cats fat and healthy under a similar régime for as long as two and a half to three years. The dogs were kept in individual cages at night, and in the daytime, except in very bad weather and on Sundays, were out-of-doors in an enclosed yard. The dogs were fed, daily, milk and a dog mash containing bread crumbs and ground-up meat. During a large part of the experimental period, they also received twice a week Hamburger steak and bones. We would like to emphasize here the importance of bones in the diet of a dog in captivity. During a part of the experimental period when the bones were omitted, the youngest of our dogs lost weight, although he was very lively and apparently perfectly healthy. As soon as bones were restored to the diet, his gain in weight was rapid.

Dosage. Zinc oxide was administered to these animals daily in the form of a dry powder mixed with the food. At the beginning of the experiments the cats were given, in milk, zinc oxide suspended in gum acacia, but this proved a less satisfactory method of dosage than to mix the dry powder with salmon.

Chemical analysis of the zinc oxide used gave the following results—figures which show it to be an extremely pure product:

	per cent
Insoluble in HCl	0.007
CO_2	0.025
Total S as SO_3	0.001
Cl	0.004
CdO	0.004
PbO	0.023
Bi_2O_3	under 0.001
CuO	under 0.001
Fe_2O_3	under 0.001
MnO	under 0.001
As_2O_3	under 0.001

Sb_2O_3	under 0.001
ZnO (diff.)	99.799
Red. Powder as SO_2	0.010
Water soluble salts dried at 110°C	0.098

The amounts of zinc oxide administered daily to the cats ranged from 175 mgm. to 1 gram. The only unfavorable symptom which appeared in our animals was a lack of appetite in cats on large doses (above 600 mgm.), a symptom which we attribute to the disagreeably sweetish taste of the oxide-containing food, and not to nausea. We have had no difficulty at all with daily doses of 600 mgm. or less. Cats taking 200 to 600 mgm. ate heartily and regularly.

The dogs received 500 mgm. to 1 gram of zinc oxide daily mixed with food, and as a rule took it readily. The daily doses administered to the individual animals, in terms both of milligrams of zinc oxide and in milligrams of zinc per kilogram of animal, may be seen in table 1.

Methods of observation and collection of specimens. The animals were weighed weekly throughout the experimental period and were observed daily for signs of vomiting, diarrhea or other indication of illness.

Urine specimens were collected from the cats as follows: The animal was fed in the morning of the day preceding the collection of a 24-hour specimen. At 9 o'clock on the following morning, he was placed in a rectangular, oblong, copper-netting cage suspended over a large enamel pan, and left there with distilled water in a copper container to drink but no food. Under circumstances such as these, cats frequently neither void nor defecate for twenty-four hours. When such was the case, at the end of the twenty-four hours the animal was placed upon a cat board. Often the excitement incident on this caused him to void at once into a glass beaker held to the urethral opening. If not, we found it relatively easy to empty the bladder completely by grasping it through the abdominal wall and squeezing it gently and firmly. The maneuver resembles closely that applied to the uterus in expelling a retained placenta. An experienced person can readily tell by its small size when the bladder is completely empty. We could depend upon it that urine specimens obtained in this way were uncontaminated with particles of zinc oxide which might be upon the mouth or fur of the animals. Occasionally the cats urinated into the pan, but all pan urine specimens were discarded for fear of contamination. On a few occasions 24-hour fecal specimens were collected and analysed for zinc.

Urine specimens were collected from the dogs as follows: For the 24-hour period of the collection the dog was kept in a freshly washed cage. If at the end of the period he had neither urinated nor defecated—and this was almost always the case—the dog was taken out-of-doors on a leash, and the urine, which the animal usually voided very promptly, collected

in an aluminum pan. *Feces* were collected similarly. At the beginning of the experiment the dogs were taught not to soil their cages, just as a pet dog is house broken, and they soon became accustomed not only to retaining urine and feces for a twenty-four hour period, but also to voiding and defecating at the end of this period into pans held in the proper position. The reasonable uniformity of volume of the specimens collected and of their zinc content have convinced us that our collections were complete and representative ones.

Urine specimens were always examined for albumin by the sulphosalicylic acid test; for sugar, with Benedict's reagent; and the sediment, for casts, pus cells, blood cells, etc.

Blood studies. Hemoglobin determinations and blood cell counts were made on each animal (except on three cats killed early in the series) just before it was killed for autopsy. Blood counts were also made on a few animals in the early part of the experimental period. More frequent blood studies were considered unnecessary because of the continuous good health of the animals.

Zinc dosage was discontinued in the case of four cats two weeks previous to autopsying them. Each one of these cats had an experimental mate which had been on the same zinc dose for the same period of time. Each of these latter animals was killed upon the same day that zinc dosing of its mate was discontinued. The object of this procedure was to determine how rapidly zinc which might be stored in the body would be eliminated.

Autopsies. The animals were killed at the end of the experiment by being bled to death under ether anesthesia, all the blood being saved for zinc analysis. Each animal was then skinned and the hide saved for analysis. Hides of zinced animals were washed with distilled water before ashing. Next, an autopsy was done, each organ being weighed, a section taken for histological study, and the rest saved for zinc determination. Large samples of muscle from the hind legs and of fat were taken for determination of zinc concentration in these tissues. In all cases, the gastro-intestinal tract from the pharynx down was slit open after removal from the animal and thoroughly washed with running water before being ashed. In the case of zinced animals, the mouth and pharynx were also thoroughly washed with distilled water as soon as the carcass was dismembered. In order to separate the bones from muscle, the carcass of each animal was cut up and boiled in distilled water for approximately six hours. The bones were then carefully picked out, ashed and analysed separately from the main body of muscle and connective tissue. This latter material was evaporated to dryness, ashed, and analysed. The zinc figures for muscle obtained in this way were checked by and averaged with analyses upon smaller samples of muscle cut from the thighs.

Chemical analyses. All tissues were dried, charred, and then ashed in an

... observations and autopsy findings upon experimentally zinced animals

Animal number and sex	Average daily dose: Zinc oxide	Average daily dose: Calculated as mgm. zinc per kgm. of animal	Duration of zinc administration	Weight: At beginning of zinc administration	Weight: At end of zinc administration	Blood findings: Week of observation	Blood findings: Hb	Blood findings: R.B.C.	Blood findings: W.B.C.	Urine findings: Number of specimens examined	Urine findings: Albumin	Urine findings: Sugar	Urine findings: Sediment	General health during experiment	Autopsy findings
	mg.		weeks	kgm.	kgm.		per cent								
Cat 9♂ (castrated)	250	33.8	10 on zinc 3 off zinc	4.8	6.4 At end 2 weeks off zinc, 6.5	12th	80	11,089,000	25,000	7	Positive (trace) twice in normal period. Negative thereafter	Negative throughout	Nothing significant	Health excellent throughout. Appetite good. No vomiting, diarrhea, muscular weakness, tremors, nor paralyses. Fur exceptionally thick and lustrous. See figure 1	All organs examined grossly and histologically and found to be normal
Cat 7♂	250	41.1	10	4.3	4.8	10th	100	12,096,000	32,900	8	Positive (trace) throughout both normal and zinc periods	Negative throughout	Nothing significant	Same as above	Same as above
Cat 3A ♀	200	44.0	31	3.8	4.1	5th 31st	— 85	12,237,000 9,978,000	17,400 15,200	5	Positive (trace) throughout	Negative throughout	Nothing significant	Same as above except for period of moderately severe respiratory infection, involving no weight loss, from which cat, to all outward appearances satisfactorily recovered	Only gross pathological finding was in posterior lobe of right lung where there was a small area containing a calcareous centre surrounded by a small amount of pus. All organs examined histologically and found to be normal except posterior lobe of right lung, which showed a patch of bronchopneumonia. Lung findings evidently incident to the respiratory infection, and not experimentally significant
Cat 2A ♀	200	52.7	31 on zinc 2 off zinc	2.8	3.6 At end 2 weeks off zinc, 3.3	5th 33rd	— 95	9,121,000 10,122,000	20,500 30,100	8	Positive (trace) throughout	Negative throughout	Nothing significant	Same as cat 3A. See figure 2	No gross pathological findings. All organs examined histologically and found to be normal except lungs which showed rather extensive peribronchial infiltration with lymphocytes, and a general increase in lymphatic tissue, finding not significant experimentally
Cat 8♀	300	64.4	11	3.4	3.7	11th	75	9,264,000	18,800	6	Positive (trace) twice in normal period. Negative thereafter	Negative throughout	Nothing significant	Four weeks after being put on zinc, 4 kittens were born to this cat. During week previous to their birth, she became severely excoriated around anus, a local irritation, unaccompanied by diarrhea or obstruction, which improved on treatment. A few days later she developed a very severe respiratory infection. After the kittens were born, the anal excoriation disappeared rapidly and there was a gradual recovery from the respiratory disease. Even though laboring under the double strain of nursing kittens and of the respiratory infection, this cat recovered remarkably well, and otherwise was in excellent health throughout the experiment	No gross pathological findings except a few small dark brown areas on outer surface of kidney. Examined histologically these proved to be very small highly cellular tumors, made up of large oval cells. Otherwise the kidney and all other organs were normal
Cat 10♂	300	66.2	11 on zinc 2 off zinc	3.0	4.1 At end 2 weeks off zinc, 4.1	13th	95	11,508,000	35,600	11	Positive (trace) throughout both normal and zinc periods	Negative throughout	Nothing significant	Excellent throughout except for a mild respiratory infection during the 4th week of zincing	All organs examined grossly and histologically and found to be normal

Animal number and sex	Zinc oxide	Calculated as mgm. zinc per kgm. of ash	Duration of zinc administration	At beginning of zinc administration	At end of zinc administration	Week of observation	Hb	R.B.C.	W.B.C.	Number of specimens examined	Albumin	Sugar	Sediment	General health during experiment	Autopsy findings
	mg.		*weeks*	*kgm.*	*kgm.*		*per cent*								
Cat 1 ♀	175 354	38.2 83.2	8 45 } 53	3.1	3.5	27th 53rd	— 85	10,152,000 11,711,000	32,300 16,700	7	A trace in three specimens	Negative throughout	Nothing significant	Health excellent throughout. There was occasional vomiting, but no more frequently than often occurs in a healthy pet cat during the course of a year. A small spot of mange, appearing over one eye, in the 11th month of the experiment yielded readily to treatment. See figure 3	All organs examined grossly and histologically and found to be normal
Cat 3 ♀	316 681	76.9 188.5	8 8 } 16	2.9	2.5					1	Positive (trace)	Negative	Nothing significant	Health excellent throughout. Occasional but not significant vomiting. This cat gained weight on the smaller dose but lost it on the larger one, apparently because the large dose of sweetish oxide rendered the food somewhat unpalatable and the cat, consequently, ate less than usual	Only gross pathological finding was the pancreas, which was markedly enlarged, hard in texture, and covered with nodules. All organs were examined histologically and found to be normal except the pancreas, which showed a generalized fibrous overgrowth affecting the entire organ. No evidence of any acute process
Cat 4 ♂	614 1000	117.4 223.0	8 13 } 21 on zinc 2 off zinc	3.8	3.1					1	Positive (trace)	Negative		Same as cat 3. An area of mange appeared on one leg but this responded readily to treatment	Same as for cat 3, except that grossly, the pancreas seemed neither so large nor so hard
Cat 5 ♂	627 1000	152.6 267.6	8 13 } 21	2.9	2.6					1	Positive	Negative		Same as cat 3. Severe mange appeared on one leg but disappeared readily under treatment	Grossly pancreas was enlarged, hard, and covered with nodules. Unfortunately no histological sections were made of the organs of this cat
Dog 4 ♂	500	36.1	19	9.9	11.9	3rd 19th	90 100	8,432,000 8,649,000	24,200 17,100	15‡	Positive (trace) in all but five, four of which were in zinc period	Negative except for one specimen in middle of zinc period	[illegible] showed [illegible] stained [illegible] cellular masses resembling [illegible] not definitely identifiable as cells or casts	Health excellent throughout. Appetite good. No intestinal nor nervous disturbances of any kind. He was sturdy and vigorous, with sleek and glossy coat. For a period during zinc administration, when bones were omitted from the diet, he lost weight, but when bones were included again, the loss in weight was fully and rapidly regained. See figure 4	All organs examined grossly and histologically and found to be normal
~~Dog 5 ♀~~	~~500~~	~~39.9~~	~~3~~	~~6.5~~	~~6.6~~	~~3rd~~	~~70~~	~~4,561,000~~	~~18,300~~	~~5~~	~~Four in normal period negative. The one specimen in zinc period +++ (one week before death)~~	~~Negative~~	Abundance of [illegible] cells in the [illegible] urine	This dog was in poor condition when we first obtained her. During the three weeks normal period preliminary to zincing, she improved markedly, gained nearly a kilo in weight and seemed quite lively and healthy, though when taken out on a leash she sometimes exhibited a peculiar sidling gait and was reluctant to run along with the other dogs. About ten days after zinc was begun she suddenly became utterly apathetic. This was her one outstanding clinical symptom. (Urine showed pus and albumin.) She practically refused to walk when taken out, although able to do so when compelled. There were no signs of intestinal disturbance, no discharges, no tremors nor paralyses. This lasted two days. In another couple of days she was apparently well. No relapses occurred. About a week later she was found dead (for some hours) in the yard without gross evidence as to the cause of death	Only gross pathological finding was slight infection of the meninges. All organs were examined histologically. The kidney showed swelling and overfilling of kidney vessels. The lung showed an exudate: much fibrin, many leucocytes: but no definite solidification. Other organs were normal. Diagnosis: *Distemper pneumonia*
Dog 2 ♀	1000	76.5	15	9.9	10.8	3rd 15th	90 100	9,176,000 8,835,000	20,900 19,100	14	Negative throughout both normal and zinc periods	Positive once in normal period and twice about middle of zinc period	Nothing significant	Same as dog 1, except that there was no weight loss when bones were omitted from the diet, a difference in reaction probably due to the fact that dog 1 was a young growing dog whereas dog 2 was adult	All organs were examined grossly and histologically and found to be normal. Five very young embryos were found

electric muffle furnace at 450°C. The residues were extracted with 1:1 distilled hydrochloric acid and hot water, filtered, and the filter paper and contents re-ashed and extracted: this was repeated as often as was necessary for the complete destruction of all the organic matter.

Tissues were ashed and analysed in their entirety, except in the case of bones, carcasses, and dog hides, where fractions of total ash solution were taken for analysis, as follows:

	BONE		CARCASS		HIDE	
	Total volume	Volume analysed	Total volume	Volume analysed	Total volume	Volume analysed
	cc.	cc.	cc.	cc.	cc.	cc.
Cats	500	50	500	100		
Dogs	2000	100	1000	100	500	500

Iron was removed from the acid ash solutions with cupferron. The zinc and 2 to 5 mgm. of added copper were then precipitated together as sulphides in neutral or alkaline solution in the presence of a buffer salt; the precipitate was well washed with water and with hot alcohol; it was then dissolved in nitric acid, the latter fumed off, and the copper removed as sulphide in hydrochloric acid solution, leaving the zinc in pure form in the filtrate. The filtrate was next evaporated to dryness and made up to a known volume. For bones, it was necessary to use large volumes (600 to 800 cc.) when precipitating the zinc and copper together, and to repeat the precipitation, in order to eliminate calcium phosphate.

When the amount of zinc involved was in the range of 1 to 2 mgm. or less (e.g., urines and small organs), the final solutions were read by the ferrocyanide turbidimetric method. When the amount was over 1 to 2 mgm. (e.g., livers, bones, carcasses), the zinc was determined by the ferrocyanide titration method. The foregoing procedure is outlined in detail by Fairhall (1926a) in a paper published from this laboratory.

RESULTS OF CLINICAL AND LABORATORY OBSERVATIONS AND OF AUTOPSY FINDINGS. The clinical and laboratory observations and autopsy findings on the individual animals in this series of experiments are summarized in table 1. This table includes also the average daily dose of each of the animals and the duration of zinc administration.

General health. In the main, the health of all the animals was excellent throughout the experimental period. Several of the cats developed acute respiratory infections of varying degrees of severity—cat "snuffles"—during the course of the winter but all recovered satisfactorily. Spots of mange in a few of the animals, appearing early in the work, yielded readily to treatment. Dog 5, an animal in poor condition when we obtained her, very thin and with a limping gait, but chosen because of her relatively

small size, died of distemper three weeks after the beginning of zincing. Lungs, kidneys and meninges were involved in this infection, the dog finally succumbing to distemper pneumonia.

With these exceptions, our animals were an unusually healthy group. There was no significant vomiting nor diarrhea, no muscular weakness,

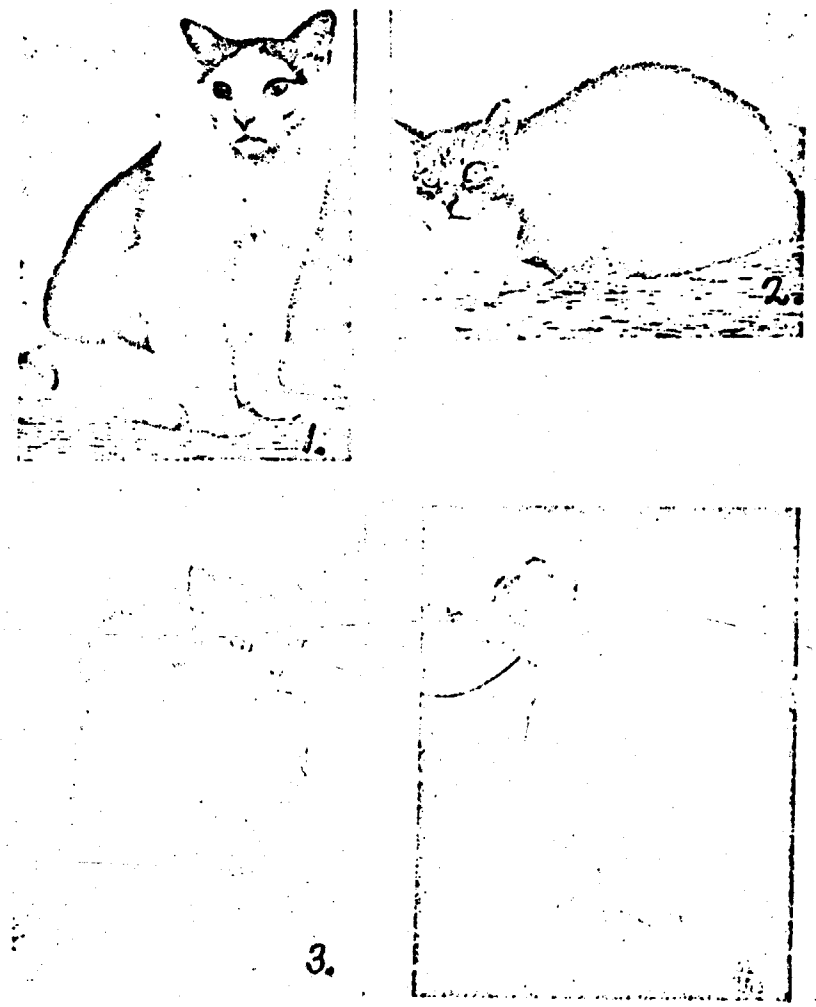

Fig. 1. Cat 9. Dose, 250 mgm. zinc oxide daily for ten weeks. Photograph taken at end of tenth week of zincing.

Fig. 2. Cat 2A. Dose, 200 mgm. zinc oxide daily for thirty-one weeks. Photograph taken at end of thirty-first week of zincing.

Fig. 3. Cat 1. Dose, 175 mgm. zinc oxide daily for eight weeks and then 354 mgm. daily for forty-five weeks. Photograph taken at end of forty-fifth week of zincing.

Fig. 4. Dog 4. Dose, 500 mgm. zinc oxide daily for nineteen weeks. Photograph taken at end of nineteenth week of zincing.

tremors, nor paralyses. The fur of the cats was exceptionally sleek, thick and lustrous; indeed, these animals were frequently shown to visitors, not as examples of the non-toxicity of zinc oxide, but as proof of the excellent health and condition in which it is possible to maintain cats in captivity over long periods of time when proper diet and housing conditions are provided.

Figures 1, 2, 3 and 4 are photographs illustrating the general healthy appearance which all of our experimental animals presented.

Weight. With the exception of three cats (nos. 3, 4 and 5) on a very heavy daily dose of zinc oxide—approximately 600 to 1000 mgm.—an amount of sweetish powder so large as to make the salmon unpalatable, all of the animals gained weight, some of them markedly, during the weeks of zincing. The three cats in question lost during sixteen, twenty-one, and twenty-one weeks of zinc, respectively, 0.4, 0.7 and 0.3 kgm.—losses not large enough to be significant. These highly zinced cats exhibited occasional vomiting, but this was neither striking nor consequential, being no more frequent than often occurs in a normal pet cat during a period of months. There was no diarrhea nor other significant evidence of digestive disturbance. Lack of appetite was the only noticeable symptom, and this we attributed to the unpalatability of the food. When one considers that these three cats were receiving during the period of lack of appetite, a daily dose of zinc oxide equivalent, in terms of a man of 70 kgm., to amounts ranging approximately between 14 and 23 grams per day, one is only surprised that they ate at all.

This excessive dose was given to a few animals, not because it was considered especially useful to determine the physiological effects of huge amounts of zinc, but rather to determine whether or not the normal zinc content of the body could be significantly increased by zinc administration, and, if so, whether or not abnormal deposits of zinc would be retained when dosing was discontinued. The results, from this point of view, will be discussed under the question of zinc storage.

Blood findings. With the exception of three cats (nos. 3, 4 and 5) which were killed first in the series and before this procedure was decided upon, blood counts and hemoglobin determinations were made upon all of the animals on the last day of the experiment. In a few instances counts were also made after a few weeks of zincing. The individual findings are given in table 1. The red cell counts (ten observations) in the cats averaged 10,728,000; the white counts, 21,500; and the hemoglobin, 88 per cent. In the dogs, the averages were as follows: red cells, 7,931,000; white cells, 19,900; hemoglobin, 90 per cent. With the exception of one red count on the dog that died of distemper (no. 5), the red cell counts on both cats and dogs were in the high range or somewhat above the high normal levels reported by other observers. A comparison of the extreme red cell counts

reported by other observers upon healthy cats and dogs with the extremes obtained by us upon zinced cats and dogs is as follows:

Cats

	R. B. C.
Goodall (1909)	7,280,000 to 8,600,000
Wells and Sutton (1915)	9,628,000 to 9,664,000
Zinced cats	9,121,000 to 12,237,000

Dogs (non-pregnant)

	R. B. C.
Dawson (1900)	6,250,000 to 8,500,000
Goodall	4,750,000 to 5,500,000
Musser and Krumbhaar (1914)	4,630,000 to 7,760,000
Wells and Sutton	6,593,600 to 7,600,000
Zinced dogs	4,564,000 to 8,835,000

These figures indicate clearly that the daily ingestion of considerable quantities of zinc by cats and dogs over long periods of time does not cause anemia in these animals.

Our white counts, though apparently high on first inspection, correspond closely to the normal figures reported by various observers. The same is true of the hemoglobin determinations. High and low normal white counts and hemoglobin determinations taken from the literature as compared with our figures are as follows:

CATS	W.B.C.	PER CENT Hb
Wells and Sutton	9,000 to 20,600	
Goodall	15,800 to 38,800	65 to 80
Zinced cats	15,200 to 35,700	75 to 100
DOGS	**W.B.C.**	**PER CENT Hb**
Dawson	11,000 to 28,000	59 to 98
Musser and Krumbhaar	8,800 to 33,050	81 to 110
Goodall	11,200 to 31,200	75 to 110
Zinced dogs	17,100 to 24,200	70 to 100

Clearly, the zinc has had no deleterious effect on the blood picture of our animals.

Urine findings. From dog 2, an animal receiving a daily dose of 1 gram of zinc oxide, fourteen specimens were collected (four controls, ten specimens during the zincing period) and all were negative for albumin with the sulphosalicylic acid test. This was the only animal, the urine of which was uniformly negative for albumin throughout both normal and zinc periods. Cats 8 and 9 showed traces of albumin in the normal period but none in the zinc period. Cats 7 and 10 showed a trace of albumin through-

out both normal and zinc periods. Control specimens of urine were, unfortunately, not obtained from cats 1, 3, 4, 5, 2A and 3A, and, with the exception of cat 1, in which a trace of albumin was found in three out of seven specimens, all of these animals showed positive sulphosalicylic acid albumin tests in all specimens collected during the zincing period. The complete absence, however, of significant findings, such as casts, white or red cells, in the sediment of the cat urines; the minute amounts of albumin detectable by the sulphosalicylic acid test; the common occurrence of small amounts of albumin in the urine of normal cats and dogs, as indicated both in the control specimens from our zinced animals and in the eight specimens collected from control cat 6, all of which were positive; and, finally, the normal kidney findings in all of the animals (except in dog 5)—these facts all indicate that the traces of albumin found in the urines of our zinced cats have no special pathological significance.

Of fifteen specimens from dog 4, four out of five were positive for albumin in the normal period, and six out of ten in the zinc period. Curiously enough, the urine of this animal in both control and zinc periods always contained bile and the sediment showed bile-stained granular masses resembling, but not definitely identifiable as cells and casts. As is so common in male dogs, a drop of pus was always evident on the tip of this dog's penis. The possibility that some sort of chronic genito-urinary infection was responsible for some of the urine findings is worthy of consideration. In any event, histological examination after death, of the liver and kidneys of this animal showed these organs to be entirely normal.

Four control urines from dog 5 were negative; one specimen collected during the zincing period, one week before her death from distemper, contained marked albumin and an abundance of pus cells—a finding which we attribute to an involvement of her kidneys in the acute intercurrent infection of which she died.

All urines of all cats were negative throughout for sugar (Benedict test). Dog 2 gave a positive Benedict once in the control period and twice about the middle of the zinc period, and dog 4 once in the middle of the zinc period. Histological examination showed normal kidneys and normal pancreases in both of these animals.

Autopsy findings. The details of the gross autopsy and of the histological findings upon all of the animals are given in detail in table 1. Two of the cats showed small pathological areas in the lungs resulting from intercurrent respiratory infection not completely resolved. The kidney of one cat showed, grossly, a few dark brown areas on the outer surface, which proved on histological examination to be small highly cellular tumors. The kidneys of dog 5 who died of distemper pneumonia showed swelling and overfilling of the vessels; the lungs of this animal showed an exudate, much fibrin, and many leucocytes, but no definite solidification. The other

With the exception of these findings, which were incidental and without experimental significance, and of fibrotic changes in the pancreases of three cats—an observation discussed in the next paragraph—all of the organs (stomach, duodenum, liver, spleen, lungs, kidneys, adrenals, reproductive glands, submaxillary gland, thyroid) were examined grossly and histologically and were found to be normal. Three cats (nos. 3, 4 and 5), on extremely large daily doses of zinc oxide (maximum, 681 to 1000 mgm.), showed at autopsy pancreases which grossly appeared harder than normal and were covered with firm nodules. Through a misunderstanding, the organs of cat 5, the first animal in the series killed, were taken in their entirety for chemical examination and no portions were saved for histological study. Presumably, however, the microscopic findings in the pancreas would have been similar to those in cats 3 and 4, since the gross appearance of each of the three organs was similar. The weight of cat 5's pancreas was 3 grams—a rather low figure. The histological reports on cats 3 and 4 are as follows:

Cat 3: Pancreas: Weight, 10 grams. Perfect acini are not common. The tissue is heavily and diffusely overgrown with fibrous elements. Fibrous trabeculae are wide and heavy and extend diffusely into the groups of acini, sometimes apparently abolishing them. No definite islets are made out. There is no distension of ducts and no evidence of any acute process—simply a generalized fibrous overgrowth affecting the entire organ.

Other organs normal.

Cat 4: Killed two weeks after discontinuance of zinc administration. *Pancreas:* Weight 3.8 grams. The gland is very firm but not so hard nor so large as the pancreas of cat 3, an animal killed during zinc administration. Histological examination shows many normal acini which are, however, sewn through with large amounts of fibrous tissue. Here and there, one can make out groups of cells constituting islands of Langerhans, but these are not very well defined. There is no hemorrhage, merely a considerable proliferation of fibrous tissue all through the stroma of the gland.

Other organs normal.

Except in the pancreases from cats 3, 4 and 5—animals receiving doses of zinc oxide far beyond any conceivable human exposure—we have never found histological evidence of pancreatic damage in zinced animals, though the number of zinced animals which we have studied in this connection includes forty individuals (rats, kittens, cats and dogs). Further discussion of the pancreatic findings in cats 3, 4 and 5 occurs in the section on zinc excretion.

Summarizing our clinical and laboratory observations and our autopsy findings upon zinced animals, we may say that we have never observed any significant clinical symptom nor obtained any significant laboratory evidence of damage resulting from the daily ingestion of zinc oxide over long periods of time. With the exception of fibrous change in the pancreases of three excessively zinced cats, histological examination following autopsy

has shown in the organs of zinced cats and dogs no damage of any sort attributable to zinc.

It is worth noting again at this point that sugar was uniformly absent from the urine of all our zinced cats, including specimens from the three

TABLE 2

Normal zinc content of urine and feces of cats and dogs

ANIMAL	DATE	URINE				FECES: ZINC CONTENT PER 24 HOURS
		24-HOUR VOLUME	Zinc content			
			Per 24 hours	Per cc. urine,	Per kgm. animal	
	1925	*cc.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
Cat 6	December 7	27	0.28	0.010	0.06	4.4
Cat 7	November 5	81	0.43	0.005	0.09	
	November 11	64	0.21	0.003	0.05	
Cat 8	November 4	64	0.11	0.002	0.04	
	November 9	36	0.14	0.004	0.04	
	November 14	86	0.06	0.001	0.02	
Cat 10	October 31	63	0.11	0.002	0.04	
	November 13	72	0.07	0.001	0.02	
Average			0.18	0.004	0.045	
Dog 2	November 21	80	0.38	0.005	0.038	
	December 4	80	0.19	0.002	0.020	25.2
	December 9	89.5	0.41	0.005	0.042	5.2
	December 15	73	0.33	0.005	0.033	
Dog 3	December 1	119.5	0.25	0.002	0.022	
	December 5	121.5	0.40	0.003	0.034	
	December 9	104.5	0.39	0.004	0.033	
	December 15	80.5	0.13	0.002	0.011	
Dog 4	November 20	195	0.55	0.003	0.064	17.6
	December 2	60	0.28	0.005	0.031	22.0
	December 9	154.5	0.59	0.004	0.060	4.2
Dog 5	November 25	100.5	0.32	0.003	0.054	
	December 9	71.5	0.37	0.005	0.056	8.0
Average			0.35	0.004	0.041	

animals with the fibrosed pancreases. Two dogs, one on one occasion, the other twice, passed urine specimens during the zinc period which were positive for sugar. One control specimen from the second dog, however,

was also positive, and both of these dogs showed normal pancreases at autopsy. The production of glycosuria by the administration of zinc acetate and malate, particularly when given intravenously, was reported in 1918 by Salant and Wise. These authors attributed the glycosuria, not to the pancreas, but to an increased permeability of the kidneys to sugar and designated the condition as a renal diabetes. Nearly thirty years previously, D'Amore and his associates reported glycosuria in their zinc oxide-fed dogs, but the probability of lead or arsenic contamination of the oxide used by these investigators leads us to discard this observation along with their other findings. Certainly, further experimental evidence is required before an association may be made between zinc and diminished pancreatic function.

ZINC EXCRETION. *Zinc in normal cat and dog urine.* The normal amount of zinc in cat urine, as indicated by our series, ranges between 0.06 and 0.43 mgm. per twenty-four hours, and in dog urine between 0.13 and 0.59 mgm., the averages for these two species of animal being 0.18 and 0.35 mgm. of zinc per twenty-four hours, respectively. Table 2 gives a series of zinc analyses upon the urines of normal cats and dogs. This detail of figures is presented because there exist in the literature no standards of normal zinc excretion in these animals.

Zinc in normal cat and dog feces. One 24-hour specimen of normal cat feces showed 4.4 mgm. of zinc while six specimens of normal dog feces showed, respectively: 25.2, 5.2, 17.6, 22, 4.2 and 8 mgm. (table 2). These figures indicate a range in the fecal zinc content of dogs very similar to that found in men on an ordinary mixed diet—i.e., approximately 3 to 20 mgm. per twenty-four hours (Drinker, Fehnel and Marsh, 1927).

Zinc in urine of zinc-fed animals. Four cats, upon daily doses of zinc oxide which ranged between 200 and 300 mgm., were included in this group, and three dogs, one of which received 1 gram of zinc oxide daily, the other two, ½ gram. The average amount of zinc excreted in zinc feeding is contrasted in figure 5 with the average daily normal urinary zinc excretion, and, in the case of the cats, with the average amount of zinc in 24-hour specimens of urine obtained from the zinced animals during a period of two weeks immediately following the discontinuance of zinc dosing. In order to show sample series of figures from which these averages were developed, the complete data upon cat 10 and dog 4 are presented in table 3, and are charted also in figure 5.

These figures show that during periods of zinc oxide feeding the amounts of zinc in the urines of cats and dogs increase markedly, and that, when zinc dosage is discontinued, urinary zinc rapidly falls to the normal level. In view of the absence of abnormal clinical findings in the urine of our zinced animals during life, and of the normal histological findings in the kidneys of these animals following autopsy, this marked increase in urinary

zinc during zinc feeding is a point especially to be noted. Batchelor, Fehnel, Thomson, and Drinker (1926) observed the same thing in their studies upon zinc excretion among zinc workmen—i.e., abnormal amounts of zinc in the urine for periods of many years, but unassociated with evidence of kidney damage.

The rapid disappearance of abnormal amounts of zinc from the urine when zinc feeding is discontinued suggests that significant abnormal

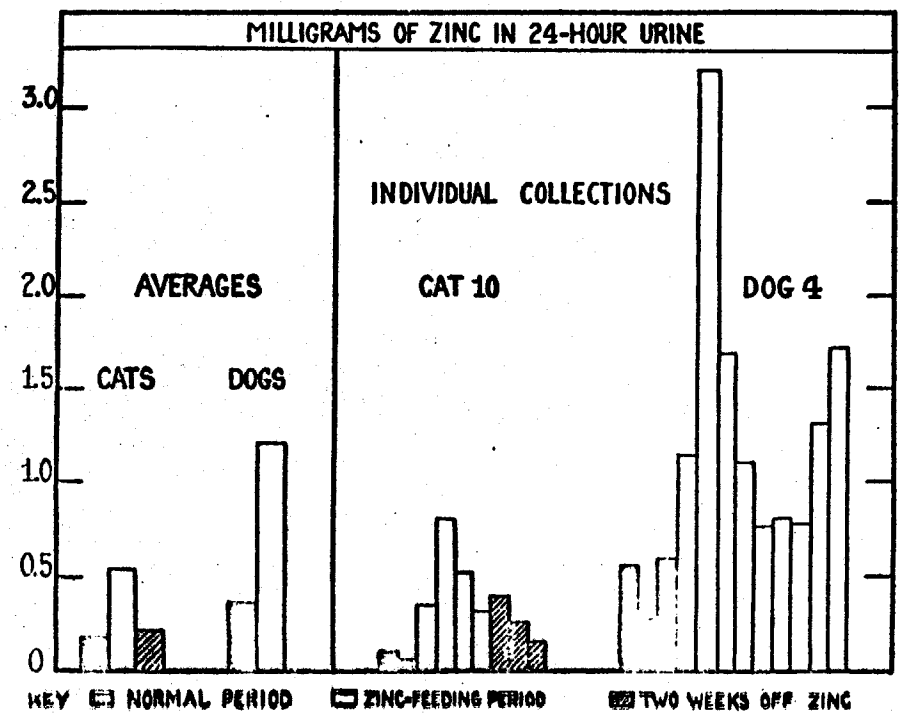

Fig. 5. Average 24-hour excretion of zinc in the urine of normal cats and dogs contrasted with the amounts of zinc excreted in the urine of the same animals during zinc feeding, and, in the cats during a 2-week period following the discontinuance of zinc feeding. Note the fall of urinary zinc to a normal level when zinc dosing is discontinued. Zinc in individual specimens from cat 10 and from dog 4 during control and experimental periods is also plotted to show 24-hourly variations in zinc excretion in spite of constant dose.

amounts of zinc are not stored in the body, but that zinc in excess of the normal body requirement is excreted approximately as rapidly as it is taken in. We have not been able to correlate the amounts of zinc in the urine with the size of the zinc dose. Dog 2, for example, on a daily dose of 1 gram of zinc oxide, excreted, as a rule, less zinc in the urine than dog 4 on a daily dose of ½ gram.

Zinc in feces of zinc-fed animals. Analyses were not made routinely

upon the feces of zinc-fed animals because, in our opinion, the results would be of little value since it would be impossible to determine how much of the zinc recovered represented zinc which had been absorbed and subsequently excreted, and how much of it represented zinc which had passed through the intestinal tract unabsorbed. A single analysis of fecal zinc

TABLE 3

Zinc in urines of cat 10 and dog 4 during control periods, during zinc feeding, and in cat 10 during two weeks following discontinuance of zinc feeding

ANIMAL	PERIOD	DATE	URINE		
			24-HOUR VOLUME	Zinc	
				In 24 hours	Per cc.
			cc.	mgm.	mgm.
Cat 10	Normal	October 31	63	0.11	0.002
		November 13	72	0.07	0.001
	Zinc feeding November 25, 1925 to February 5, 1926 300 mgm. ZnO daily	December 23	42	0.35	0.008
		January 7	52	0.80	0.015
		January 16	47	0.52	0.011
		January 27	40	0.32	0.008
	Off zinc	February 6	71	0.40	0.005
		February 12	100	0.26	0.003
		February 16	50	0.16	0.003
Dog 4	Normal	November 20	195	0.55	0.003
		December 2	60	0.28	0.005
		December 9	154.5	0.59	0.004
	Zinc feeding December 17, 1925 to April 30, 1926 0.5 gram ZnO daily	December 30	114	1.14	0.010
		January 15	122	3.20	0.026
		January 27	80	1.68	0.021
		February 6	135	1.10	0.008
		February 13	125	0.77	0.006
		March 3	95	0.81	0.009
		March 12	150	0.78	0.005
		March 30	116	1.32	0.011
		April 27	270	1.72	0.006

made on a 24-hour specimen from cat 9 (daily dose, 250 mgm. zinc oxide) gave 265.8 mgm. zinc, a figure which indicates, obviously, that ingested zinc leaves the body in major part in the feces. No special significance need be attached to the fact that in this particular instance, the zinc output for one day was greater than the daily dose. Our experience with

rats[2] indicates that a comparison of daily zinc intake with daily zinc output will reveal small lags and small spurts in zinc excretion, but that, when a comparison of the two processes over a long period of time is made, they will be found to be in an approximate equilibrium.

Pancreas as a possible factor in excretion of zinc. The fibrous changes observed in the pancreases of three excessively zinced cats (nos. 3, 4 and 5) have already been discussed. When analysed chemically, the three pancreases in question yielded amounts of zinc greatly in excess of the normal. The normal concentration of zinc in cat pancreas (see table 4) is 0.026 mgm. per gram of tissue. The concentrations in the pancreases of cats 3, 4 and 5 were, respectively, 0.410, 0.233, 0.260. Our less heavily zinced cats and dogs, when killed during the course of zinc administration, also showed a slight increase of zinc in the pancreas. At the end of two weeks following the discontinuance of zinc, the zinc content of the pancreas, in three cats subjected to this procedure, had, however, fallen practically to a normal level.

These observations, together with some unpublished ones, in which the zinc content of pancreases of animals subjected to the inhalation of zinc was found to double or even treble within three hours and to reach the high levels of cats 3, 4, and 5 within one to four days following exposure to zinc fume, have lead us to conceive of the possibility that the pancreas may serve normally as one of the agents by which zinc, or possibly other heavy metals, are excreted into the gastro-intestinal tract, and that the fibrosis in the cases under discussion may have resulted from an excessive demand upon the pancreas to exercise this function.

This suggestion is obviously a tentative one, and depends for its proof upon confirmation of Delezenne's finding of zinc in normal pancreatic juice, upon the establishment of normal standards of zinc content in this fluid, and upon the recovery of abnormal amounts of zinc in the pancreatic juice of zinced animals—an experiment which we plan to undertake at a future time. We are the more inclined to venture this hypothesis in regard to the pancreas because of the fact that, with the exception of the pancreas and the bone, the only organs which show any significant increase in zinc concentration following zinc feeding (see figs. 6 and 7), are organs taking part in the excretion of this metal, viz., liver, gall bladder and bile (analysed together), gastro-intestinal tract, and kidney. The zinc concentration of bone falls to a normal level in cats on discontinuance of zinc feeding (see fig. 6), while that of the pancreas, like the liver, falls—but not within two weeks to the normal level—thus suggesting an excretory mechanism still at work.

In summary, we may say that our evidence in regard to the excretion of

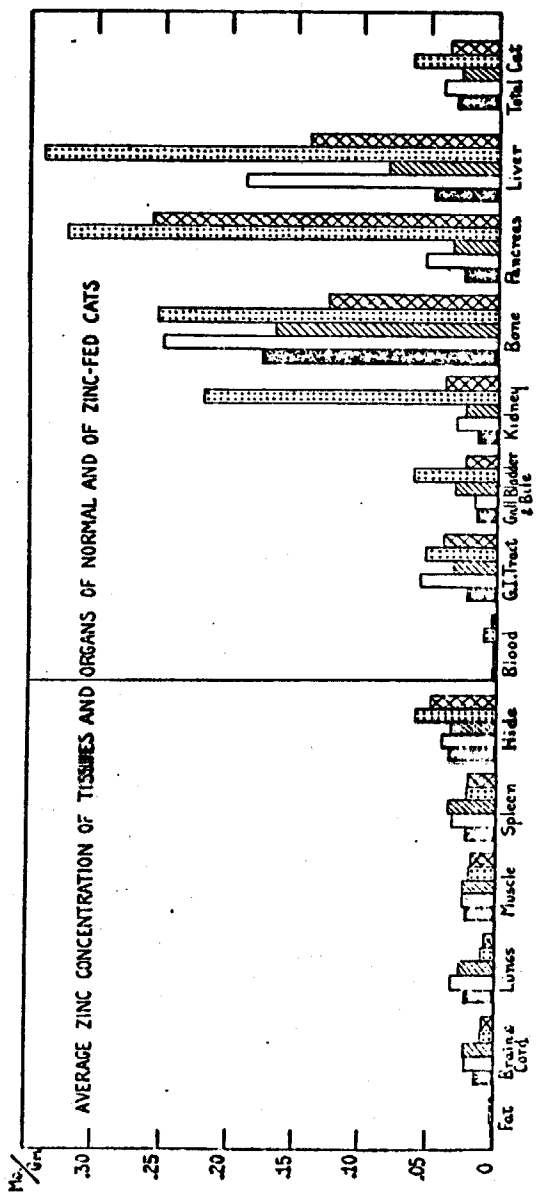

Fig. 6. Average zinc concentration of tissues and organs of normal cats (black columns); of cats receiving 200 to 304 mgm. zinc oxide daily and killed at height of zinc administration (white columns); of cats receiving similar doses for similar periods of time but not killed until two weeks after discontinuance of zinc dosing (single cross-hatched columns); of cats receiving excessively large doses of zinc oxide (681 to 1000 mgm.) and killed during zinc administration (dotted columns); and of one cat receiving a similar dose for a similar period of time but not killed until two weeks after discontinuance of zinc (double cross-hatched columns). See text for comment on noteworthy points.

zinc confirms that in the literature,—namely, that a small fraction of absorbed zinc leaves the body in the urine, but that the main bulk of it is excreted into the alimentary tract—some of it directly, some of it by the liver into the bile, and possibly some of it by the pancreas—and ultimately leaves the body in the feces.

ZINC STORAGE. *Zinc concentrations in tissues of normal cats and dogs.* Table 4 contains data upon the normal zinc concentration in the tissues and organs of cats and dogs. These figures include average normal concentrations of cat organs and tissues obtained by Lutz (1926) in this laboratory with his urobilin method (Lutz, 1925). Using the turbidimetric method of zinc analysis, we have, in a few instances (notably, gall bladder and bile, spleen, submaxillary gland, bone, brain and cord), obtained somewhat higher results than those reported by Lutz. In the main, however, our

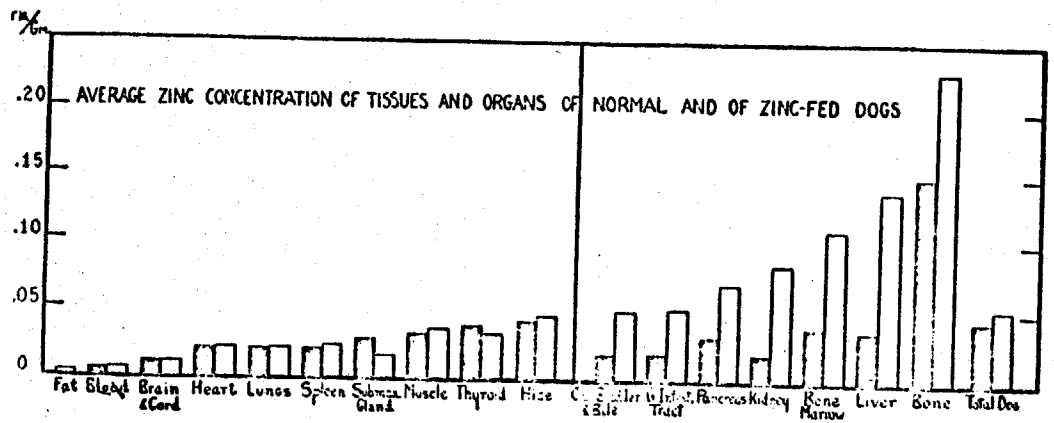

Fig. 7. Average zinc concentrations of tissues and organs of normal dogs (black columns) plotted against average zinc concentrations of tissues and organs of zinc-fed dogs (white columns). See text for comment on significant points.

figures and those of Lutz closely conform. In plotting normal zinc concentrations in cat tissue we have used an average normal obtained by averaging our normal figure with Lutz's average for the same tissue. The data upon normal dogs, however, are entirely new and are presented in detail (table 4) because of the absence in the literature of adequate normal standards of zinc concentration in dog tissues, and because of their usefulness in investigations not only upon the physiological action of abnormal amounts of zinc but upon the normal zinc metabolism as well. The percentage distribution of the total zinc content of a normal dog is plotted in figure 8. Of a total of 556.55 mgm. of zinc recovered from this animal, approximately 52 per cent was localized in muscle, 26 per cent in bone, and 13 per cent in hide.

Zinc concentrations in tissues of zinced animals. Ten cats and three dogs

TABLE 4

The normal concentration of zinc in the tissues of cats and dogs, expressed in milligrams of zinc per gram of tissue

ANIMAL	ADRENALS	BLOOD	BONE	BONE MARROW	BRAIN AND CORD	FAT	GALL BLADDER AND BILE	GASTRO-INTESTINAL TRACT	HEART	HIDE	KIDNEY	LIVER	LUNGS	MESENTERIC LYMPH GLANDS	MUSCLE	PANCREAS	REPRODUCTIVE TRACT: Ovaries	REPRODUCTIVE TRACT: Testes	SPLEEN	SUBMAXILLARY GLANDS	THYROIDS	TOTAL ANIMAL
Cats (♂): Lutz* (1926)	*	0.005	0.126		0.009		0.006	0.019		0.039	0.014	0.041	0.015		0.021	0.025		*	0.013	0.010	0.038	0.033
average..... Our average..	*	0.002	0.227		0.020	0.001	0.024	0.025		0.030	0.013	0.055	0.028	0.016	0.022	0.027		*	0.033	0.038	0.052	0.031
Average.....	*	0.004	0.177		0.015	0.001	0.015	0.022		0.035	0.014	0.048	0.022	0.016	0.022	0.026		*	0.023	0.024	0.045	0.032
Dogs: Dog 1 ♀	0.016		0.11		0.009		0.014	0.013	0.024		0.011	0.040			0.036	0.021	0.034		0.015			
Dog 3 ♀	0.046	0.003	0.19		0.012		0.009	0.024	0.017	0.043	0.034	0.033	0.026		0.030	0.034	0.120		0.016			0.045
Dog 6♂	0.071	0.006		0.038			0.030				0.012		0.015	0.036		0.041		0.023	0.032	0.028	0.039	
Average.....	*	0.005	0.15	0.038	0.011		0.018	0.019	0.021	0.043	0.019	0.037	0.021	0.036	0.033	0.032	*	*	0.021	0.028	0.039	0.0[illegible]

* Average not significant because of wide range of concentrations in different individuals: e.g., cat adrenals, 0.033–0.194; cat testes, 0.005–0.046.

are included in this section, the daily doses of zinc oxide varying from 200 to 1000 mgm. and the duration of zinc administration from three to fifty-three weeks. For convenience in analysis, the cats have been divided into four groups—i.e., four cats (nos. 1, 8, 7 and 3A, see table 1) receiving large daily doses of zinc oxide (200 to 354 mgm.) and killed at the height of zinc administration; three mate cats (nos. 10, 9 and 2A) receiving similar doses for similar periods of time but not killed until two weeks after discontinuance of zinc administration; two cats (nos. 3 and 5) receiving excessively large daily doses of zinc oxide (681 to 1000 mgm.) and killed during zinc

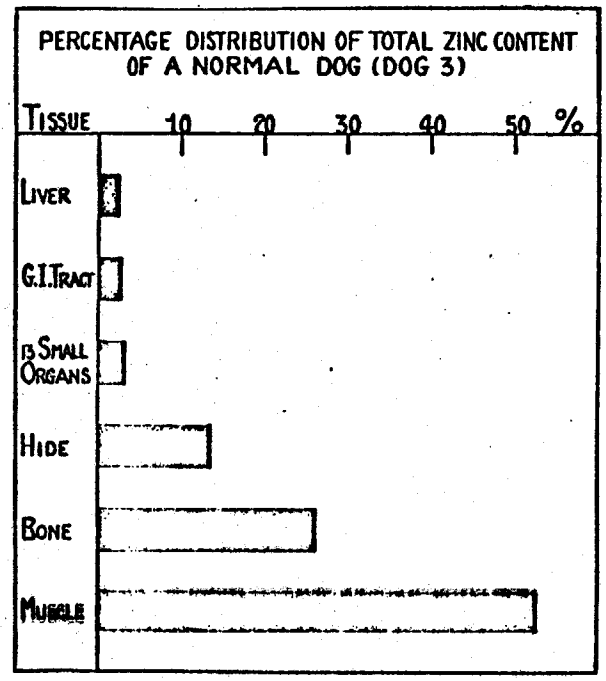

Fig. 8. Percentage distribution of the total zinc content (556.55 mgm.) of a normal dog (dog 3).

administration; one cat (no. 4), a mate to cat 5, receiving a similar dose for a similar period of time but not killed until two weeks after the discontinuance of zinc dosing. Details as to size and duration of dose for individual animals may be obtained from table 1.

The average zinc concentrations in the organs and tissues of the cats in each of these groups is plotted in figure 6 against the average normal zinc concentration of cat tissues and organs. Our studies upon zinc storage include, besides these cats, three dogs, two of which received ½ gram of zinc oxide daily for three weeks (dog 5) and for nineteen weeks (dog 4), respectively, and one which received 1 gram daily for fifteen weeks (dog 2).

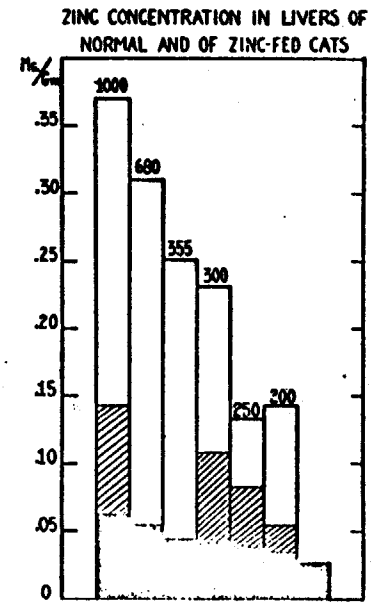

Fig. 9. Zinc concentrations of livers of six zinc-fed cats (200 to 1000 mgm. zinc oxide daily) killed at height of zinc administration (white columns), and of four cats on similar doses for similar periods of time but killed two weeks after discontinuance of zinc dosing (cross-hatched columns), plotted against the zinc concentration of the livers of seven normal cats (black columns). Note the proportion between size of dose and zinc concentration in the liver, and the rapid fall in liver zinc concentration when zinc dosing is discontinued. The numbers at the tops of the columns indicate daily zinc doses in milligrams of zinc oxide.

Dogs 2 and 4 experienced no ill health and were killed the day following the last dose of zinc. Dog 5 died of an intercurrent distemper pneumonia after only three weeks of zincing. The average concentrations of zinc in the organs of these three dogs are plotted in figure 7 against the average normal zinc concentration of dog tissues and organs.

The significant facts upon zinc storage brought out in figures 6 and 7 may be summed up as follows:

a. The ingestion of zinc oxide over long periods of time, even in very large amounts, does not increase the zinc concentration of fat, brain and cord, heart, hide, lungs, muscle,[3] spleen, submaxillary or thyroid glands. The slight apparent increase in cat hide concentrations shown in figure 6 in the heavily zinced cats is so probably due to slight contamination of the fur with zinc oxide of the dose that we attribute no significance to it.

b. Zinc concentration in the blood of cats and dogs is not significantly increased unless the amount of zinc oxide ingested be excessively large, and a blood increase depending on excessive ingestion rapidly falls to a normal level when zinc dosing is discontinued.

c. The zinc concentrations of organs involved in the excretion of zinc—i.e., liver, gall bladder with its content of bile, gastro-intestinal tract and kidney—are increased as a result of zinc oxide ingestion, the increase in general varying

[3] The figures for muscle used in the charts were derived by averaging the zinc figure obtained from analysis of an approximately 100-gram sample of pure muscle of a particular animal with the figure obtained from the analysis of the great mass of muscle, ligaments, and bits of fat left after complete removal of organs and bones (following prolonged boiling in water). The zinc concentrations of pure muscle and of this muscle mass were so nearly identical as to make this procedure a justifiable one.

directly with the size of the dose. The zinc concentrations in these organs, however, fall rapidly to or towards the normal level when zinc administration is discontinued. This fact is graphically shown in figure 9, in which are plotted the zinc concentrations of the livers of ten zinc-fed cats (four of them off zinc for two weeks) against normal zinc figures for cat liver (seven observations).

d. The zinc concentration of the pancreas is increased by zinc feeding, the increase being greatest in the animals receiving excessive doses, but falls off on discontinuance of zinc administration. A possible excretory function of the pancreas is suggested in this connection.

e. Bone marrow in dogs (not analysed in cats) and total bone in both cats and dogs show, as a result of zinc feeding, an increase in zinc concentration. This increase in bone returns to normal when zinc is discontinued.

f. The total zinc concentration of zinc-fed animals increases slightly with moderate zinc dosing and somewhat more markedly with excessive dosing, but falls to a normal level when zinc dosing is discontinued. This fact is a striking and significant one.

The zinc concentrations of the adrenals and of the sex glands in the various control and experimental animals are not charted because the normal figures for these organs, as well as the figures from the zinced animals, show such great variability as to suggest that the zinc content of these glands may be dependent upon functional activity,[1] varying with the state of the gland at the time it happens to be taken for analysis. The following figures illustrate, for example, the variable zinc concentrations found in the adrenals of different animals:

	Mgm. of zinc per gram of tissue
Normal control male, cat 6	0.194
Castrated male, zinced cat 9	0.020
Female, zinced cat 1	0.100
Male, zinced cat 10	0.208
3-weeks old kitten (nursing zinc-fed mother)	0.750
8-weeks old kitten	0.470

The following are a few figures upon the zinc content of ovaries of different zinced cats. Unfortunately we have no normal ovarian figures.

	Mgm. of zinc per gram of tissue
Cat 1	0.273
Cat 3A	0.077
Cat 2A	0.523*
Cat 8	0.370
Cat 3	0.036†

* Mate to 3A and off zinc for two weeks. Note, however, the much higher zinc figure.

† Excessively zinced cat.

[1] For discussion of zinc in its relation to functional activity, see Drinker and

In a consideration of figure 7, plotted from analyses of the organs of zinced dogs 2, 4 and 5, it is worthy of comment that the zinc concentrations of the bone, bone marrow, combined gall bladder and bile, gastro-intestinal tract, hide, kidneys and liver in dog 5 were markedly higher than the zinc concentrations in the same organs of dogs 2 and 4, both of which had ingested zinc, and dog 2 in double the dose, for a much longer period of time. The contrasting figures in milligrams per gram of tissue are as follows (dog 5 versus the average of dogs 2 and 4): bone marrow, 0.26 *vs.* 0.035; bone, 0.28 *vs.* 0.21; gall bladder and bile, 0.076 *vs.* 0.050; gastro-intestinal tract, 0.075 *vs.* 0.040; hide, 0.063 *vs.* 0.039; kidney, 0.183 *vs.* 0.035; liver, 0.224 *vs.* 0.097; total dog, 0.068 *vs.* 0.046.

Three possible explanations of these higher zinc concentrations in dog 5 present themselves: the possible existence of exceptional animals with a greater tendency than the average to store zinc; the possibility that the normal tendency to excrete zinc is interrupted or impeded by intercurrent disease or that a leucocytic infiltration of the affected parts increases their zinc content (cf. Delezenne's finding of high zinc content in leucocytes); or, to our minds, the much more probable explanation that, when an animal's zinc intake is markedly increased, a definite interval of time is required for an approximate equilibrium between zinc intake and zinc excretion to be established, and that during this early interval of adjustment the zinc concentration in certain organs probably reaches a much higher level than is the case later when the excretory mechanism has adapted itself to its new requirements. With the exception of dog 5, all of our zinced animals have been killed after many weeks of zinc administration, so we have no confirmatory evidence of this explanation of the discrepancy between dog 5 and dogs 2 and 4, and are able to present it as a suggestion only.

SUMMARY AND CONCLUSIONS

Cats and dogs have been fed with daily doses of zinc oxide ranging from 175 to 1000 mgm. and for periods of time varying from three to fifty-three weeks. Clinical and laboratory studies made upon these animals include observations upon general health during the course of the experiment (weight determinations, appetite, nervous and digestive symptoms, condition of fur, etc.); hemoglobin and blood cell determinations; urine findings; gross and microscopic examination of tissues following autopsy; and zinc determinations in urine during the course of the experiment and in all organs and tissues after death.

As a result of these studies, we have never observed any significant clinical symptom nor obtained any significant laboratory evidence of damage resulting from the daily ingestion of zinc oxide over long periods of time. With the exception of fibrous change in the pancreases of three cats sub-

jected to doses of zinc very greatly in excess of any possible human exposure, histological examination following autopsy has shown in the organs of zinced cats and dogs no damage of any sort attributable to zinc.

The normal amount of zinc excreted daily in cat urine is shown to range between 0.06 and 0.43 mgm.; in dog urine, between 0.13 and 0.59 mgm.; in dog feces, between approximately 5.0 and 25.0 mgm.

Our studies upon zinc excretion in zinc-fed animals indicate that a small fraction of absorbed zinc leaves the body in the urine, but that the main bulk of it is excreted into the alimentary tract—some of it directly, some of it by the liver into the bile, and possibly some of it by the pancreas—and ultimately leaves the body in the feces. The amount of zinc in the urine of zinc-fed animals is markedly increased over the normal, though the total amount of zinc in the urine is but a small fraction of the total excreted. It is worthy of note that our animals excreted abnormal amounts of zinc through the kidneys for months without any histological evidence of kidney damage.

Figures are presented upon the normal zinc concentrations in the tissues and organs of cats and dogs, upon the zinc concentrations in the tissues and organs of zinced animals, and of zinced animals from which zinc administration had been discontinued for two weeks. These data indicate that the zinc concentrations of organs involved in the excretion of zinc (liver, gall bladder and bile, gastro-intestinal tract, and kidney) are increased as a result of zinc oxide ingestion, but that these abnormal concentrations fall rapidly to the normal level when zinc dosing is discontinued. Zinc concentrations in bone, bone marrow, and pancreas are also increased by zinc feeding—increases which fall towards the normal level on discontinuance of zinc. A possible excretory function of the pancreas is suggested in this connection.

The total zinc concentration of zinc-fed animals increases slightly with moderate zinc dosing and somewhat more markedly with excessive zinc dosing, but falls practically to a normal level within a short time (two weeks) after zinc dosing is discontinued.

A possible connection is suggested between the zinc content and the functional activity of the adrenals and of the sex glands.

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AN INVESTIGATION OF THE EFFECT UPON RATS OF LONG-CONTINUED INGESTION OF ZINC COMPOUNDS, WITH ESPECIAL REFERENCE TO THE RELATION OF ZINC EXCRETION TO ZINC INTAKE

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Received for publication February 7, 1927

In order to obtain more precise and accurate information than now exists in the literature, upon the absorption, storage and excretion of zinc, and upon the physiological and pathological action of zinc and its compounds upon the animal body, the authors have administered daily by mouth to rats, cats and dogs varying doses of non-corrosive zinc compounds for long periods of time, and have made, during the course of the experiments, clinical and laboratory studies, which have included observations upon general health (weight determinations, appetite, nervous and digestive symptoms, etc.) and upon fertility; hemoglobin and blood cell determinations; urine findings; exact determinations of zinc intake and zinc excretion; and, finally, gross and microscopic examination of all tissues and organs following autopsy, with determinations of their zinc content. The results of these procedures with cats and dogs, together with a review of the literature upon zinc absorption, transportation, storage and excretion, and upon the effects of zinc oxide feeding have already been published (Drinker, Thompson and Marsh, 1927), and a report has been made as to the negative effect of zinc feeding upon the fertility of rats (Thompson, Marsh and Drinker, 1927).

In the experiments reported in this paper, which deal with the results of the feeding of zinc compounds to rats, we have been especially concerned with the exact observations upon the relation of zinc excretion to zinc intake and upon the effects of the ingestion of organic zinc compounds (acetate, citrate, malate) as compared with zinc oxide ingestion. The importance of the action of the organic zinc compounds at the present time depends upon the fact that it is, in the main, the organic rather than the inorganic compounds of zinc which are present as contaminations when acid foods or liquids are cooked or stored in zinc-lined containers.

The physiological effects following the administration of zinc compounds vary enormously depending upon the method of administration and especially upon the compound administered. The action of the heavy

metals consists of two parts, the local effects induced at the point of application and the general effects which follow the absorption of the metal into the blood and tissues. The most corrosive salts of any metal are those which are most completely dissociated into ions, that is, the chlorides and nitrates, provided they are soluble. The sulphates are much less irritant because they are less completely dissociated, and perhaps because the sulphuric acid formed hydrolytically may fail to penetrate the cells. The iodides are generally regarded as less irritant than the chlorides, but are less frequently used and less well known.

The least corrosive of the salts of the metals are those formed with the weakly dissociated organic acids, such as the acetates, tartrates or citrates. When these are united with a metal which is not itself an active poison, such as zinc, they are almost purely astringent. The insoluble salts of the metals come into less intimate contact with the tissues and have much less effect. Many of them, however, are slowly taken up and may then act as astringents or irritants. (Cushny, 1924.)

Like the other heavy metals, the soluble salts of zinc precipitate proteins and therefore possess an astringent action, or in large quantities act as irritants and corrosives. The sulphate is the soluble salt most commonly used in medicine, but the chloride, ingested accidentally or with suicidal intent, has frequently given rise to corrosive poisoning. The sulphate is much less irritant and more astringent than the chloride, which is used only as a caustic and disinfectant. The more insoluble zinc oxide (Drinker, Thompson and Marsh, 1927) and the carbonate (Lehmann, 1897) do not cause irritation even when ingested in considerable amounts over long periods of time.

Experimental observations upon the action of organic zinc compounds began as early as 1818, when Devaux and Dejaer (quoted by Helpup, 1889, and by Sacher, 1893) administered zinc acetate and zinc citrate to Spanish prisoners at Lüttich. These early investigators found that large doses of the acetate caused vomiting and mild diarrhea, but that 2 to 4 grams of either the citrate or the acetate were without effect.

Falck (1860, 1861) gave zinc acetate by stomach tube to rabbits (1 to 2 gram doses) and injected zinc acetate in solution into the esophagi of fasting pigeons in doses varying from 0.5 to 2 grams. The rabbits died within 24 hours and displayed at autopsy corrosion and inflammation of the stomach and small intestines. The pigeons exhibited vomiting and diarrhea and, if vomiting was prevented by tying the esophagus above the point of injection, died in from 1½ to 23 hours, depending on the size of the dose. Section showed corrosion of the mucous membrane in the crop and marked inflammation in the upper part of the small intestine. The doses selected by Falck were enormous, especially when one considers the size of his animals and the fact that he was using a soluble salt—so enormous, indeed,

that the whole investigation seems hardly worthy of consideration as a physiological experiment.

Rosow (1862) repeated Falck's experiments upon two pigeons with the exception that his doses of zinc acetate (1 and 1.5 grams) were mixed in the form of a powder with bread and were fed to the pigeons instead of being introduced into the esophagus in solution. The pigeons died in a short time but did not exhibit the vomiting and diarrhea which Falck reported with his birds.

Brandl and Scherpe (1899), investigating the effects of zinc malate, because of their interest in the possible harmful effects of dried apples contaminated with zinc from being dried on zinc trays, fed guinea pigs, rabbits and dogs for periods of 2½ to 4 months small amounts of zinc malate in pill form. With none of the animals on small doses was there any appearance whatever of illness—no loss of appetite, no constipation, no diarrhea, neither during nor after the experiment—and the animals all increased normally in weight. With larger doses, the authors reported local irritation with the occurrence of either diarrhea or persistent constipation.

Salant (1920) reported occasional disturbances of renal function in rats fed daily for 4 months 10 to 15 mgm. of zinc acetate, but no effect upon general health. Cats fed 50 mgm. of zinc daily, as malate, for periods of 10 days to 2 months, also suffered no injury to general health.

The local action of the double salt of sodium zinc tartrate is similar to that of the malate (Sacher, 1893; Brandl and Scherpe, 1899). In small doses it has no untoward effect; in large doses it is irritating to the gastro-intestinal tract, causing, according to these authors, nausea, vomiting and diarrhea, with hyperemia and even ecchymosis and ulceration of the mucosa. Zinc albuminate, however, may be ingested in large daily doses without effect. Rosow (1862), for example, administered zinc albuminate (8 to 9 parts of zinc in 100 parts of the albuminate) without harmful effect to pigeons in daily doses up to 8.5 grams of the albuminate; to dogs in a wide range of dose (2 to 32 grams daily); and to men in doses varying from 3 to 16 grams daily, one patient receiving as much as 278 grams in 36 days without exhibiting digestive symptoms of any sort.

I. Experimental methods. The experiments reported in this paper deal with the results of the feeding of gum acacia suspensions of zinc oxide and solutions of zinc acetate, zinc citrate and zinc malate to rats in daily doses varying from 0.5 to 34.4 mgm. of zinc, and for periods of time varying from 35 to 53 weeks. The investigation was conducted on a total of thirty-five white rats (Mus norvegicus albinus), including thirteen control animals. The rat was selected as an experimental animal because we believe that 9 or 10 months of its relatively short life—one year in a rat's life, as worked out by Donaldson (1924), being approximately equivalent

to 30 years in the human cycle—would give ample opportunity for the appearance of any chronic damage which zinc might be capable of producing. The rats were divided into three groups as follows:

Group I. This group included *a*, eight male rats on varying doses of zinc oxide suspended in 3.5 per cent gum acacia (0.5 to 22.8 mgm. of zinc daily) for from 36 to 43 weeks; *b*, two male rats on zinc acetate solution (1.9 and 3.6 mgm. of zinc daily) for 53 and 48 weeks, respectively; and *c*, two control male rats, one on 3.5 per cent gum acacia solution and one on distilled water, both for 34 weeks. Four of the zinc oxide rats served also as controls of the first 6 weeks of the experiment, receiving during this time distilled water instead of the zinc oxide suspension. Group I rats were caged individually, their fluid intake was measured, the amount of food consumed and its zinc content determined, and all excreta collected and analysed for zinc.

Group II. This group included *a*, three male rats caged together and ingesting for 35 to 36 weeks average daily doses of 34.4 mgm. of zinc in the form of zinc oxide suspended in 3.5 per cent gum acacia solution; and *b*, five male control rats which were caged together and received as their only liquid for 34 to 35 weeks 3.5 per cent gum acacia solution.

Group III. This group included *a*, three males on zinc malate solution (12.4 mgm. zinc per rat per day); *b*, three females on zinc acetate solution (4.4 mgm. zinc daily); *c*, three females on zinc citrate solution (9.7 mgm. zinc daily; *d*, six control rats, three male and three female, on distilled water —all for 29 weeks. At the end of this period, group III rats were mated and continued on zinc, but some not on their original solutions, for from 6 to 18 weeks longer. The controls were also mated and continued on distilled water. Before mating, group III rats were caged together in groups of three, each group being of the same sex.

The rats in groups I and II were a very pure healthy stock obtained from a Philadelphia colony bred expressly for experimental purposes. Group III rats were of a different and less pure strain, some of the original rats and many of their offspring showing pale brown markings. Group III rats tended also to reach a higher maximum weight than did the rats in groups I and II.

Group I rats were put on zinc at the age of 12 weeks; group II rats at 22 weeks, and group III rats at 5 to 7 weeks. Previous to zincing, all groups had a preliminary adaptation period on the diet used during the experimental period. During the course of the experiment, each rat was weighed weekly or fortnightly; at the conclusion of the experiment each rat was autopsied, gross and microscopic studies and chemical analyses for zinc being made on all tissues. The exact zinc dosage, the form of the dose, and the period during which zinc was administered to each rat in the three groups may be seen in table 1.

TABLE 1

Summary of amounts and duration of zinc doses and of blood findings just before autopsy in three groups of zinced rats

RAT GROUP AND NUMBER	FORM OF DAILY DOSE	AVERAGE DAILY DOSE CALCULATED AS ZINC	NUMBER OF WEEKS ON ZINC	BLOOD FINDINGS		
				Per cent hemoglobin	Red blood cells	White blood cells
		mgm.				
Group I						
2	Zn acetate	3.6	48	90	10,168,000	13,400
3	Zn acetate	1.9	53	90	9,857,000	12,300
1	Zn oxide	0.5	42	90	9,673,000	21,200
4	Zn oxide	2.7	42	100+	8,032,000	11,800
5	Zn oxide	5.4	42	90	9,862,000	7,600
6	Zn oxide	7.0	43	90	9,537,000	7,200
7	Zn oxide	10.4	39	90	10,071,000	9,100
8	Zn oxide	13.7	37	90	12,013,000	11,400
9	Zn oxide	17.9	36	90	11,797,000	11,300
10	Zn oxide	22.8	38	90	11,464,000	8,500
11 (Control)	3.5 per cent gum acacia	0	34	95	11,536,000	16,000
12 (Control)	Distilled water	0	34	85	10,116,000	17,100
Group II						
13	Zn oxide	34.4	35	85	12,269,000	10,800
14*	Zn oxide	34.4	36			
15	Zn oxide	34.4	35	85	10,921,000	7,000
16 (Control)	3.5 per cent gum acacia	0	34	80	10,626,000	6,300
17 (Control)	3.5 per cent gum acacia	0	34	80	10,824,000	15,500
18 (Control)	3.5 per cent gum acacia	0	34	95	12,852,000	11,800
19 (Control)	3.5 per cent gum acacia	0	34	90	11,967,000	9,300
20 (Control)	3.5 per cent gum acacia	0	35	90	11,356,000	9,900
Group III†						
21	Zn malate	12.4	29			
		16.5	12	90	11,335,000	13,500
22	Zn malate	12.4	29			
		11.1	13	100	9,808,000	8,800
23	Zn malate	12.4	29			
	Zn acetate	6.3	18	90	9,033,000	12,500

TABLE 1—*Concluded*

RAT GROUP AND NUMBER	FORM OF DAILY DOSE	AVERAGE DAILY DOSE CALCULATED AS ZINC	NUMBER OF WEEKS ON ZINC	BLOOD FINDINGS		
				Per cent hemoglobin	Red blood cells	White blood cells
		mgm.				
24	Zn citrate	9.7	29			
	Zn malate	11.1	14	90	9,727,000	17,900
25	Zn citrate	9.7	29			
	Zn oxide	13.0	14	100+	11,[illegible]0,000	7,500
26	Zn citrate	9.7	29			
	Zn acetate	2.0	14	90	10,[illegible]3,000	17,300
27	Zn acetate	4.4	29			
		6.3	18	90	9,[illegible]6,000	10,600
28	Zn acetate	4.4	29			
	Zn malate	16.5	13	80	9,[illegible]5,000	11,300
29*	Zn acetate	4.4	29			
	Zn oxide	13.0	4			
	Zn acetate	3.5	2			
30 (Control)	Distilled water	0	41	90	9,[illegible]7,000	16,000
31‡ (Control)	Distilled water	0	43	100	8,[illegible]8,000	9,800

* Died of intercurrent respiratory disease.

† The experimental life of group III rats was divided into two parts, the one previous to, the other following mating. The zinc doses and, in many cases, the zinc compound administered was changed when the second period began—a fact which explains the double dosing in this group.

‡ Four other water controls were included in group III but are omitted from this table since no blood counts were made on them.

HOUSING AND METHOD OF COLLECTING EXCRETA: Group I rats were housed in individual cages of the type shown in figure 1. This type of cage, together with our method of collecting excreta, is a slightly modified copy of an apparatus and a method demonstrated to us by Dr. James L. Gamble of the Children's Hospital, Boston—an apparatus resulting from a number of alterations by Doctor Gamble and by Dr. P. G. Shipley of Baltimore, of a metabolism cage for rats originally described by Ackroyd and Hopkins (1916). In order to prevent zinc contamination, the cages were of pure copper, all unions being made with copper rivets instead of solder. Every few months, after careful cleaning with acid, and drying,

the cages were heavily lacquered with a nitrocellulose lacquer and the baked. This was done in order to prevent the formation of the green basic carbonate with which the parts of the cage exposed to wetting tend to become spotted.

A in figure 1 is a tunnel, partially obstructed inside by a small solid fence (on the inside of the tunnel at point *B*), over which only the rat's head can go, and leading to a detachable food can, *C*. This arrangement of the food compartments prevents gross contamination of the excreta with food and, because of the detachable can, provides an easy method of weighing the food supplied and food residues. A calibrated burette, *D*, contains the solution or suspension of zinc to be administered and provides the only source of liquid. The rat is thus forced to consume a daily dose of zinc, the amount of which varies with the concentration of the suspension. After 2 or 3 weeks' observation one can estimate closely how much of a given suspension a rat will drink per day, the average daily consumption being remarkably uniform. Though one cannot by this method administer a selected exact dose, one can, by varying the strength of the suspension offered, ensure that the rat will consume an average daily dose very close to the selected one. *E* is a detachable wire mesh floor which rests upon supports hanging from the bottom of the cage.

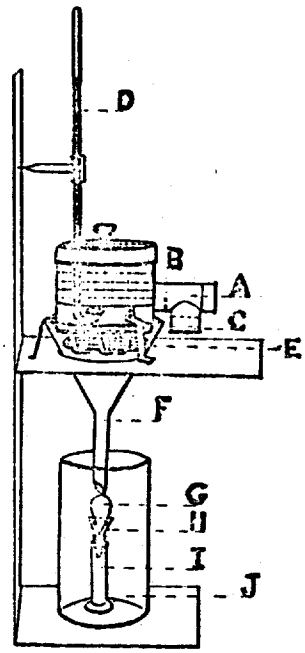


Fig. 1. Diagram of apparatus used in quantitative collection and separation of rat urine and feces. See text for explanation of letters.

The cage rests on a large funnel, *F*, down which the urine runs onto a pear-shaped glass bulb, *G*, which is supported about ½ inch clear of all its surfaces in a small glass funnel, *H*, by means of a few glass spicules projecting from its sides. Urine reaching this bulb flows down the funnel into a small graduate, *I*, containing a little powdered thymol. The daily urine volume is thus self-registered. The amount of food consumed is determined by weighing the food can and its contents at the beginning and at the end of a given period, and, similarly, the fluid consumption by burette readings.

The mesh of the wire floor is sufficiently large to allow rat feces to fall through without sticking. The falling fecal masses strike the sides of the large funnel, pass down the funnel stem, and strike the surface of the glass

beneath. Being formed and dry, they bounce off into the large surrounding glass cylinder, *J*, and are collected weekly.

All of the apparatus used in our experiments was carefully washed with distilled water weekly and the washings—chiefly food dust, hair and dried urine—saved and analysed for zinc. All apparatus was then thoroughly cleaned with soap and water, and either a new collection period started or the collection period under way at the time continued, as the case might be.

At first analyses were made of the zinc content of a week's excreta from group I rats. After 10 weeks, however, the collection periods were lengthened to 2 and, finally, to 4 weeks.

Diet. *Group I rats.* The composition of the stock food was as follows:

	Parts
Rolled oats	5.0
Hominy	3.0
Dried milk-powder (Klim)	1.5
Beef scraps	2.5
Salt	trace
Yeast	0.36

Each rat also received 5 grams of lettuce twice weekly. Whenever a new stock food was made up, two 100-gram samples were analysed for zinc. The average figure obtained was 0.059 mgm. of zinc per gram of food. Single analyses of lettuce gave an average zinc concentration of 0.0013 mgm. of zinc per gram. With these data and with the record which was kept of the food consumed by each rat in group I, the amount of zinc ingested by each individual with his food could be determined, and added to the zinc dose to obtain a figure for the total zinc ingested.

Groups II and III rats. These rats received the same stock food as group I rats, except that yeast was omitted. They also received molasses stock feed (a mixture of alfalfa, corn, oats and molasses) and dog biscuit with lettuce twice weekly.

METHOD OF PREPARATION AND ADMINISTRATION OF ZINC DOSES. Zinc was administered in liquid form, rather than as a dry powder mixed with food, in order to reduce the danger of contaminating with zinc the food of group I rats.

Zinc oxide suspensions. A primary stock suspension was made by suspending about 200 grams of pure zinc oxide[1] in approximately 5 liters of 10

[1] Chemical analysis of the zinc oxide used in these experiments gave the following data:

	Per cent
Insoluble in HCl	0.007
CO_2	0.025
Total S as SO_3	0.001

per cent zinc-free gum acacia solution. This was decanted several times, and the final supernatant liquid, now approximately 3.5 per cent gum acacia, was analysed in order to obtain its exact zinc concentration per cubic centimeter.

If a fortnightly collection period was running for group I rats, the amount of fluid a rat would drink in this length of time was liberally estimated—about 250 cc. Into this volume of 3.5 per cent gum acacia solution was introduced the number of cubic centimeters of the primary stock suspension that would supply an amount of zinc amply sufficient to give the rat concerned a daily dose of zinc within the range allotted to him.

From these secondary stock suspensions, the graduated siphon burettes in each cage were filled whenever necessary, meticulous care being exercised to avoid any loss of zinc in the process. Readings were then made of each rat's daily fluid consumption. In order to obtain accurate figures for the amount of zinc actually ingested by each group I rat, all of the suspension left at the end of a collection period in the burette and in the secondary stock bottle was analysed, and the figure obtained was subtracted from the accurately known zinc allowance made up for the rat in question. Group II rats were supplied in a similar manner.

Organic zinc salt solutions. The salts concerned (acetate, citrate and malate) were synthesized in this laboratory by Dr. Lawrence Fairhall, were then made up in stock solutions of known zinc content, and were administered in graduated siphon burettes, the amount of zinc ingested being calculated from the fluid consumption.

In groups II and III one burette supplied three rats, so the dose figures for individuals in these groups represent one-third of the average total amounts of zinc ingested by all members of the group.

URINALYSES AND BLOOD COUNTS. Collections of urine for urinalysis were made from group I rats eight or nine times during the course of the investigation, each time immediately after the apparatus had been thoroughly cleaned for the week. During the period of the collection—at first 5 to 6 hours, later 18 hours—the rats were given no food so that there

Cl	0.004
PbO	0.023
CdO	0.004
Bi_2O_3	under 0.001
CuO	under 0.001
Fe_2O_3	under 0.001
MnO	under 0.001
As_2O_3	under 0.001
Sb_2O_3	under 0.001
ZnO (Difference)	99.799
Reducing power as SO_2	0.010
Water soluble salts dried at 110°C	0.098

could be no chance of dust contamination from this source. The burettes, however, were filled as usual. In this manner, with the longer collection period, approximately 5 cc. of uncontaminated urine were obtained from each group I rat. This was tested qualitatively for albumin with the sulphosalicylic acid test and for sugar with Benedict's reagent. When albumin was determined quantitatively, Esbach's reagent was used.

Just before autopsy, blood for red and white cell counts and for hemoglobin determinations was obtained from the rat's tail vein. Counts were made with pipettes standardized by the U. S. Bureau of Standards.

Autopsies. Immediately preceding autopsy, the animal was anesthetized with chloroform and then at once decapitated over a weighed porcelain evaporating dish. The blood, which poured freely from cut vessels in the neck, was weighed and used for zinc analysis.

The rat was then skinned, particular care being taken to make the removal of the hide complete since, in zinced rats, incomplete removal around the feet was a possible source of zinc contamination of the carcass. Hides, after weighing, were thoroughly washed in distilled water to remove any extraneous zinc that might be adherent. The viscera were next removed from chest and abdomen, the gastro-intestinal tract from the throat down being taken out intact, slit up, and the contents washed out with running water. In the case of zinced animals, the mouth and throat were also washed out with water. All organs were dissected clean and weighed in tared evaporating dishes, small sections of each being taken for histological study. Of the reproductive tract, only the glandular tissues were analysed, i.e., the testes, seminal vesicles and accessory glands in the male, and the ovaries in the female. A 10-gram sample of muscle was removed from the hind quarters, and, finally, the brain and cord were removed. The rest of the rat—practically all skeleton and muscle, with an insignificant amount of tissues not considered worth analysing, such as bladder, omentum, blood vessels, etc.—was weighed and analysed *en masse* under the name of "carcass."

Chemical analysis. All samples were dried, then charred on a hot plate and ashed in a muffle furnace at about 450°C.

Analysis of zinc oxide doses and of dose remains. The ash of samples of zinc oxide doses and the ash of the dose remains were dissolved in 5 cc. of 1:1 hydrochloric acid and 20 cc. of water and titrated with potassium ferrocyanide solution.

Analysis of feces and rat carcasses. The ash of feces and of carcasses was treated with 1:1 hydrochloric acid and the residue returned to the muffle repeatedly until all the carbon had disappeared. Iron was removed in acid solution by cupferron. On account of the excessive amount of calcium present, the filtrate was made up to large volume—about 600 cc. It was then neutralized with ammonium acetate, and the zinc and added

copper brought down as sulphides. (If any calcium phosphate was in evidence at this stage, it was necessary to reprecipitate.) These sulphides were well washed with water, then several times with hot alcohol, and were finally dissolved in nitric acid. The latter was thoroughly fumed off, the residue taken up with hydrochloric acid and water, and the copper removed as sulphide. The filtrate containing the zinc in pure form was evaporated to dryness and titrated as described above.

Analysis of food and hides. The method of analysis of food samples and of hides was the same as for feces and carcasses, except that the neutralized filtrate after the removal of iron with cupferron was made up approximately to 100 instead of to 600 cc.

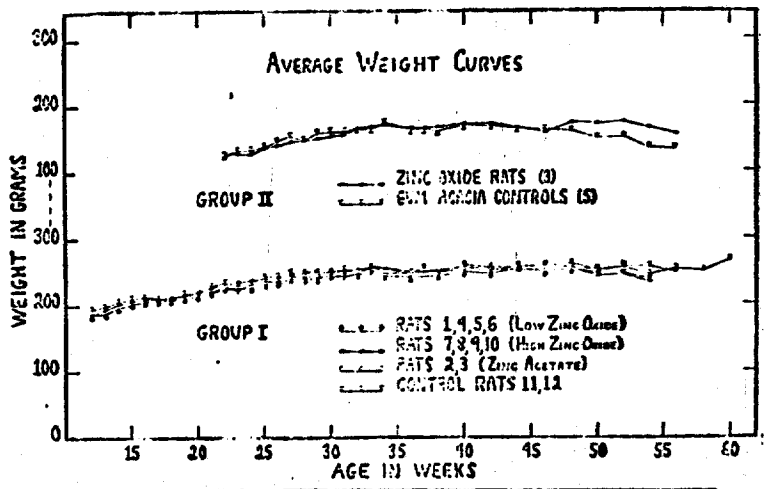
Analysis of urine and tissues. The analytical procedure in the case of urine and tissues was the same as with food and hides until the last stage was reached. At this point the pure zinc residue was dissolved in 10 cc. of water. Zinc determinations on the tissues were made by the ferrocyanide turbidimetric method; on the urines, for the most part, by the urobilin colorimetric method (Lutz, 1925). A detailed description of the above procedures has been given by Fairhall (1926).

II. Results of clinical and laboratory observations and of autopsy findings. *General health and weight.* All of the rats continued in good health until June, the experiments having begun for the most part in December. During the summer, however, a chronic respiratory infection—the first symptom of which was a noisy respiration, a sort of croaking—gradually spread through the colony, affecting approximately half of both the control and the zinced rats. Gradually some loss in weight occurred, relatively little in some rats, greater in others. Two rats eventually died of this disease, the diagnosis at autopsy being chronic bronchopneumonia. The gum acacia control rats in group II were earlier and rather more severely affected than any of the zinced rats, the next most severely involved lot being the zinc oxide rats in group II, which were housed in an adjoining cage. Group I rats were later and to a less extent involved.

Except for this final pneumonia epidemic, the health of all of the rats was normal throughout the 34 to 53 weeks of the experiment. The animals showed normal appetite and activity, healthy lustrous fur and normal gain or normal maintenance of weight. There were no signs of intestinal or of nervous disturbances. The weekly food and liquid consumption of individual rats remained astonishingly uniform throughout the experimental period. The zinced rats were as healthy and gained weight as rapidly or maintained it as normally as did the controls. As a result of the unpalatability of the zinc doses, however, especially the higher ones, the zinced animals were inclined to drink less than the controls.

In figures 2 and 3 the weight curves of various groups of zinced rats are

compared with similar curves from control animals. It is obvious from these figures that in so far as weight gain and weight maintenance are concerned the zinced rats were the peers of the control animals. The



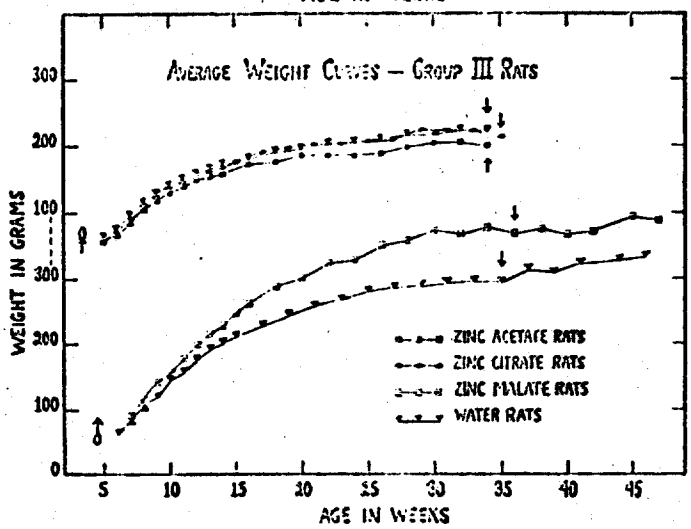


Fig. 2 (Upper). Average weight curves of rats in groups I and II.

Fig. 3 (Lower). Average weight curves of rats in group III, each curve representing the average of three rats. Arrows indicate points at which animals were mated and fertility experiments begun.

final respiratory epidemic explains the gradual falls shown in figure 2 in the average weight curves of the gum acacia controls during the last 10 weeks of the experiment and of the zinc oxide rats (group II) during the

last 4 weeks. Some of the weight curves of the rats in group I also show at the very end, but to a less marked degree, the effect of the epidemic.

BLOOD FINDINGS. Table 1 shows the blood findings on each rat just before autopsy. The following tabular statement presents the extreme and the average red cell and white cell counts and hemoglobin determinations in the zinced rats as compared, not only with figures from similar observations upon our control animals, but also with blood studies reported by Goodall (1909) upon a series of normal rats.

	Extremes	*Averages*
	Red Cells	
Zinced rats	8,032,000-12,269,000	10,308,000
Control rats	8,938,000-12,852,000	10,872,000
Goodall's normals for rats		8,100,000
	White Cells	
Zinced rats	7,000-21,200	11,600
Control rats	6,300-17,100	12,400
Goodall's normals for rats		10,600
	Per Cent Hemoglobin	
Zinced rats	80-100 +	90.5
Control rats	80-100	89.4
Goodall's normals for rats		110.0

It is clear from these figures that the ingestion of zinc, even in large doses over long periods of time, causes no significant variation from the normal in the blood picture of rats.

URINALYSES. During the course of the experiment, eight or nine analyses were made of the urine of each of the rats in group I. Albumin is a normal finding in rat urine—an Esbach quantitative analysis, for example, showed 0.3 gram per liter in a 24-hour specimen of the urine of control rat 11—and was always present in the urine of both control and zinced rats, the amount varying from a faint trace to a distinct cloud. In the case of two rats, however,—zinced rat 7 and control rat 12—there was always a heavy precipitate of albumin with the sulphosalicylic acid test. Neither of these rats exhibited any untoward clinical symptoms ascribable to the kidney, and both showed normal kidneys at autopsy. Sugar was never found and casts were so few and infrequent as to be of no significance.

The weekly urine volume varied with different rats depending upon the fluid intake, the heavily zinced rats drinking much less of the unpalatable suspensions which provided their only source of fluid, than did the control and low-dose zinc rats, and excreting, consequently, less volume of urine. During the entire course of the experiment, however, the volume, both of fluid intake and of urine output in individual rats, remained remarkably constant; significant variation did not occur in a single instance.

AUTOPSY FINDINGS. *Gross findings.* With the following exceptions, which are plainly due to intercurrent disease, the gross autopsy findings were negative. The lungs of zinced rats 9, 13, 15, 21, 25, 26 and 27 and

of control rats 11, 18 and 20 showed small consolidated areas and pus sacs, mainly confined to the upper lobes. In some instances adhesions to the pleura were present. In zinced rats 7, 8, 14 and 29 and in control rats 17 and 19 these processes were more extensive, being present in patches throughout the lungs. Rat 3 showed a pus pocket at the external meatus of the bladder which exuded a light green pus when the skin was cut. Parasitic cysts were found in the livers of rats 3, 8 and 14.

Histological findings. The histological findings in the lungs of the rats reported above as showing gross changes varied in degree, extent and stage of disease process, but in all instances was there evidence of bronchopneumonia or its sequelae. Many sections showed only small patches, others larger areas of solid leucocytic infiltration; other sections showed atelectatic cavities and dilated bronchi filled with exudate; still others showed definite encapsulated, chronic abscesses, which undoubtedly had their origin in pneumonic foci. The livers of rats 14 and 21 showed some cloudy swelling, a degree of disturbance no more than one might expect from the infection in the lung.

With the exceptions noted, histological studies upon the organs of the zinced rats revealed, throughout, normal tissues. Sections of stomach, intestines, kidney, pancreas, spleen, adrenal and reproductive glands were normal in all instances and, with the exception of the two cases of cloudy swelling already mentioned, the livers of all zinced rats were normal.

In brief, we may say of our autopsy findings, that approximately one-half both of control and of zinced rats showed evidence of chronic bronchopneumonia or its sequelae, the degree, extent and stage of disease process varying in different animals. With the exception of these evidences of bronchopneumonia or its consequences and of parasitic cysts in three livers, gross and microscopic autopsy studies showed normal organs in all of the zinced rats. In no instance was there any evidence of damage due to zinc.

Summarizing our clinical and laboratory observations upon zinced rats, we may say that we have never observed any significant clinical symptom nor obtained any significant laboratory evidence, either during life or from autopsy findings, of damage resulting from the long-continued daily ingestion of zinc oxide or of zinc acetate, citrate or malate. These results are in accord with our experiences, already reported, upon the long-continued feeding of zinc oxide to cats and dogs (Drinker, Thompson and Marsh, 1927).

III. ZINC EXCRETION. *Zinc in normal rat urine and feces.* The normal amount of zinc in the urine (not including cage washings) of rats on an ordinary mixed rat diet, such as the one used by us, ranges approximately between 0.02 and 0.20 mgm. per week; that in rat feces, between 1.25 and 7 mgm. It must be remembered, however, in connection with the figure

TABLE 2

Specimen figures of zinc ingested weekly in food and of weekly zinc excretion in urine and feces of normal rats on a constant quality diet

RAT NUMBER	EXPERIMENTAL WEEK*	TOTAL ZINC INGESTED IN FOOD (CALCULATED)	ZINC EXCRETED			
			In urine	In feces	In cage washings†	Total†
		mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*
7	4	3.6	0.12	3.3		3.42
	5	3.2	0.10	3.0		3.10
	6	3.6	0.15	3.9		4.05
		10.4				10.57
8	5	3.4	0.10	3.0		3.10
	6	3.6	0.15	3.9		4.05
		7.0				7.15
9	4	3.5	0.04	3.3		3.34
	5	3.2	0.07	3.0		3.07
	6	3.6	0.12	3.3		3.42
		10.3				9.83
10	4	3.3	0.02	3.3		3.32
	5	3.1	0.02	3.3		3.32
	6	3.6	0.12	3.3		3.42
		10.0				10.06
11	13 and 14	10.6	0.14	8.8	0.08	9.02
	15 and 16	10.4	0.09	9.9	0.13	10.12
	17 and 18	10.6	0.19	13.6	0.09	13.88
	19 and 20	10.4	0.07	10.0	0.20	10.27
	21 and 22	11.5	0.07	10.9	0.20	11.17
	23 and 24	11.7	0.10	10.9	0.20	11.20
	25 and 26	9.6	0.10	9.1	0.20	9.40
		74.8				75.06
12	11 and 12 (9 days only)	6.7	0.05	6.4	0.03	6.48
	15 and 16	10.6	0.11	9.9	0.09	10.10
		17.3				16.58

* Experimental collection periods lasted at first a week, then 2 weeks, then 3, and finally 4 weeks. Note that figures upon rats 11 and 12 cover 2-week periods.

† For the first 6 weeks of the investigation, zinced rats, 7, 8, 9 and 10 were used as controls, receiving distilled water instead of zinc oxide suspensions. During this time, however, cage washings were not saved for zinc analysis, so a small fraction of the zinc excreted by these rats during this time is not included in the table. From the seventh week on, all cage washings were saved.

TABLE 3

Specimen figures of zinc ingested and zinc excreted by rats on varying daily doses of zinc oxide

RAT NUMBER	EXPERIMENTAL WEEK	ZINC INGESTED		ZINC EXCRETED			TOTAL ZINC INGESTED	TOTAL ZINC EXCRETED
		In dose	In food	In urine	In feces	In case washings		
		mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	11 and 12	5.6	9.0	0.15	15.8	0.29	14.6	16.24
	13 and 14	4.6	9.6	0.09	16.8	0.41	14.2	17.30
	15 and 16	5.7	9.2	0.14	15.4	0.46	14.9	16.00
	17 and 18	8.5	9.6	0.18	14.1	0.26	18.1	14.54
	19 and 20	8.5	10.3	0.12	17.3	0.32	18.8	17.74
	21 and 22	5.7	9.7	0.12	15.1	0.32	15.4	15.54
	23 and 24	6.6	11.0	0.13	15.9	0.37	17.6	16.40
	25 and 26	6.9	9.6	0.13	15.8	0.37	16.5	16.30
	27, 28 and 29	8.2	13.8	0.20	20.9	0.54	22.0	21.64
							152.1	**151.70**
4	11 and 12	38.0	9.6	0.13	43.7	0.29	47.6	44.12
	13 and 14	35.3	10.5	0.14	49.8	0.54	45.8	50.48
	15 and 16	31.5	9.8	0.17	43.1	0.40	41.3	43.67
	17 and 18	41.3	10.2	0.21	43.0	0.39*	51.5	43.60
	19 and 20	35.9	9.5	0.14	47.9	0.39	45.4	48.43
	21 and 22	44.2	10.3	0.14	53.6	0.39	54.5	54.13
	23 and 24	36.4	10.8	0.18	47.3	0.39	47.2	47.87
	25 and 26	34.1	9.9	0.18	42.7	0.39	44.0	43.27
	27, 28 and 29	50.2	13.6	0.20	60.9	0.57	63.8	61.67
							441.1	**437.24**
5	13 and 14	73.8	10.3	0.26	88.2	0.38	84.1	88.84
	15 and 16	71.1	10.3	0.17	90.0	0.86	81.4	91.03
	19 and 20	74.9	10.3	0.14	91.3	0.29	85.2	91.73
	21 and 22	76.0	10.7	0.14	85.4	0.29	86.7	85.83
	23 and 24	65.4	10.9	0.16	69.9	0.39	76.3	70.45
	25 and 26	67.1	9.9	0.16	70.5	0.39	77.0	71.05
	27, 28 and 29	98.5	14.0	0.20	116.4	0.52	112.5	117.12
							603.2	**615.05**
10	11 and 12	342.0	9.3	0.31	304.1	0.79	351.3	305.20
	13 and 14	324.0	9.7	0.20	314.0	0.49	333.7	314.69
	15 and 16	312.0	9.9	0.24	357.5	0.40	321.9	358.14
	19 and 20	333.7	9.9	0.30	331.8	0.77	343.6	332.87
	21 and 22	332.7	10.4	0.29	350.0	0.77	343.1	351.06
	23 and 24	303.7	11.0	0.30	374.5	0.60	314.7	375.40
	27, 28 and 29	444.0	13.6	0.20	388.0	0.63	457.6	388.83
							2365.9	**2356.19**

* Analysis lost. Figure estimated.

for zinc in urine that always some urine dried on the wire of the cage floors and on the funnels, with the result that its zinc content was included in the figure for cage washings instead of in urinary zinc. Actually, therefore, the normal total weekly excretion of zinc in urine by rats ranges somewhat higher than 0.02 to 0.20 mgm. We have not added the figure for cage washings to that of urinary zinc to secure a figure for total zinc in urine because of the fact that, undoubtedly, at times the cage washings included not only dried urine but also small fragments of feces which had stuck to the wire mesh of the floor and which could be completely removed only in the final washing. Table 2 gives specimen figures of the amounts of zinc ingested weekly in food by six normal rats during control periods, and of the amounts of zinc excreted in urine and feces by these same rats during the same periods. It is obvious from these figures that the great bulk of zinc normally passing through the body is excreted through the gastro-intestinal tract, a very minor amount through the kidneys. This observation on rats merely confirms previous ones on cats, dogs and men (Drinker, Thompson and Marsh, 1927; Drinker, Fehnel and Marsh, 1927).

Table 2 also shows that while the weekly excretion of zinc may lag a little behind or may somewhat exceed the weekly ingestion of zinc, nevertheless, during longer periods of time—several weeks—the total amounts of zinc ingested and of zinc excreted by adult rats are practically the same. Group I rats upon which these observations were made were 12 weeks old and between 180 and 200 grams in weight at the beginning of the experiments. We have no similar data upon very young, very rapidly growing rats. It is highly probable, however, that in the early weeks of life there is a considerable excess of zinc intake over zinc excretion, since the zinc concentration of rats per gram of animal tends to be the same, regardless of age (Drinker and Collier, 1926).

Zinc in the urine and feces of zinced rats. Table 3 gives specimen figures of the amounts of zinc ingested in food and in zinc doses of varying amounts by four zinced rats during total experimental periods of 15 and 19 weeks. The same table also contains data upon the amounts of zinc excreted in the urine and feces and in the cage washings of these same rats during the same experimental periods.

A number of significant facts are to be derived from this table. Considering the figures upon zinc ingested in food, for example, as an index of the total amount of food eaten, we have in them evidence that the daily ingestion of large doses of zinc had no effect upon the total food consumption. Rat 10, for example, on an average weekly dose of 159.6 mgm. of zinc, consistently ate as heartily as rat 1 on an average weekly dose (3.5 mgm.) less than the normal zinc content of his food. Had digestive disturbances existed in the highly zinced rats, this clearly would not have been the case. The uniformity of the weekly amounts of zinc ingested in

food by individual rats, indicating as it does uniform weekly total food consumption, is also graphic evidence of good health. The quantity of food eaten depended on the rat's appetite; he was always provided with an excess. Had the zinc been a source of gastro-intestinal irritation, undoubtedly as the experiment progressed there would have been a decrease in the amount of food consumed by individuals, especially by the highly zinced animals.

The figures in table 3 show clearly that the gastro-intestinal tract in rats is the chief avenue of excretion of abnormal amounts of ingested zinc, as it is the main route of excretion in the normal zinc metabolism. Highly zinced rats show increases over the normal in urinary zinc but these increases are relatively small, much smaller than is the case with highly zinced cats and dogs. Rat 10, for example, during 3 control weeks on distilled water, averaged 0.053 mgm. of urinary zinc and 3.3 mgm. of fecal zinc per week. During 15 experimental weeks on an average weekly zinc dose (for these weeks) of 159.5 mgm., the zinc in this rat's urine averaged 0.12 mgm. per week; in his feces, 163.3 mgm.—that is to say, urinary zinc increased 2.26 times, fecal zinc 49.5 times. Cats and dogs on doses of zinc even less per kilogram of animal than the dose of rat 10, showed average increases in urinary zinc of approximately three, five and even seven times the amount normally excreted in 24 hours. Similarly, zinc in the urine of zinc workmen, exposed to the inhalation of zinc oxide and of fine metallic zinc dust, shows a greater percentage increase over the normal level than occurs in the urine of zinc-fed rats (Batchelor, Fehnel, Thompson and Drinker, 1926).

Whether these differences in the excretion of zinc by the kidneys in cats, dogs and men, as compared with rats, are due to different functional capacities of the kidneys in the respective species—the normal presence of albumin in rat urine should be considered in this connection—or whether a higher concentration of hydrochloric acid in the stomachs of the carnivora ensures the solution and absorption of more of the ingested zinc than occurs in the herbivora, with a consequently greater proportional increase in the urinary zinc, we are not prepared to say. If the latter factor be the determining one, a large fraction of the zinc oxide ingested by rats must pass unabsorbed, possibly unchanged, through the gastro-intestinal tract.

The data in table 3 upon total zinc ingested and total zinc excreted by four representative rats on varying zinc doses, during illustrative periods of 15 and 19 weeks, show that rats are able to excrete as much zinc as they ingest, even when the zinc dose is very large. We are not able to present ingestion and excretion data upon individual animals for 10 consecutive months, although the experiments were carried on for that length of time. Accidental contamination or loss of occasional specimens during the course of collection or analysis was inevitable during so long a period, and pre-

vented our obtaining an unbroken series of figures. It is clear, however, from the large number of excretion figures which we have—specimens of which are presented in table 3—that rats do not tend to store zinc in amounts significantly above the normal, but that their excretion of this metal tends to keep pace with its ingestion. The next section contains further data upon the question of zinc storage.

In summary, we may say that our evidence upon the excretion of zinc by rats indicates an insignificant normal excretion of zinc by the kidney, with a relatively small increase in urinary zinc following the ingestion of even very large amounts of the metal. The great bulk of ingested zinc, both in the normal metabolism and following zinc dosing, is excreted by the gastro-intestinal tract. Excretion of zinc in rats tends to keep fairly close pace with ingestion—a fact true whether the ingested zinc be an organic compound or the more insoluble zinc oxide.

IV. Zinc storage. In figure 4 the average zinc concentrations of the different tissues and of the entire bodies of seven control rats are compared with the zinc concentrations of eleven rats fed varying doses of zinc oxide daily for from 35 to 43 weeks and with the zinc concentrations of eleven rats fed daily doses of various organic zinc compounds for from 35 to 53 weeks. Zinc concentrations are expressed in milligrams per gram of tissue.

It should be mentioned here that, in general, our figures for normal zinc concentrations in rat tissues, obtained by the turbidimetric method of analysis, average somewhat higher than those reported by Lutz (1926) with his urobilin method. We have repeatedly found instances of concentrations within the ranges reported by Lutz but, in the main, the range of our figures is more extensive, especially at the upper end, and our average normal figures higher. For this reason, we include the following tabular statement of the average zinc concentrations obtained by us in the tissues of seven control rats:

	Mgm. zinc per gram of tissue
Blood	0.007
Hide	0.036
Muscle	0.038
Carcass (bone and muscle)	0.041
Gastro-intestinal tract	0.045
Liver	0.047
Lungs	0.048
Kidneys	0.050
Brain and cord	0.053
Testes and seminal vesicles	0.082
Ovaries	0.090
Pancreas	0.173
Adrenals	0.180
Spleen	0.228
Total rat	0.010

The significant facts upon zinc storage in rats brought out in figure 4 may be summed up as follows:

The ingestion of zinc oxide or of organic zinc compounds by rats, over long periods of time and even in large amounts, does not significantly increase the zinc concentration in the tissues and organs of this species of animal. The only increases worth commenting on are the increase in the zinc concentration of the blood of rats ingesting organic zinc compounds and the increase in the zinc concentration of the kidneys of the zinc-fed animals. The other increases shown in figure 4 are trifling and would probably disappear if the series were larger.

The blood of the organic zinc salt rats, with a single exception, was always higher in zinc than was the blood of control animals or of rats fed

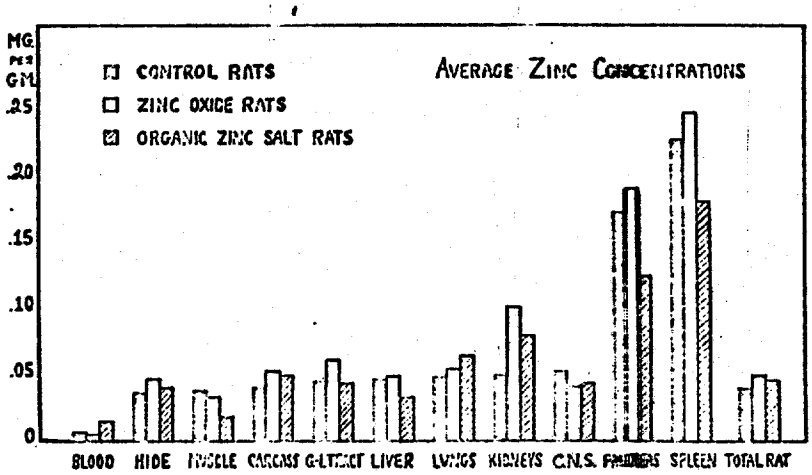


Fig. 4. Final average zinc concentrations in tissues and organs of control rats and of two groups of rats fed, the one, zinc oxide, the other, organic zinc salts, over a long period of time.

zinc oxide, the average blood zinc concentration of the organic salt rats being 0.015 mgm. of zinc per gram of blood as compared with 0.007 for the control rats. The zinc oxide rats were uniform at the lower end of the normal range, their average blood zinc concentration being 0.005 mgm.

This increase in the blood zinc of the organic salt rats suggests the probability that proportionally more of the organic zinc compounds were actually absorbed than was the case with the oxide. Assuming this fact to be true, it did not, however, promote zinc storage since the organic zinc salt rats showed, in general, a somewhat lower zinc concentration of tissues than did the zinc oxide rats.

The increase in the zinc concentration of the kidneys of the zinc-fed animals is no more than one would expect to find in an organ concerned in

the excretion of zinc. Our experience with cats and dogs (Drinker, Thompson and Marsh, 1927) leads us to believe that this increase would disappear in a short time were zinc feeding discontinued. One might expect to find a decided increase in the zinc concentration of the gastro-intestinal tract since this tract, rather than the kidney, is the chief organ of excretion of zinc. Whether the relatively slight increase which does occur—and this only in the zinc oxide-fed animals—is to be explained by a rapid passage of zinc through the gastro-intestinal walls and a consequently relatively small increase in tissue zinc concentration at any one time, or whether it is due to the washing of the tract necessary before analysis in order to get rid of intestinal contents and possible contamination from unabsorbed zinc, we are unable to say.

The concentration of the total rat in the case of the zinc-fed animals is slightly but not significantly greater than that of the controls (controls, 0.040 mgm. of zinc per gram of rat; zinc oxide rats, 0.050 mgm.; organic zinc salt rats, 0.046 mgm.). In terms of total zinc content, this increase represents a few milligrams of zinc only—a total increase of 2.5 mgm., for example, in a 250-gram rat, which is less than the daily dose of all except two of the zinced animals. Trifling increases of this sort in a normal constituent of all body tissues are clearly not significant in terms of storage, especially when one takes into account the many months of zinc feeding. The smallness of the increase in total zinc concentration in the zinc-fed animals is the more noteworthy in that the daily doses of zinc were, in many cases (see table 1), very large in proportion to the size of the animals.

SUMMARY AND CONCLUSIONS

Daily doses, ranging from 0.5 to 34.4 mgm. of zinc, in the form of gum acacia suspensions of zinc oxide and of aqueous solutions of zinc acetate, zinc citrate and zinc malate have been administered by mouth to rats for periods of time varying from 35 to 53 weeks. Clinical and laboratory studies made upon these animals include observations upon general health during the course of the experiment (weight determinations, appetite, nervous and digestive symptoms, condition of fur, etc.); amount of food and of fluid intake; volume of urine output; urinalysis; hemoglobin and blood cell determinations; gross and microscopic examination of tissues following autopsy; and, finally, determinations of ingested zinc, of zinc excreted in urine and feces, and of zinc content of organs and tissues after death.

As a result of these studies, we have never observed any significant clinical symptom nor obtained any significant laboratory evidence of damage resulting from the daily ingestion by rats, during long periods of time, of zinc oxide, zinc acetate, zinc citrate or zinc malate. Gross and histological

examination of tissues following autopsy has shown in the organs of zinced rats no damage of any sort attributable to zinc.

The normal amount of zinc excreted in the urine of rats is shown to range approximately between 0.02 and 0.20 mgm. per week; that in rat feces, between 1.25 and 7 mgm.

Our studies upon zinc excretion both in control and in zinc-fed rats indicate that a small fraction of absorbed ingested zinc leaves the body in the urine but that the main bulk of it is excreted through the gastro-intestinal tract. Highly zinced rats show increases over the normal in urinary zinc but these increases are relatively smaller than we have previously shown to be the case with highly zinced cats and dogs. It is worthy of note, nevertheless, that for months rats may excrete abnormal amounts of zinc through the kidneys without any clinical or histological evidence of kidney damage.

Figures are given upon total zinc ingested and total zinc excreted—figures which indicate that rats are able to excrete as much zinc as they ingest, even when the zinc dose is very large. This fact is true whether the ingested zinc be an organic zinc compound or the more insoluble zinc oxide.

Data are presented upon the zinc concentrations in the tissues and organs of control and of zinced rats. These data indicate that the ingestion by rats of zinc oxide or of organic zinc compounds over many months and even in large amounts does not significantly increase the zinc concentration in this species of animal—that is to say, rats do not tend to store zinc in amounts significantly above the normal but their excretion of this metal tends to keep pace with its ingestion.

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396.9 D73

Proc. Soc. Exptl. Biol. Med. 83:625-627 (1953)

Effect of Zinc on Cytochrome Oxidase Activity. (20439)

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Excess dietary zinc causes a hypochromic, microcytic anemia in rats(1-3). The anemia is wholly or partially prevented by feeding additional copper(2,3). The need for copper in hemoglobin formation was discovered by Hart, Steenbock, Waddell and Elvehjem(4). Cohen and Elvehjem(5) and Schultze(6,7) reported that cytochrome oxidase activity was decreased in livers, hearts and bone marrow of rats fed a copper-deficient diet. Schultze and Kuiken(8) found that liver catalase activity was also decreased in copper deficiency. Thus, it is evident that copper is concerned with the formation of iron-porphyrin compounds.

Since zinc has been shown to induce a copper deficiency state in rats, this investigation was undertaken to determine the effect of dietary zinc on the activity of cytochrome oxidase in heart tissue.

Experimental. Animal studies. Rats originating from the Sprague-Dawley strain were started on experimental diets at the age of 21 to 25 days. Six rats (3 males and 3 females) were placed on each of 7 treatments. The basal diet was whole milk powder* to which mineral supplements of ferrous sulfate, cupric sulfate and manganous sulfate had been added. The rats on the basal diet received approximately 0.06 mg of copper, 0.38 mg of iron and 1 mg of manganese per day as a result of this supplementation. The following diets were fed *ad libitum* for a period of 5 weeks: 1) Basal, 2) Basal + 1.0% zinc, 3) Basal + 1.5% zinc, 4) Basal + 0.03% copper, 5) Basal + 1.0% zinc + 0.03% copper, 6) Basal + 1.0% zinc + 1 mg % folic acid, 7) Stock diet.† A-D Percomorph Liver Oil was administered weekly to each rat by dropper. Growth and hemoglobin were determined at the end of the experimental period. The animals were then sacrificed and cytochrome oxidase activity of the heart tissue was measured.

Enzyme studies. The cytochrome oxidase activity was determined as follows. The animal was decapitated and the heart removed at once. It was divided longitudinally and one half of it placed in 5.0 ml of ice cold 0.1 M potassium phosphate buffer, pH 7.2. The tissue was homogenized for 2 minutes in a Potter-Elvehjem glass homogenizer surrounded by an ice-water bath, and the sample was then diluted to the desired concentration with 0.1 M phosphate buffer. The diluted homogenate was allowed to stand exactly 5 minutes in an ice-water bath to permit large particles to settle out before pipetting into the reaction vessels. The enzyme activity was determined by the manometric method of Slater(9) with some minor modifications of reagent concentrations. Four concentrations of cytochrome *c* (Sigma Chemical Co.) were used at each of two levels of enzyme for a complete determination. The ferrocytochrome *c* was standardized spectrophotometrically using a molecular extinction coefficient at 550 mμ of 27.840(10). The test system consisted of 1.0 ml of 0.2 M potassium phosphate buffer (pH 7.2), 0.3 ml of 4 x 10^{-3} M aluminum chloride, 0.3 ml of 0.114 M sodium ascorbate, 1.0, 0.8, 0.7 or 0.6 ml of 1.94 x 10^{-4} M cytochrome *c*, and 0.2 or 0.3 ml of enzyme suspension containing approximately 1 mg of organic matter per ml. The volume of the reaction mixture was made to 3.0 ml by additions of heavy-metal-free water. The enzyme suspension was added last. Sodium hydroxide was found to be unnecessary in the center well of the Warburg flask. After a 10-minute equilibration period, oxygen consumption was measured at 10-minute intervals for a 40-minute period in a Warburg apparatus at 37°, with air as the gas phase. The activity was expressed as oxygen uptake in microliters

* Klim, The Borden Co., New York City.

† Rar Red G.L.P. Dog Food Pellets, Cooperative G.L.F. Marketing Service, Inc., Cooperstown, N. Y.

TABLE I. Effect of Treatment on Growth, Hemoglobin Level and Cytochrome Oxidase Activity of Rats.*

Treatment	Wt change, g	Hemoglobin, g/100 ml	Cytochrome oxidase activity, Qo_2†
1. 0‡	149	14.1	1699
2. 1.0% zinc	101	5.5	500
3. 1.5% "	80	4.5	282
4. 0.03% Cu	146	13.8	1774
5. 1.0% zinc + 0.03% Cu	113	9.7	2304
6. 1.0% zinc + 1 mg% folic acid	111	5.0	391
7. Stock diet	161	14.2	1885

* Duration of animal experiment was 5 wk. All values are the mean for 6 animals, 3 males and 3 females.

† Qo_2 = µl of oxygen uptake/hr/mg of organic matter calculated to infinite concentration of cytochrome *c*. The tissue used was heart.

‡ Mineralized whole milk powder.

(µl) per hour per mg of organic matter. The *organic matter* was determined by the method of Johnson(11), using a 0.1% solution of sucrose as the standard. Duplicate 0.4 ml aliquots of the suspension were taken at the time of pipetting the homogenate into the Warburg vessels, and placed in pyrex test tubes. The color developed was read in a filter colorimeter using Corning filters 3389, 3389, 5113 (½ std. thick) with a transmission peak at 440 mµ with a spread of approximately 75 mµ at the 1% level. The Qo_2 (organic matter) was determined at each level of cytochrome *c*. The reciprocals of the four Qo_2 values were plotted against the reciprocals of the corresponding cytochrome *c* concentrations on rectangular coordinate paper and extrapolated to zero [infinite cytochrome concentration](9).

Results and discussion. The adverse effect of excess dietary zinc on growth and hemoglobin formation in the young rat has been confirmed in this study. These results are presented in Table I. A marked growth retardation was observed at both the 1.0 and 1.5% levels of zinc ($P < 0.01$). The addition of copper to the diet containing 1.0% zinc improved growth significantly ($P < 0.05$), although this improved growth was still considerably below that obtained for the animals on the basal diet. Since Albert(12) has shown folic acid to be a good chelating agent for heavy metals, this compound was included in one of the treatments to learn if it would reduce the toxic effect of zinc. The results presented indicate that it was without effect.

A level of 1.0% zinc in the diet resulted in a reduction in hemoglobin concentration of about 40% of normal ($P < 0.01$) and the addition of 1.5 zinc caused a further significant decrease ($P < 0.05$). The addition of 0.03% copper to the diet containing 1.0% zinc was effective in partially preventing the anemia ($P < 0.01$). Folic acid had no effect on the hemoglobin level, as might be expected in a microcytic anemia.

The effect of excess dietary zinc on cytochrome oxidase activity is presented in Table I. The results show the enzyme activity was decidedly lower in the presence of excess dietary zinc ($P < 0.01$). When copper alone was added to the basal diet, no change in enzyme activity occurred; however, the addition of copper to the 1.0% zinc diet actually caused an increase in cytochrome oxidase activity above that of the basal animals ($P < 0.01$). The following is a possible explanation of this increase. An anemic animal with a low concentration of hemoglobin must circulate the blood more frequently in an attempt to supply the tissues with the oxygen needed for metabolism. This necessitates a marked increase in heart action. The higher-than-normal enzyme activity may have been the result of the increased need on the part of the heart tissue itself for those enzymes involved in energy production. In this group of rats (copper and zinc) a moderate anemia existed, although the copper supply was adequate for high cytochrome oxidase activity. It is evident from these results that the synthesis of cytochrome oxidase takes precedence over that of hemoglobin. This was also an earlier observation of Schultze(7).

Folic acid did not alter the effect of zinc on cytochrome oxidase activity, and hence these values serve as additional data confirming the deleterious effect of zinc on the activity of this enzyme.

The cytochrome oxidase activity observed in the rats on the stock diet was greater than that of the rats on the basal diet. It is improbable that this difference was due to a higher copper content in the stock diet, since the enzyme activity was not changed when 0.03% copper was added to the basal diet.

It was believed important to eliminate the possibility that the presence of ionic zinc in the homogenates was the cause for the low cytochrome oxidase activity. The concentration of zinc in the heart tissue of the animals on the 1.0% zinc diet was determined by the method of Parks, *et al.*(13). This level of zinc, 80 µg per g of heart tissue (dry weight), was added *in vitro* to heart homogenates of normal rats. No reduction in cytochrome oxidase activity was observed, indicating that the effect of zinc was not occurring in this manner. It is of interest to note that the copper content (13) of the hearts of animals receiving 1.0% zinc was 40% of the normal value. [Normal, 27 µg per g; 1.0% zinc, 10 µg per g (dry weight).]

The coefficient of correlation was determined for the relation between the hemoglobin values and cytochrome oxidase activities. There was a highly significant correlation between these sets of values, $r = 0.79$.

The exact nature of the action of zinc on hemoglobin and cytochrome oxidase formation is not known, although sufficient evidence exists to show that excess dietary zinc induces a copper deficiency state. Since many metals form chelates with protoporphyrin, it may be that zinc is competing with copper in some manner in the formation of iron porphyrins.

Addendum. While this paper was in preparation an abstract was published in the *Fed. Proc.*, *12*, 283 (1953), describing similar studies by Van Reen and Pearson. Their findings, although on rat liver instead of heart tissue, are in agreement with the results of the studies presented here.

Summary. 1. Excess dietary zinc resulted in lowered growth, hemoglobin concentration and cytochrome oxidase activity in the rat. 2. The addition of copper to the zinc diet increased growth and hemoglobin, but to suboptimum levels, whereas the cytochrome oxidase activity was raised to a greater than normal level. 3. A highly significant correlation was found between hemoglobin level and cytochrome oxidase activity. 4. The inhibitory effect of zinc was not due to the presence in the homogenate of free zinc ions, since the *in vitro* addition of inorganic zinc to normal rat hearts in amounts equivalent to that found in the zinc-toxic hearts had no effect on the activity of cytochrome oxidase.

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Received June 22, 1953. P.S.E.B.M., 1953, v83

AGRIC. RES. [INDIA] 2(4): 307. (1962).

TOXICITY OF ZINC IN RATS

Histopathological studies of tissues of rats fed 0·2, 0·3 and 0·4 per cent zinc along with stock diet were completed. Such studies revealed hyperkerotosis of mucous membrane of cardiac end of stomach. Liver in the three groups showed focal areas of disorganisation of liver cells and degenerative changes (B. Dutt and Vasudevan).

Acta physiol. scand. 1958. 43. 275—291.

From the Pharmacological Institute, University of Uppsala, Sweden.

The Effect of Zinc and Certain Other Bivalent Metal Ions on the Isometric Tension Development of Glycerol-extracted Muscle Fibre Bundles.

By

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Received 25 April 1958.

In addition to magnesium (~ 10 mmoles/kg, HAMM 1955, EDMAN unpublished) and calcium (~ 1 mmole/kg, HAMM 1955, EDMAN unpublished), striated muscle contains zinc in a rather high concentration (~ 0.8 mmole/kg, KOGA 1934, HAMM 1955, KOCH *et al.* 1956, EDMAN unpublished).[1] While the effects of calcium and magnesium on the contractile mechanism have been extensively investigated, no thorough investigation has, up to the present, been performed with zinc. The present work comprises a comparative study of the effects of zinc and certain other bivalent metals on the isometric ATP-induced contraction of glycerol-extracted muscle fibre bundles. The investigation has also shown that certain metals can produce a reversible relaxation of fibre bundles previously contracted by adenosinetriphosphate (ATP). The relaxing effect of zinc in the presence of ATP was described earlier in a preliminary report (EDMAN 1956).

[1] The values represent content per kg of wet tissue.

Methods.

Fibre bundles from rabbit psoas muscle were used. The extraction and preparation of these bundles as well as the technique for isometric recording of the tension development were described in an earlier publication (EDMAN 1957). The length of the fibre bundles was 10 mm; the cross section was 75—159 × 131—281 μ.

All the substances studied were dissolved in a KCl solution with pH 7.30 containing 100 mM K, 10 mM veronal (diethylbarbituric acid) and 1 mM Mg. Hereafter this solution will be called the "veronal buffer". In the control experiments the veronal buffer alone was used as medium. In the cases of manganese, cobalt and mercury a new stock solution was prepared for each day's experiments; for the rest of the metals the stock solution was used for two weeks at most.

A 0.2 mM stock solution of mercury was prepared by dissolving $HgCl_2$ in bidistilled water and subsequently diluting 50 times with the veronal buffer. The pH of the stock solution was 7.2.

The water used in preparing the solutions and washing the glass vessels had been twice distilled in borosilicate glass. The pH determinations were performed with a glass electrode.

The pH of the final solutions was checked, being allowed to range from 7.20 to 7.30. For the highest concentrations of cadmium (> 1 mM) studied it was necessary to adjust the pH with KOH.

Veronal, diethylbarbituric acid, crystal, Ph. S. XI was used. All other chemicals used were of pro analysi quality. According to an analysis performed by the Analytical Department of the University of Uppsala, one gram of the diethylbarbituric acid substance contained 180 μg ash, 40 μg sodium, 4 μg calcium, < 3 μg magnesium and ≤ 0.5 μg zinc.

ATP was obtained in the form of dibarium salt, "chromatographically pure", from Schwarts Laboratories, Lot no. 5204, and Pabst Laboratories, Lot no. 129. The purity of the ATP preparations and their concentration of ATP, ADP and AMP were checked as described earlier (EDMAN 1957). According to this analysis the amount of total nucleotide in 51 mg of the substance was:

Schwartz, Lot no. 5204: 0.0546 mmole.
Pabst, Lot no. 129: 0.0573 mmole.

The relative nucleotide concentration was as follows:

Schwartz, Lot no. 5204: ATP 89 %, ADP 4 %, AMP traces only, and an unknown fraction, possibly uridine nucleotide, 1 % at most. Pabst, Lot no. 129: ATP 92 %, ADP 2 % and AMP traces only.

The potassium salt of ATP was prepared from Ba_2ATP for each day's experiments. After 92 mg of the Ba_2ATP substance was suspended in 5 ml of bidistilled water, 1 N HCl was added drop by drop until all of the substance had dissolved. Then 1.50 ml of K_2SO_4 solution (25.15 mg/ml) was added. After centrifugation an additional drop of K_2SO_4 solution was added to the ATP solution to ensure a slight excess of SO_4^{--}. Finally, the ATP solution was neutralized to pH 7.3 with 1 N KOH and diluted with the veronal buffer to 60 ml.

In those series of experiments where the effects of metal ions on the course of the *contraction* were studied, control and test experiments were performed alternately. The fibre bundles for the controls and tests were dissected from the same muscle preparation and were treated throughout in a similar manner. After the fibre bundle had been prepared and fastened between the two platinum loops as described earlier (EDMAN 1957), it was placed in a Petri dish containing 60 ml of the control or test solution for two hours immediately before the start of the experiment. This solution was used for 16 fibre bundles at most. The volume of the bath solution during the recording of the contraction was always 40 ml. The contraction was induced by exchanging the glass vessel with bath solution for another containing the same solution, but with ATP added. Not more than 8 experiments were performed with the same bath solution. The initial tension of the fibre bundle was 17.2 mg. The details on the recording of an isometric contraction are given in an earlier publication (EDMAN 1957).

The fibre bundles used in the studies of the *relaxing effect* of metal ions were washed for about one hour after dissection in veronal buffer only. The fibre bundles were given an initial tension of 22.2 mg and were contracted by ATP (0.37—0.40 mM) in a bath of the veronal buffer solution. After 2—5 min., when the contraction was almost finished, the vessel with bath solution was exchanged for another containing the test solution with ATP (0.37—0.40 mM). The volume of the bath solution was 40 ml in the relaxation experiments also. For the data on the concentrations of the metals studied, see Results.

In order to study the relaxing effect of the metal in the absence of ATP the fibre bundle was first immersed in ATP-free veronal buffer solution for 10—15 min. after a contraction. The tension decreases somewhat within the first 5 to 10 min. after removal of the ATP from the bath.

For all experiments presented in this paper the deviation from strict isometry in the recording device was less than 0.06 mm. *i. e.* less than 0.6 % of the length of the fibre bundles.

The experiments were performed at room temperature, 20—22° C.

If not otherwise stated, the data on the concentrations refer to the total concentration in the bath solution.

Results.

1. *The effect of metal ions on the isometric contraction of glycerol-extracted muscle fibre bundles.*

The contraction was induced by 0.22—0.24 mM ATP. The effects of the metals on the entire course of the contraction were studied. Some results are illustrated in Fig. 1. This figure shows the tension developed after different intervals from the start of contraction.

Since the cross section differs from bundle to bundle and ATP can only penetrate into a peripheral layer of the fibre bundle, the tension developed is expressed in relation to the circumference of the fibre bundle. As shown earlier (EDMAN 1957) there is a direct proportionality between the tension developed and the circumference of the fibre bundle under the given conditions. Five minutes after the start of contraction the tension attains a practically constant level. The last values in the figures may therefore be assumed to represent the final tension in the fibre bundle. The slope of the contraction curve is an expression of the "*absolute* rate of contraction" (EDMAN 1957), defined as tension increase per unit of circumference and unit of time. Each figure also shows the time required to reach 40 % of the final tension for test and control experiments respectively.[1] The reciprocal of this time value is an arbitrary measure of the "initial *relative* rate of contraction" (EDMAN 1957), *i. e.* a measure of the fraction of the final tension developed per unit of time during the initial phase of the contraction. The relative rate was used in order to compensate for changes in the rate of contraction brought about by a mere change in the contractility (EDMAN 1957). The "initial *absolute* rate of contraction", as used hereafter, is the mean tension increase per unit of fibre bundle circumference and unit of time over the interval 0—40 % of final tension. It is an approximation to the initial tangent of the curve.

Table I summarizes the results observed in the study of the effects of the metal ions on the initial relative and absolute rates of contraction and on the final isometric tension of the fibre bundle.

[1] The original contraction curve contains small irregularities in the rapid, initial phase. Thus, in order to get a more accurate value for the time required to reach 40 % of the final tension this value was calculated for each experiment as the arithmetical mean of the times for reaching 30, 40 and 50 % of the final tension.

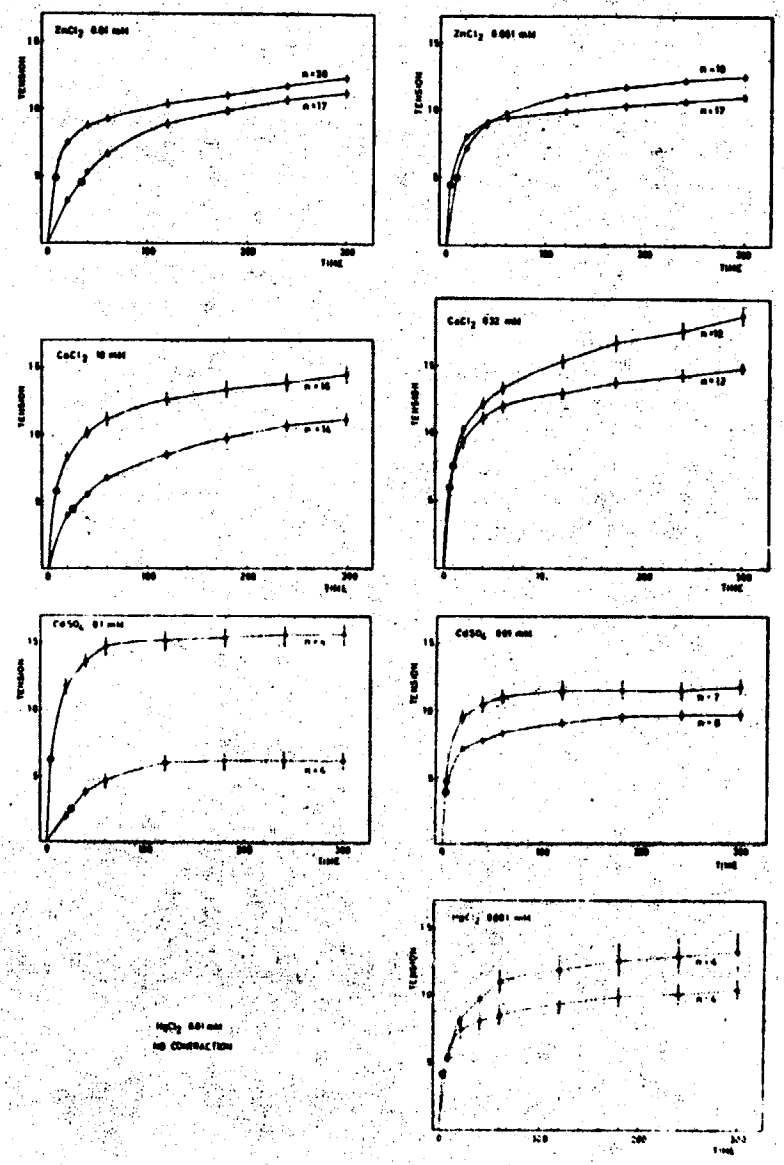

Fig. 1. Effect of high and low concentrations of zinc, calcium, cadmium and mercury on the isometric contraction of glycerol-extracted muscle fibre bundles. ATP concentration 0.22 mM. Cross section of the fibre bundles: 75—159 × 131—281 μ. Ordinate: tension in mg per 100 μ of fibre bundle circumference. Abscissa: time in sec. after immersion in ATP. Filled circles: test experiments, open circles: control experiments, both representing the mean tension developed after different time intervals. The standard error of the mean is indicated with a bar. The number of statistical units is indicated at the respective curves. The average time for reaching 40 % of the final tension is indicated with ⊗ for control curves, and with ⊙ for test curves. The standard error of these latter values does not exceed the size of the symbols.

Zinc in a concentration of 0.025 mM or more completely abolishes the tension development with the magnesium and ATP concentrations used. At 0.01 mM zinc there is still a manifest inhibition of both the relative and the absolute initial rate of contraction and an insignificant lowering of the final tension. In lower concentrations (0.001 mM), zinc still inhibits the initial phase, but increases the final tension. The zinc effects are illustrated in Fig. 1.

Among the other metals studied, calcium, cadmium and mercury (Fig. 1) have an effect similar to that of zinc on the contraction. Thus, in relatively high concentrations (calcium 10 mM, cadmium 0.1 mM, mercury 0.01 mM) they inhibit the entire isometric contraction. Mercury in 0.01 mM or higher concentrations completely abolishes the contractility of the fibre bundle. In low concentrations, calcium (0.32 mM) is without significant effect on the initial phase, but, on the other hand, increases the final tension. Low concentrations of mercury (0.001 mM) and cadmium (0.01 mM) also seem to produce an increase in the final tension, but in these cases, with the few experiments performed, the effects are insignificant.

As pointed out earlier, the magnesium concentration used in these studies was 1 mM. In a series of experiments, however, the effect of higher (10 mM) and lower (0.2 mM) magnesium concentrations was studied. The 1 mM magnesium concentration was taken as basis of reference. An insignificant decrease of 10—20 % in the final isometric tension was obtained with 10 mM and 0.2 mM magnesium as compared with the 1 mM concentration. Magnesium in 10 mM concentration brings about a significant increase of about 94 % in the initial relative rate.

No noteworthy effect during any part of the contraction could be established with cobalt (0.1 and 1 mM), manganese (0.1 and 1 mM) and strontium (0.1, 1 and 10 mM).

II. *The relaxing effect of metal ions on glycerol-extracted muscle fibres.*

As already shown some metal ions in adequate concentrations inhibit the isometric contraction. Therefore, it would seem of interest to determine if these metal ions are also able to relax fibre bundles that have been previously contracted by ATP. The effects were studied in the absence of ATP and in the presence

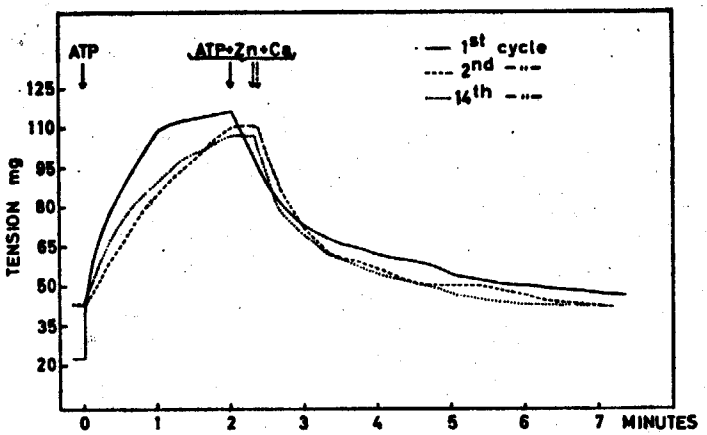

Fig. 2. Repeated contraction-relaxation cycles produced in the same fibre bundle by 0.1 mM zinc and 10 mM calcium in the presence of 0.37 mM ATP. Ordinate: Tension in mg. Abscissa: Time in min. after the start of the respective contraction cycle. The initial tension of the bundle: 22.2 mg. Contraction induced by 0.37 mM ATP. Relaxation brought about by 0.1 mM $ZnCl_2$ + 10 mM $CaCl_2$ + 0.37 mM ATP. Between the different cycles the fibre bundle is washed with buffer solution for 30 min. Cross section of the fibre bundle: 140 × 257 μ.

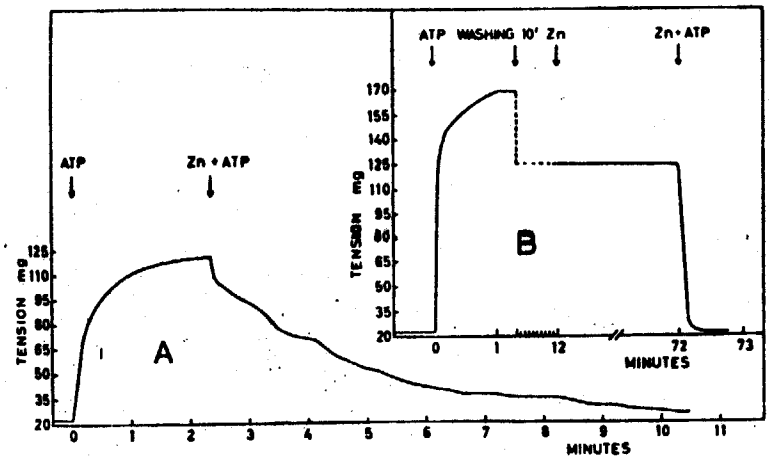

Fig. 3. Effect of zinc on a contracted fibre bundle in presence and absence of ATP. Ordinate: tension in mg. Abscissa: time in min. Initial tension of the bundle: 22.2 mg. Contraction induced by 0.40 mM ATP.

A. Relaxation produced in a contracted fibre bundle by 0.05 mM $ZnCl_2$ and 0.40 mM ATP without pretreatment with zinc. Cross section of the fibre bundle: 108 × 182 μ.

B. After contraction the fibre bundle is washed with buffer solution for 10 min. and immersed in 0.05 mM $ZnCl_2$ alone for 60 min; the zinc alone causes no relaxation. The solution is then exchanged for 0.05 mM $ZnCl_2$ + 0.40 mM ATP, which relaxes the bundle. Cross section of the fibre bundle: 108 × 206 μ.

Note the rapid relaxation by zinc and ATP after pretreatment of the fibre bundle with zinc as compared with the slow relaxation in Fig. 3 A.

of 0.37—0.40 mM ATP. For the performance of the experiments, see Methods. The results are summarized in Table I.

1. *Zinc.*

Figures 2 and 3 show the relaxing effect of zinc in the presence of 0.4 mM ATP. Under the conditions applying in the present study, 0.025—0.05 mM is the lowest concentration of zinc in the bath solution at which complete relaxation of the fibre bundle occurs. (Complete or total relaxation signifies return to the initial tension.)

After about 15 minutes' washing with zinc-free buffer solution following the relaxation, the fibre bundle is again able to contract upon ATP application. In this way the contraction-relaxation cycle may be repeated several times. In one experiment (Fig. 2) 14 cycles were performed in the same fibre bundle. After each relaxation the bundle was washed for 30 minutes with zinc-free buffer solution. No decrease in the ability to relax could be established in this experiment; the tension always returned to the same level during the relaxation. After 13 cycles the ability of the fibre bundle to develop tension had decreased by 10 % of the maximum tension obtained during the first contraction. It is not clear whether this decrease in the contraction ability is due to a decrease in the contractility of the single elements or possibly to damage of the fibre bundle, *e. g.* at the site of the knots, through the repeated exchange of the bath solution.

Application of zinc alone in concentrations $\leq$ 0.88 mM to a contracted fibre bundle deprived of ATP (see Methods) does not bring about any decrease in tension. Only when ATP is also present in the bath, does zinc, in the concentrations studied, induce relaxation. This is illustrated in Fig. 3 for 0.05 mM zinc. Fig. 3 also shows that the course of the relaxation is much more rapid if the fibre bundle is treated with zinc for some time before ATP is added than if zinc and ATP are applied together to the contracted fibre bundle.

2. *Cadmium.*

In the presence of ATP, cadmium produces relaxation of a contracted fibre bundle similarly to zinc. However, in order to obtain complete relaxation by cadmium it is necessary to use a more than 4 times higher concentration.

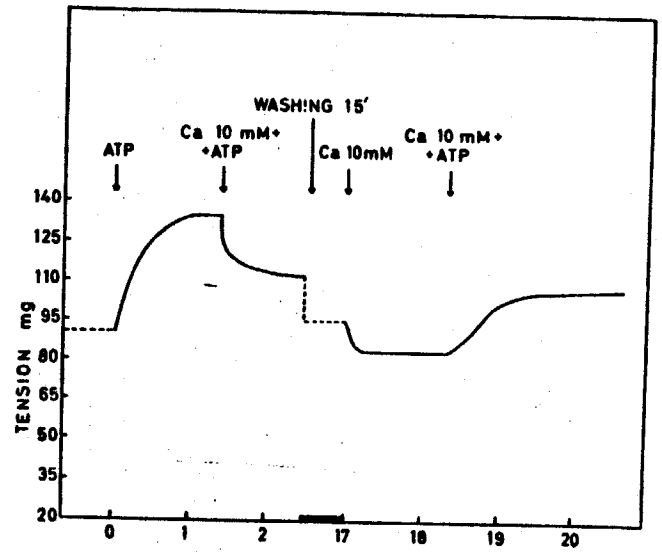

Fig. 4. Effects of 10 mM calcium on a contracted fibre bundle in presence and absence of ATP. Ordinate: Tension in mg. Abscissa: Time in min. Initial tension of the bundle: 22.2 mg. Cross section of the fibre bundle: 112 × 280 μ. At the first arrow the fibre bundle has been washed for 15 min. with calcium-free buffer solution subsequent to a previous contraction-relaxation cycle produced by calcium and ATP.

Note that the tension developed after immersion into calcium + ATP is about the same regardless of the previous tension.

The contraction-relaxation cycle produced by cadmium is repeatable after 30 minutes' washing with cadmium-free buffer solution subsequent to the relaxation. The repeatability, however, is not as complete as in the case of zinc, *i. e.* the contractility decreases after repeated relaxations with cadmium. A marked lowering (about 25 %) of the final tension is already evident after four cycles (for a fibre bundle treated with 1 mM cadmium for 5—6 min. during each relaxation period and washed for 30 min. with cadmium-free buffer solution after the relaxation).

3. *Mercury.*

A slow relaxation is obtained with 0.1 mM mercury in the presence of ATP. About 15 min. is usually required to attain total relaxation. When mercury and ATP are applied together to a contracted fibre bundle that has lost some of its tension through

Table 1.

The effect of bivalent metal ions on the isometric tension development of glycerol-extracted muscle fibre bundles.

Metal	Bath concentration mM	A				B						
		Contraction (0.22—0.24 mM ATP)				Relaxation						
						In presence of 0.37—0.40 mM ATP			In absence of ATP			Reversibility
		Initial relative rate	Initial absolute rate	Final tension	Number of pairs of expts.	Range	Mean	Number of expts.	Range	Mean	Number of expts.	
$ZnCl_2$ ...	0.88											
	0.1	—100***	—100***	—100***	4	49—100	82	5	0	0	3	+
	0.025	—100***	—100***	—100***	4	75—100	93	20	0—10	2	6	+
	0.01	—81***	—82***	—9*	17	93—100	96	4	0	0	4	+
	0.001	—68***	—64***	+14***	17	—16—(—57)	—27	6	0—8	1	10	
	0.0001	—24	—26	—1	14							
$CaCl_2$ ...	10	—68***	—75***	—23***	14	16—30	22	12	5—78	45	6	+
	1	—8	+6	+14	12							
	0.32	—31*	—16	+26***	12							
$CdSO_4$ ..	5											
	1											
	0.1	—82***	—94***	—60***	4	54—100	86	9	0—52	18	4	+
	0.01	+1	+29	+21*	7	71—100	91	11	0	0	2	+
$HgCl_2$ ...	0.1	—100***	—100***	—100***	2	41—100	86	17	50—80	64	5	0
	0.01	—100***	—100***	—100***	3							
	0.001	—65	—61	+27	4							
	0.0001	—18	—7	+16	5							
$MgCl_2$ ...	10	+94***	+65	—17	8							
	0.2	—40	—43**	—10	12							
$CoCl_2$ ...	10											
	1	+5	—21	—27*	7	27—42	35	3	0—23	12	4	—
	0.1	+43	+64**	+12	4	6—14	10	2				
$MnCl_2$ ...	10											
	1	—12	—14	—5	7	14—49	26	3	0	0	1	+
	0.1	+11	+11	+1	4	9—26	17	2				
$SrCl_2$ ...	10	—41	—42	—6	8	5—(—21)	—6	8	0—53	32	7	—
	1	—37	—36	+1	5							
	0.1	—26	—17	+5	5							

Part A: The effect on the contraction given as the difference between the mean of the tests and the mean of the controls in percentage of the controls. The degree of significance has been calculated using Fisher's t-test; *** = $P < 0.001$, ** = $0.001 < P < 0.01$, * = $0.01 < P < 0.05$.

Part B: The relaxing effect on the fibre bundles previously contracted by 0.37—0.40 mM ATP. The relaxation, the drop in tension, is expressed in percentage of the preceding tension obtained in the one series in the presence of 0.37—0.40 mM ATP and in the other series after removal of ATP by washing. Negative values refer to increase in tension. Reversibility: + reversible, 0 irreversible.

removal of ATP (see Methods), there is an initial rise in tension followed by a slow relaxation. This course is probably due to the slowness of the accumulation of mercury in the fibre bundle.

In the absence of ATP, 0.1 mM mercury brings about a lowering of the tension in addition to the decrease in tension that follows the removal of ATP from a contracted fibre bundle.

After 15 minutes' treatment of the bundle with 0.1 mM mercury the lost contractility cannot be restored by washing with mercury-free buffer solution. The washing has been performed with 40 ml solution for time intervals ranging from 30 min. to 3 hrs.

4. *Calcium and strontium.*

Both calcium and strontium in 10 mM concentration are able to produce relaxation of a contracted fibre bundle in the absence of ATP. In the presence of 0.4 mM ATP, a smaller relaxation (see Table I) is obtained with 10 mM calcium, while 10 mM strontium is without relaxing effect. Fig. 4 illustrates typical effects of calcium.

The contraction-relaxation cycle produced by calcium and strontium is repeatable; the contractility is restored after 5—10 minutes' washing with buffer solution subsequent to the relaxation.

5. *Manganese and cobalt.*

A slight relaxation is obtained with cobalt and manganese (1 mM and 10 mM) in the presence of 0.4 mM ATP.

Discussion.

At relatively high concentrations the metals zinc, calcium, cadmium and mercury inhibit the entire course of the contraction. This in itself is not a surprising effect. At lower concentrations, however, zinc and calcium significantly increase the final tension, while they inhibit the initial development of tension (zinc) or have no significant effect on the initial phase (calcium).

Thus, there is a qualitatively different action on the final tension at high and low concentrations of zinc and calcium. However, both effects may be explained by a simultaneous decrease in tension of the single element and an inhibition of the ATPase activity. Taking the tension of the bundle as a whole, the decrease in tension developed by the single element will then be compensated to a certain degree or even overcompensated by activation of deeper layers of the fibre bundle made possible by inhibition of the ATPase activity and consequent deeper penetration of ATP. The effect through activation of deeper layers will be negligible in the most initial phase of the contraction but will grow increasingly significant during the continued course up to the final tension. This may explain the differences in the effect of the individual metals at low concentrations (zinc 0.001 mM and calcium 0.32 mM) on the initial phase and on the final tension.

The effect of metals on the initial phase of the contraction may

further be complicated by the inconstancy of the free metal ion concentration within the fibres during the contraction. Before the addition of ATP the fibres contain the metal in a certain concentration, which is in equilibrium with the metal concentration of the bath. With the addition of ATP, however, the concentration of free metal ions in the bath solution decreases, owing to formation of an ATP-metal complex (Martell and Schwarzenbach 1956, Raaflaub 1956, Walaas 1957, G. Weizel, personal communication concerning zinc, calcium and magnesium) and the equilibrium between the free metal ion in the bath and the protein-bound metal in the fibres is changed. This will cause a change in protein-bound metal unless the protein is completely saturated even at the lower concentration of free ions. The distribution of free metal ions within the fibres is further complicated by the fact that ATP has only a certain, limited depth of diffusion into a fibre and a fibre bundle. Moreover, ADP and AMP are also able to form metal complexes although in a lesser degree than ATP (Martell and Schwarzenbach 1956, Raaflaub 1956, Walaas 1957). In consequence, the distribution of free metal ions within the fibres will not be uniform even at steady state. The complications caused by the change in amount of protein-bound metal occurring during the development of the contraction has not been considered in earlier studies of metal ion effects in these systems.

The final tension is insignificantly lower with 10 mM and 0.2 mM than with 1 mM magnesium. This agrees with observations by Ranney (1955) working with thick fibre bundles from dog heart trabecular muscle. He found an optimum at 3 mM magnesium. Korey (1950) working with thick rabbit psoas fibre bundles found 10 mM magnesium to be optimal.

Cobalt and manganese can compensate for the decrease in function that arises in the actomyosin thread (Gerendas 1941, Bowen 1952) or the glycerol-extracted muscle fibre (Watanabe and Sleator 1957: manganese) when the magnesium concentration is reduced. According to Bowen (1952), the stimulating effect on the shortening of actomyosin threads is equally great for magnesium, cobalt and manganese when these metals are used in equimolar concentrations. This does not prove, however, that the mechanism of action is the same for the three metal ions or that magnesium can be fully replaced by cobalt and manganese. In fact, according to the foregoing discussion, a comparison of

the total metal concentrations in the bath does not necessarily give a decisive answer to the question whether or not a metal can be replaced atom for atom by another metal at the strategic sites of the contractile protein or of the substrate. Furthermore, a strictly quantitative comparison of the effects of different metals on whole fibres or fibre bundles is difficult because of the aforementioned fact that a change in the ATPase activity modifies the depth of diffusion of ATP into the fibres.

HASSELBACH (1953) has shown that zinc in a comparatively high concentration, > 4 mM, reversibly inhibits the tension development of glycerol-extracted muscle fibres and fibre bundles.[1] As shown in the present work, the inhibiting effect of zinc is quite evident even at 0.01 mM concentration in the bath solution. At 0.025 mM zinc no contraction can be induced by 0.23 mM ATP.

According to HASSELBACH (1953) and HASSELBACH and A. WEBER (1955) mersalyl is the only one of the ions and compounds of heavy metals studied [Ag^+, Fe^{++}, Fe^{+++}, Hg^{++}, Cu^{++}, Zn^{++}, $KMnO_4$, K_3FeCN_6, Cu-glycine, Salyrgan® (mersalyl), Oxarsan® (m-amino-p-hydroxyphenyl arsinous oxide) and p-chloromercuribenzoic acid] which produces relaxation of contracted fibres and fibre bundles in the presence of ATP.[1] As demonstrated in the present work, however, relaxation can be produced by several bivalent metal ions. The discrepancy probably depends upon differences in the concentrations of the various components used in our systems.

Among the relaxation-inducing metal ions studied, zinc seems to be of particular interest. Zinc is effectively relaxing even at a bath concentration as low as 0.025—0.05 mM in the presence of 1 mM magnesium and 0.4 mM ATP. Furthermore, the contraction-relaxation cycle is repeatable many times. The physiologic content of zinc in skeletal muscle is comparatively high, *i. e.* according to KOGA (1934) 0.5 mmole, HAMM (1955) 0.6 mmole, KOCH *et al.* (1956) 1.1 mmole, EDMAN (unpublished) 0.8 mmole; all values represent content per kilogram wet tissue.

As shown in this work, mercury in rather low bath concentrations relaxes a contracted fibre. On the other hand, LAKI and BOWEN (1955) reported that HgI_2 (150—500 mM) + KI (300—1 000 mM) caused shortening and tension development in glycerol-extracted rabbit psoas bundles and myosin-B threads. These findings, however, are not completely comparable with the results presented in the present study since the mercury concentrations in the experiments of LAKI and BOWEN were very much higher than those used in this work.

[1] According to a personal communication from Dr. HASSELBACH the bath solution was unbuffered having a pH of 7.0 and containing 80 mM KCl and 5 mM magnesium. The ATP concentration was 5 mM.

It has been proposed (WEBER and PORTZEHL 1952, 1954) that the relaxing effect of the organic mercury compound mersalyl is due to its inhibition of the ATPase activity, thereby allowing ATP to act only as a "plasticizer". A similar mechanism of action is also possible for the relaxing effects obtained in the present work by zinc, cadmium and mercury. However, in the case of mercury, it is improbable that the action is due only to an inhibition of the ATPase activity, since mercury is also able to relax a fibre bundle in the absence of ATP. This suggests a relaxing action of mercury itself by its coupling to the protein. An interesting finding in this connection is the ability of mercury in the absence of ATP to produce a viscosity drop in actomyosin solutions (KUSCHINSKY and TURBA 1951, TURBA and KUSCHINSKY 1952: mersalyl) and to inhibit the combination between actin and myosin (BAILEY and PERRY 1947: p-chloromercuribenzoate, KUSCHINSKY and TURBA 1951, and TURBA and KUSCHINSKY 1952: mersalyl). In view of the high affinity of mercury for SH-groups, blocking of these groups has been held responsible for the effect of mercury compounds on the contractile system. This assumption may be correct. However, at least in the case of zinc and cadmium it is still uncertain if coupling to SH-groups is of *functional* relevance considering the affinity of these metals, albeit much smaller, for other groups in the protein as well, *e. g.* the imidazole groups (KLOTZ 1953, GURD and WILCOX 1956).

A more detailed study of the effects of zinc on glycerol-extracted fibres will be published separately.

Summary.

1. The effect of several bivalent metal ions on the ATP-induced contraction of glycerol-extracted rabbit psoas fibre bundles has been studied under highly isometric conditions. The relaxing effect of these metals on previously contracted fibre bundles in the presence of 1 mM magnesium and in the presence or absence of ATP has also been investigated.

2. Zinc ($\geq$ 0.01 mM), cadmium ($\geq$ 0.1 mM), calcium ($\geq$ 10 mM) and mercury ($\geq$ 0.01 mM) partly or wholly inhibit the contraction. In lower concentrations, however, zinc (0.001 mM) and calcium (0.32 mM) produce an increase in the final tension but have no significant effect (calcium) or an inhibiting action (zinc) on the initial phase of the contraction.

3. In the presence of 0.4 mM ATP, 0.025—0.88 mM zinc induces relaxation of a previously contracted fibre bundle. The contraction-relaxation cycle can be repeated many times. In the absence of ATP, no relaxing effect could be demonstrated for zinc ($\leq$ 0.88 mM).

4. A less reversible relaxation, as compared with that of zinc, is obtained with cadmium (5 mM and 1 mM) in the presence of 0.4 mM ATP. Mercury (0.1 mM) produces a slow relaxation together with 0.4 mM ATP; the relaxation is irreversible after 15 minutes' incubation with mercury, however. Calcium, cobalt and manganese (10 mM) bring about a slight decrease in tension of a contracted fibre bundle in the presence of 0.4 mM ATP.

5. A certain decrease in tension can be demonstrated for calcium (10 mM), strontium (10 mM) and mercury (0.1 mM) in the absence of ATP.

6. In the discussion it is stressed that the complexing of metal ions by ATP greatly complicates the interpretation of findings obtained in studies of the interactions of metals, ATP and contractile proteins.

This investigation was supported by grants from the Medical Faculty of the University of Uppsala, the Magnus Bergvall Foundation and the Medical Research Fund of Svenska Personal-Pensionskassan.

I wish to express my gratitude to Professor Ernst Bárány for his advice and stimulating interest in this investigation.

I also wish to thank Mrs Birgit Andersson for valuable technical assistance.

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Relaxation of Glycerol-extracted Muscle Fibre Bundles Induced by Zinc in the Presence of ATP and Other Polyphosphates.

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Received 24 November 1958.

Abstract.

EDMAN, K. A. PAUL. Relaxation of glycerol-extracted muscle fibre bundles induced by zinc in the presence of ATP and other polyphosphates. *Acta physiol. scand.* 1959. *46.* 62—87. — A survey of factors affecting the relaxing effects of zinc on glycerol-extracted psoas fibre bundles has been made with isometric recording technique. No relaxation is obtained with zinc alone. In the presence of 0.05—0.4 mM adenosinetriphosphate (ATP) and 1 mM magnesium complete relaxation of a contracted fibre bundle is obtained with 0.1—0.4 mM zinc. The relaxing effect decreases at high ATP concentrations, probably due to a decrease in the free zinc ion concentration in the bath caused by complex formation between zinc and ATP. The ATPase activity of the fibre bundles is abolished during relaxation with 0.1 mM zinc + 1 mM magnesium + 0.2 mM ATP. Magnesium and zinc have mutually antagonistic effects on contractility, but the relaxing effect of zinc is probably not due to a simple competition with magnesium. Calcium (10 mM) increases the rate of relaxation induced by zinc + ATP. Relaxation can be induced by 0.2 mM zinc in the presence of 1 mM magnesium and 0.4 mM inosinetriphosphate (ITP) or uridinetriphosphate instead of ATP. The visco-elastic effects of zinc + ITP are much more pronounced than those of zinc + ATP in equimolar bath concentrations. Zinc does not modify the relaxing effect of inorganic pyrophosphate and tripolyphosphate, nor the effect of adenosinediphosphate.

As demonstrated in an earlier study (EDMAN 1958), it is possible to produce relaxation of a contracted glycerol-extracted muscle fibre bundle under isometric conditions with certain bivalent metal ions in the presence of ATP.[1] The relaxing effect of zinc appears even at low zinc concentrations (0.025 mM) in the presence of 1 mM magnesium and 0.1 mM ATP. The zinc effect is fully reversible; the contraction-relaxation cycle can be repeated numerous times without a noteworthy decrease in the contractility. In a later investigation (EDMAN 1959) the relaxing effect of zinc + ATP was studied under isotonic conditions, and, in addition, the effect of zinc + ATP on the visco-elastic properties of resting fibre bundles was recorded.

The present study is aimed at further investigation of the contraction-inhibiting and relaxing effects of zinc, including among other aspects the influence of magnesium and calcium, ATP and other nucleotides (ITP, UTP, ADP) and inorganic polyphosphates. The effects of zinc on the ATPase activity in the fibre bundles will also be presented, as well as determinations of the effect of zinc + ITP on the visco-elastic behaviour of resting fibre bundles subjected to stretch.

Methods.

I. *Contraction-relaxation experiments.*

Glycerol-extracted fibre bundles from rabbit psoas were used. The technique used in extraction and preparation of these bundles as well as in the isometric recording of the tension development has been described (EDMAN 1957). The length of the fibre bundles was 10.0 mm; the cross section was 65—150 × 145—285 μ. The fibre bundles were given an initial tension of 17.2 mg in the experiments where only contraction was studied. In the relaxation experiments the initial tension was 22.2 mg.

[1] The following abbreviations will be used: ADP, adenosinediphosphate; ATP, adenosinetriphosphate; CTP, cytidinetriphosphate; EDTA, ethylenediamine tetraacetate; GTP, guanosinetriphosphate; ITP, inosinetriphosphate; PP, inorganic pyrophosphate; TPP, inorganic tripolyphosphate; UTP, uridinetriphosphate.

A potassium chloride solution (pH 7.30), containing 10 mM Veronal (diethylbarbituric acid), 100 mM potassium and 1 mM magnesium, was used throughout as medium after the glycerol-extraction. Hereafter, this solution will be called "veronal buffer". The magnesium concentration will not be specially mentioned in the rest of the communication unless the concentration was different from 1 mM.

All nucleotides used were obtained as "chromatographically pure" substances: ATP, dibarium salt, Pabst, lot. no. 129 (for analytical data, see EDMAN 1958); ATP, disodium salt, Pabst, lot. no. 109 A (only used in the visco-elasticity measurements and in the determinations of the ATPase activity; for analytical data, see EDMAN 1959); ADP, monosodium salt, Sigma, lot. no. 18—671; ITP, trisodium salt, Pabst, lot. no. 2601; UTP, trisodium salt, Pabst, lot. no. 706. $Na_4P_2O_7 \cdot 10\ H_2O$ and $Na_5P_3O_{10} \cdot 6\ H_2O$, pro analysi, were provided by the courtesy of Chemische Werke Albert, Wiesbaden-Biebrich. For data on the quality of the other chemicals used, see EDMAN (1958). The water used for washing and for preparation of solutions was double distilled in borosilicate glass. The pH determinations were performed with a glass electrode. The experiments were carried out at room temperature (20—22° C).

If not otherwise stated, the concentrations given refer to *total* concentration in the bath solution. In calculating the concentrations of free zinc, magnesium and ATP the same stability constants (log K) were used as earlier (EDMAN 1959), viz. 4.06 for the MgATP complex and 4.76 for the ZnATP complex.[1]

Fisher's t-test was used in determining the statistical significance.

II. *Determination of the ATPase activity in the glycerol-extracted muscle.*

The aforementioned veronal buffer was used as medium. About 3 g glycerol-extracted psoas musculature was first reduced to a pulp in a porcelain mortar with about 5 ml buffer solution. After this coarse pretreatment the muscle tissue was homogenized manually in a Potter-Elvehjem homogenizer with a glass pestle. The homogenization took place at + 4° C during 5-min periods with 10-min intervals. After 8 to 10 such treatments the muscle mass consisted of an undifferentiated brei without observable fibre structures on microscopic inspection (× 120). After being washed three times with 40 ml buffer solution the muscle mass was suspended in 15 ml 50 % water solution of glycerol containing 10 mM sodium phosphate (pH 7.0), *i. e.* the same solution as used for the initial glycerol-extraction of the muscle bundles (EDMAN 1957). The muscle suspension was subsequently stored at — 20° C. Before use 1 ml muscle suspension was washed three times with 20 ml veronal buffer by means of gentle shaking for 5 min followed by 15 to 20 min centrifugation (3,500 rpm). Finally, the suspension was diluted with veronal buffer to 40 ml, and the muscle tissue was further homogenized in this volume by a few minutes' treatment in the homogenizer. Washing and centrifugation took place at + 4° C.

[1] The stability constants obtained by Prof. G. WEITZEL (pers. comm.) have recently been published (WEITZEL and SPEHR 1958).

The ATP solution for test and control experiments was prepared from the same stock solution of Na_2H_2ATP adjusted to pH 7.30 with KOH; the veronal buffer (pH 7.30) was used as solvent. The ATP solution for the tests also included zinc. Each incubation experiment was made up of 2 ml muscle suspension (~ 0.5 mg N), 6 ml veronal buffer and 4 ml ATP solution. The incubation was carried out at + 25° C during slight continuous shaking. The reaction was stopped after 5 min by addition of 4 ml trichloracetic acid to a final concentration of 2.5 %. The mixture was then centrifuged for 15 min (3,500 rpm) at + 4° C.

Two aliquots of 1.5 ml of the supernatant fluid were taken for determination of inorganic phosphate. The method of MARTIN and DOTY (1949) was used, modified to permit use of one-tenth the volume of reagents with micropipettes. The determination was carried out in cuvettes with 1 cm light path in a Beckman model B spectrophotometer.

Because of the spontaneous hydrolysis of ATP, parallel experiments in which trichloracetic acid was added to the muscle suspension before the addition of ATP were carried out both in the presence and in the absence of zinc. The phosphate value in these experiments constituted the zero level in the determination of the ATPase activity.

For determination of the dry weight 10 ml of the final muscle suspension was used. Desiccation took place at + 105° C. The nitrogen content was determined with micro-Kjeldahl technique.

III. *Visco-elasticity measurements.*

The visco-elasticity determinations with zinc + ITP were carried out in the manner described for 0.2 mM zinc + 0.4 mM ATP in an earlier study (EDMAN 1959). The bundles, given an initial tension of 22.2 mg, were first contracted isometrically with 0.4 mM ATP for 2 min and were thereafter washed with veronal buffer alone for 2 min and treated with 0.2 mM zinc for an additional 5 min. Isometric relaxation was then induced with 0.2 mM zinc + 0.4 mM ITP and the tension was recorded for 5 min, when constant tension had certainly been reached. After having been immersed in the solution of 0.2 mM zinc + 0.4 mM ITP for 60 min, the fibre bundle was stretched by 0.83 % of its length. The stretching was achieved in 2 sec. The tension was recorded for 5 min, when a constant level had been reached. The control experiments in the visco-elasticity determinations with zinc + ATP (EDMAN 1959) were carried out on the same fibre bundle preparation as used in the present study with zinc + ITP and have therefore been used as controls in the experiments with zinc + ITP also.

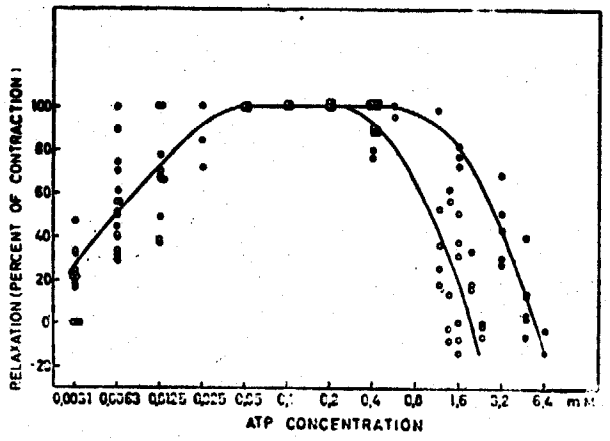

Fig. 1. Relaxing effect of 0.1 mM (open circles) and 0.4 mM (filled circles) zinc in the presence of different concentrations of ATP. Fibre bundles contracted with 0.4 mM ATP before start of relaxation (see this page for details)

Ordinate: relaxation, drop in tension, expressed in percentage of previous contraction.

Abscissa: ATP concentration during relaxation. Curves fitted by sight.

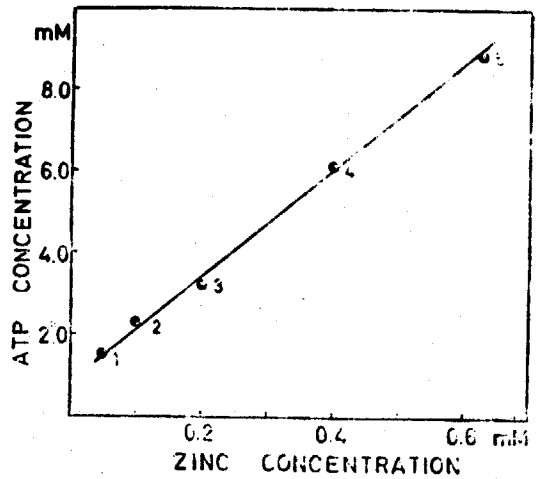

Fig. 2. Relation between zinc concentration (abscissa) and lowest ATP concentration (ordinate), just nullifying the relaxing effect of zinc in fibre bundles previously contracted by 0.4 mM ATP alone. The values represent the mean of four experiments. Concentrations (mM) of free Zn, Mg, ATP and the complexes of ZnATP and MgATP existing at the points numbered 1–5 are given in Table I.

Results.

1. Relaxing effect at varied concentrations of zinc and ATP.

Relaxation induced by 0.1 mM and 0.4 mM zinc at varied ATP concentrations.

Fig. 1 illustrates the relaxing effect of zinc in two different concentrations (0.1 mM and 0.4 mM) with variation of the ATP concentration over a wide range (0.0031 to 6.4 mM). The fibre bundles were first contracted by 0.4 mM ATP for about 2 min. When the ATP concentration during relaxation exceeded 0.4 mM, the zinc + ATP combination was applied to the fibre bundle in direct connection with the contraction. As shown earlier (Edman 1958), the tension in a contracted fibre bundle is decreased by the mere removal of the ATP by washing. Therefore, in order to determine the relaxation with zinc at ATP concentrations below 0.4 mM the fibre bundle was first immersed in buffer solution containing the lower ATP concentration for 10 to 15 min after the contraction before zinc + ATP was applied; in some experiments the bundles were treated instead with ATP-free buffer solution after the contraction. With the addition of zinc + high concentrations of ATP, the tension initially increases beyond that obtained with 0.4 mM ATP alone and thereafter decreases to an equilibrium level (for interpretation, see later). The tension was always recorded until equilibrium had definitely been reached.

As shown by Fig. 1, complete relaxation was obtained with both 0.1 mM and 0.4 mM zinc in the presence of ATP in concentrations between 0.05 mM and 0.2– 0.4 mM. (Complete relaxation signifies return to initial tension.)

The relaxing effect decreases with decreasing ATP concentration; no relaxation at all is obtained in the total absence of ATP. An ATP concentration as low as 0.006 mM is sufficient, however, to give a distinct relaxation with 0.1 and 0.4 mM zinc. At these ATP concentrations, suboptimal in respect to relaxation, the effects of 0.1 mM zinc and 0.4 mM zinc do not differ significantly from each other (left side of Fig. 1).

As shown in the right side of Fig. 1, the relaxing effect of zinc also decreases at high concentrations of ATP. If the ATP concentration is increased sufficiently while the zinc concentration

Table I.

Concentrations of free zinc, magnesium and ATP and of MgATP and ZnATP at the different points in Fig. 2.

Number of point in Fig. 2.	Concentration mM				
	Free Zn^{++}	Free Mg^{++}	Free ATP	MgATP	ZnATP
1	*0.0014*	0.14	0.60	*0.86*	0.049
2	*0.0014*	0.073	1.3	*0.93*	0.099
3	*0.0017*	0.047	2.0	*0.95*	0.20
4	*0.0015*	0.021	4.7	*0.98*	0.40
5	*0.0015*	0.014	7.2	*0.99*	0.62

remains constant, the relaxing effect of zinc is completely abolished, and, with even higher ATP concentrations, the tension increases beyond that obtained with 0.4 mM ATP alone. The higher the zinc concentration the higher the ATP concentration must be for the relaxing effect to begin to decrease. This is illustrated in Fig. 1 for zinc concentrations of 0.1 mM and 0.4 mM.

Mechanism underlying the decrease in relaxing effect of zinc at high ATP concentrations.

Fig. 2 shows the relation between the zinc concentration and the lowest ATP concentration that proved able to completely abolish the relaxing effect of zinc in fibre bundles previously contracted by 0.4 mM ATP. (The zinc concentrations tested, 0.05—0.62 mM, give complete relaxation in the presence of 0.4 mM ATP.) As may be seen, there is a direct proportionality between the total concentration of zinc and the total concentration of ATP necessary to prevent relaxation. Table I presents the concentrations of free zinc, magnesium and ATP as well as the concentrations of the MgATP and ZnATP complexes existing at the different points in Fig. 2. As is seen, the concentration of free zinc ions is approximately constant, 0.0015 mM. The MgATP concentration is also roughly constant (0.86—0.99 mM); almost all magnesium occurs as MgATP complex.

What makes the tension the same at the different points in Fig. 2? The tension cannot be determined solely by the concentration of MgATP since complete relaxation is obtained at

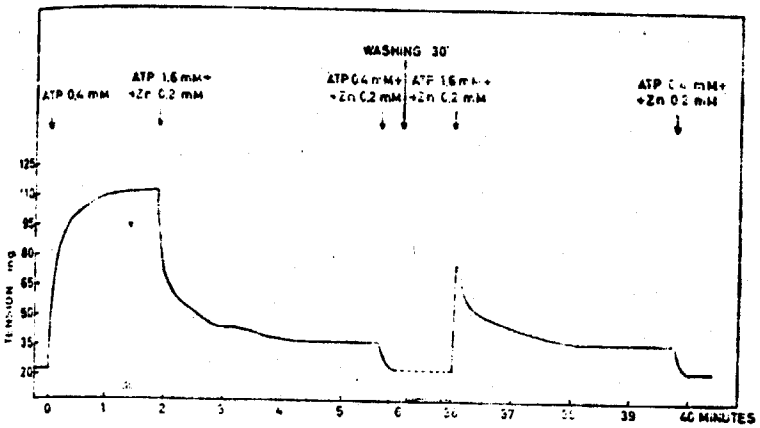

Fig. 3. Effect of 0.2 mM zinc — 1.6 mM ATP on the same fibre bundle in contracted and resting states. Washing before [illegible] treatment with 0.2 mM zinc — 1.6 mM ATP was performed with [illegible] Ringer solution alone. Note that the same equilibrium tension is obtained irrespective of the previous tension of the fibre bundle. Cross section of the fibre bundle: 84 × 178 μ.

0.95 mM MgATP when 0.017 mM free zinc is present, as demonstrated on page 71. That the tension does not drop instantaneously after the addition of the zinc — ATP mixture but initially increases and thereafter decreases to an equilibrium level (see earlier) indicates that the relaxing effect of zinc is dependent upon an accumulation of zinc in the fibre protein. This in turn will be determined by the free zinc ion concentration in the medium. Therefore, it seems probable that the relaxation-inhibiting effect of ATP in high concentrations is due to the decreased concentration of free zinc ions. With an adequate increase in the ATP concentration the zinc ion concentration in the bath solution drops to such an extent that the accumulation of zinc in the fibre protein becomes insufficient for relaxation. Since the free zinc ion concentration is constant at the various points in Fig. 2, it is conceivable that the inhibition of contractility is also the same at these points. That constant tension is obtained in spite of differences in the concentration of free ATP is probably due to the fact that ATP is optimal for contraction within the concentration range studied (cf. Fig. 6, EDMAN 1957 and Table 8, WEBER and PORTZEHL 1954). The 0.0015 mM concentration of free zinc ions is accordingly just below the threshold with respect to relaxation under the conditions used in these experiments.

[1] At the arrows in this and the other figures the previous bath solution was completely exchanged for the new one.

Comparison of the relaxing effects of zinc at suboptimal and superoptimal ATP concentrations.

As shown above, partial relaxation of a contracted fibre bundle can be obtained by application of zinc with either low (suboptimal) or high (superoptimal) concentrations of ATP. With *superoptimal* concentrations the same equilibrium tension is reached for a particular zinc + ATP combination regardless of whether the previous tension in the fibre bundle is higher or lower. A typical example is presented in Fig. 3. It may be seen that 0.2 mM zinc + 1.6 mM ATP brings about a partial decrease in tension in a contracted fibre bundle to a certain level. The same equilibrium tension is attained when the zinc + ATP combination is applied to the fibre bundle after it has been totally relaxed and subsequently washed with zinc-free buffer solution for 30 min. The initial, rapid increase in tension that takes place when zinc + ATP are applied to the relaxed and washed fibre bundle probably depends upon the slowness of the accumulation of zinc in the fibre bundle. These observations show that the changes induced by zinc + ATP in the contractile system are fully reversible. This agrees with the good repeatability of a contraction-relaxation cycle induced with zinc + ATP, as demonstrated earlier (EDMAN 1958).

With *suboptimal* ATP concentrations, zinc in the presence of ATP is able to decrease the tension in the fibre bundle to a certain level only, but it is not able, on the other hand, to increase the tension to the same level by contracting a fibre bundle that has lower tension previously. This is not surprising since the ATP concentration in the suboptimal range is too low to induce contraction even in the absence of zinc. The threshold concentration of ATP for induction of isometric contraction of glycerol-extracted fibre bundles is approximately 0.02 mM in a zinc-free medium containing 1 mM magnesium.

Relaxation induced by 2 mM zinc in the presence of 5 mM ATP.

In accordance with the foregoing observations, it is necessary to increase the total concentration of zinc in the bath solution to a corresponding degree if relaxation is to be obtained with zinc at high ATP concentration. When the total concentration of ATP is 5 mM, as is needed for relaxation with the Marsh-

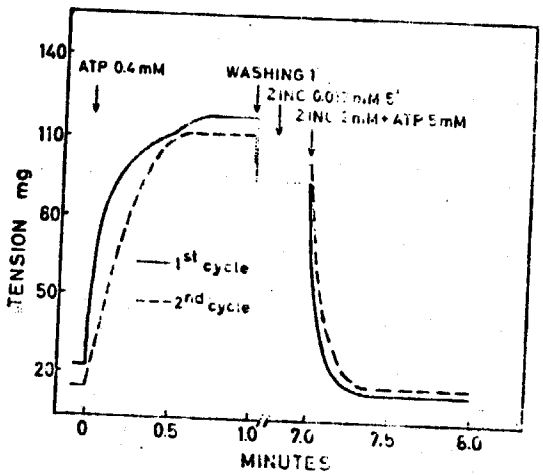

Fig. 4. Two contraction-relaxation cycles produced in the same fibre bundle with 2 mM zinc + 5 mM ATP. Washing at height of contraction performed with veronal buffer solution only. After the first relaxation the fibre bundle was washed with veronal buffer solution for 30 min before the second contraction was started. Cross section of the fibre bundle: 103 × 182 μ.

Bendall factor for instance (HASSELBACH and WEBER 1953, WEBER and PORTZEHL 1954), it is necessary to have about 2 mM total concentration of zinc for complete relaxation in the presence of 1 mM magnesium. The concentration of 2 mM zinc was found empirically and is close to the lower limit giving complete isometric relaxation. With the combination 2 mM zinc (+ 1 mM magnesium) + 5 mM ATP the free zinc ion concentration is 0.017 mM, the concentration of free ATP 2.1 mM, and that of MgATP 0.95 mM. A typical example of the isometric relaxation with this zinc + ATP combination is given in Fig. 4. The fibre bundle was pretreated 5 min with 0.017 mM zinc so the zinc had time to accumulate to an effective concentration before addition of ATP. (When zinc + ATP is applied without pretreatment with zinc, the fibre bundle responds with an initial contraction before the relaxation starts.) The time required to attain 50 per cent of total relaxation is 4.2 ± 0.7 sec (mean time from 6 relaxation experiments). The contraction-relaxation cycle is fully repeatable after 30 min washing with buffer solution in the manner described earlier for 0.025 — 0.88 mM zinc + 0.4 mM ATP (EDMAN 1958).

Table II.

Effect of 0.1 mM zinc on the splitting of 0.22 mM and 2.6 mM ATP in glycerol-extracted psoas muscle brei.

Series of experiments			μmole P/mg N/min	Test × 100/control
I	0.22 mM ATP + 1 mM Mg (control)	a	0.547	0
		b	0.559	
	0.22 mM ATP + 1 mM Mg + 0.1 mM Zn	a	0	
		b	0	
II	2.6 mM ATP + 1 mM Mg (control)	c	0.705	73.0
		d	1.036	
	2.6 mM ATP + 1 mM Mg + 0.1 mM Zn	c	0.489	
		d	0.793	

Corresponding tests and controls are indicated by the same letters.

2. *Determination of the ATPase activity.*

A prerequisite for induction of complete isometric relaxation with lower ATP concentrations than that used for the previous contraction of the fibre bundle is inhibition of the ATPase activity. Otherwise ATP would not be able to penetrate sufficiently deeply into the fibre bundle (EDMAN 1957) and would not have a possibility of relaxing the deeper layers that were contracted at a higher ATP concentration.

Table II presents the results of experiments that were carried out for determination of the ATPase activity under conditions corresponding to the contraction-relaxation experiments. The muscle pulp was thoroughly homogenized (see Methods) to allow complete penetration of ATP.

As may be seen from Table II, the ATPase activity is completely abolished with the 0.1 mM zinc + 0.22 mM ATP combination, which gives full relaxation (Fig. 1). On the other hand, with 0.1 mM zinc + 2.6 mM ATP, where the ATP concentration is clearly superoptimal for relaxation (see Fig. 1 and 2) there is only a partial reduction of the ATPase activity as compared with 2.6 mM ATP alone. The decrease in inhibition of the ATPase activity at the high ATP concentration is probably due to the decrease in free

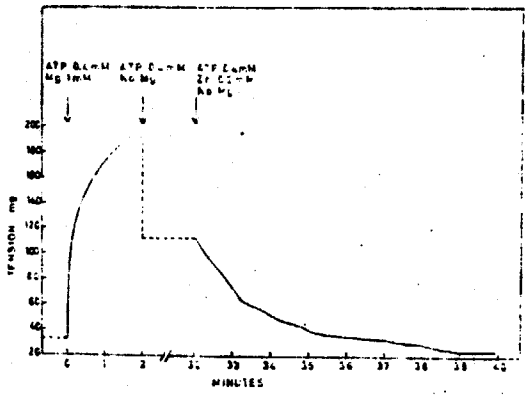

Fig. 5. Partial relaxation produced by removal of magnesium from the medium at unchanged ATP concentration. Complete relaxation follows addition of 0.2 mM zinc. Cross section of the fibre bundle: 150 × 220 μ. Two contraction-relaxation cycles preceded the cycle illustrated.

zinc ion concentration in the bath caused by complex formation between zinc and ATP as described above.

Effects of zinc on the ATPase activity of contractile proteins in solution have been reported and are in agreement with the results on glycerol-extracted muscle demonstrated in this work. Thus according to MARYAMA (1958), 1 mM zinc completely abolishes the ATPase activity of crayfish myosin B at + 30° C in the presence of 1 mM ATP, 10 mM calcium and 600 mM potassium. GILMOUR and GRIFFITH (1958) report that zinc in concentrations below 0.015 mM stimulates slightly, while in concentrations above 0.015 mM zinc inhibits the ATPase activity of rabbit myosin at + 25° C in the presence of 1.13 mM ATP, 10 mM calcium and 100 mM potassium; at 0° C even the low zinc concentrations are inhibiting. DICKENS and GLOCK (1951) demonstrated a slight inhibition of the ATPase activity of rat myosin by 0.02 mM zinc at + 37° C in the presence of 3.2 mM ATP, 5 mM calcium and 425 mM potassium.

3. *Significance of magnesium in the relaxation induced by zinc and ATP.*

The magnesium ion stimulates the ATP-induced contraction of glycerol-extracted fibres with optimal effect at 1–10 mM concentration. Reduction of the magnesium in a contracted fibre

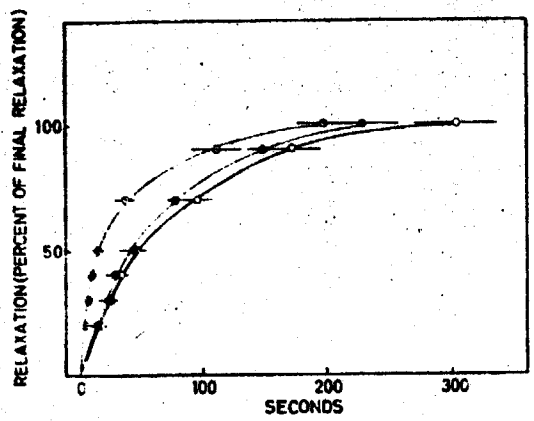

Fig. 6. Effect of magnesium on the rate of the zinc + ATP- induced relaxation of glycerol-extracted fibre bundles.

Ordinate: relaxation expressed in percentage of total drop in tension.

Abscissa: time in sec from start of relaxation.

Contraction induced by 0.4 mM ATP, relaxation by 0.2 mM zinc + 0.4 mM ATP in presence of, respectively, 0.1 mM (○), 1 mM (●) and 10 mM (◐) magnesium in the medium during contraction and relaxation.

Number of experiments: 5. The standard error of the mean indicated with a horizontal bar. The p-value for the difference between the 0.1 mM and 1 mM magnesium curves at 70 % relaxation is < 0.01. The mean tension in excess of the initial tension in mg/100 μ fibre bundle circumference before and after relaxation was:

before	after
15.0 ± 1.1	1.2 ± 0.6 at 0.1 mM magnesium
25.5 ± 2.0	0.5 ± 0.5 at 1 mM magnesium
24.1 ± 1.8	0.9 ± 0.7 at 10 mM magnesium

bundle by washing lowers the tension appreciably despite the presence of ATP in unchanged concentration (0.4 mM). This is illustrated in Fig. 5. The washing was carried out in magnesium-free veronal buffer for 30 min. Total relaxation was obtained by 0.2 mM zinc + 0.4 mM ATP.

The following experiments were performed in order to investigate whether there is any antagonism between zinc and magnesium,

and, whether the relaxing effect of zinc may be due to a competition with magnesium. The effect of a certain concentration of zinc was studied in the presence of constant ATP concentration but with varied concentrations of magnesium. The experiments were performed with two zinc concentrations, 0.2 mM and 0.0076 mM. At 0.2 mM concentration the relaxation course was studied, at 0.0076 mM concentration the contraction.

In the experiments with 0.2 mM zinc three contraction-relaxation cycles were induced in the same fibre bundle. The contraction was induced with 0.4 mM ATP in the presence of 0.1 mM, 1 mM and 10 mM magnesium. The relaxation was induced with 0.2 mM zinc in the presence of 0.4 mM ATP and magnesium in the same concentration as during the previous contraction. Between each relaxation and the succeeding contraction the fibre bundle was washed for 30 min in veronal buffer containing 0.1 mM magnesium. The order of the different contraction-relaxation cycles was varied. The results obtained with five fibre bundles are presented in Fig. 6. The absolute tension at the beginning and end of relaxation is given in the figure text. The relaxation is expressed as fraction of maximum relaxation. By this means the slope of the curves, which represents the rate of relaxation, becomes independent of the absolute tension. As pointed out earlier (page 69) zinc must probably accumulate to a certain concentration in the fibre protein before the contractility becomes inhibited. In fibre bundles, — like those used in these experiments —, not pretreated with zinc the rate of relaxation is probably determined by the rate of accumulation of zinc in the protein (Fig. 3, Edman 1958); the relaxation rate is thus an expression of the time necessary to reach an effective concentration of zinc in the fibre protein. If a simple competitive antagonism exists between zinc and magnesium, an increase in the magnesium concentration would be expected to increase the need for accumulation of zinc in the bundle, and, consequently, cause a slower relaxation.

As is evident from the results in Fig. 6, the contracted fibre bundles are relaxed almost completely to the initial level by 0.2 mM zinc + 0.4 mM ATP at all three magnesium concentrations studied, *i. e.* not even the highest magnesium concentration used (10 mM) is sufficient to inhibit the final zinc effect. The relaxation takes place more slowly in the presence of 1 mM and 10 mM magnesium than with 0.1 mM magnesium. On the other hand, there is no significant difference in rate of relaxation between 1

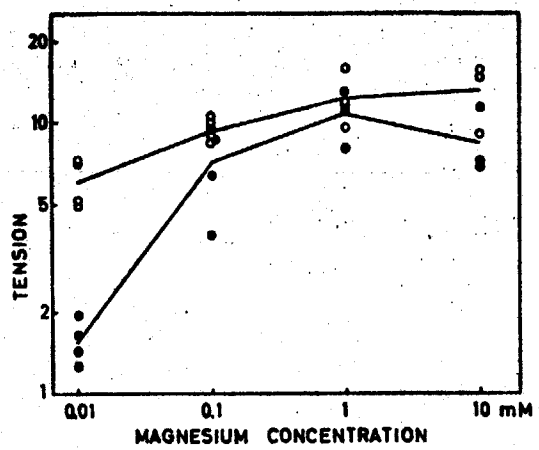

Fig. 7. Effect of 0.0076 mM zinc on the isometric contraction of glycerol-extracted fibre bundles induced by 0.24 mM ATP at different concentrations of magnesium.

Ordinate: tension in mg/100 μ fibre bundle circumference.

Abscissa: magnesium concentration (mM).

Filled circles: 0.0076 mM zinc; open circles: control experiments without zinc. Each symbol represents one experiment.

mM and 10 mM magnesium. However, if the relaxing effect of zinc were solely due to a competition with magnesium, the increase in magnesium from 1 mM to 10 mM would be expected further to inhibit the relaxation rate; in fact, this further inhibition would be expected to be much greater than that obtained with the comparatively small increase in magnesium from 0.1 mM to 1 mM. Hence, it seems improbable from these results that the relaxing effect of zinc is due to a simple competition with magnesium.

Fig. 7 shows the tension developed in a series of isometric contraction experiments carried out with 0.0076 mM zinc[1] and 0.24 mM ATP in the presence of magnesium in concentrations varied from 0.01 mM to 10 mM. The zinc concentration was selected so that almost complete inhibition of the contraction was obtained in the presence of the lowest magnesium concentra-

[1] The veronal buffer solution used as solvent in these experiments contained 0.14 μmole zinc/l according to an analysis performed by the Analytical Department of the University of Uppsala.

tion. It is evident that the inhibition of contraction by zinc decreases with increasing magnesium concentration. In the presence of 1 mM and 10 mM magnesium, 0.0076 mM zinc causes no definite inhibition of contraction.

In a quantitative evaluation of the effects of zinc at the different magnesium concentrations demonstrated in Fig. 7, the following complicating factors must be considered:

1. Inhibition of the ATPase activity increases the depth of penetration of ATP which more or less compensates for the inhibition of the contractility. According to GESKE, ULBRECHT and WEBER (1957), the ATPase activity of suspended glycerol-extracted myofibrils is maximal at 1 mM magnesium (pH 7.6, μ = 0.1, ATP 0.4 mM). It is therefore conceivable that the contraction-inhibiting effect of zinc at 0.01 mM magnesium may be underestimated because of deeper penetration of ATP. 2. With an increase in the magnesium concentration, a greater portion of the zinc will appear as free ions in the medium due to displacement of zinc from ATP by magnesium. Therefore, at high magnesium concentrations the contraction-inhibiting effect of zinc might be overestimated, since the effect is probably dependent on the free zinc ion concentration (see page 69).

4. *The minimum zinc concentration producing total relaxation.*

The zinc-induced relaxation is influenced by both the ATP concentration and the magnesium concentration in the bath. The following experiments were performed to determine the minimum free zinc ion concentration necessary for producing complete relaxation of a contracted fibre bundle in the absence of magnesium in the medium. The fibre bundle was first contracted with 0.4 mM ATP in the presence of 1 mM magnesium. After the contraction the bundle was washed with magnesium-free buffer solution for 10 min and was then immersed in zinc solution (without magnesium) for 30 min after which the solution was exchanged for another with the same composition but, in addition, containing ATP. A series of decreasing zinc concentrations were tested, and for each zinc concentration the optimal concentration of ATP for obtaining relaxation was determined. The lowest total zinc concentration at which complete relaxation could be established in the absence of magnesium was 0.0125 mM, if ATP was present in 0.04—0.08 mM concentration. This corresponds to a free zinc ion concentration of only 0.0025 mM in the medium.

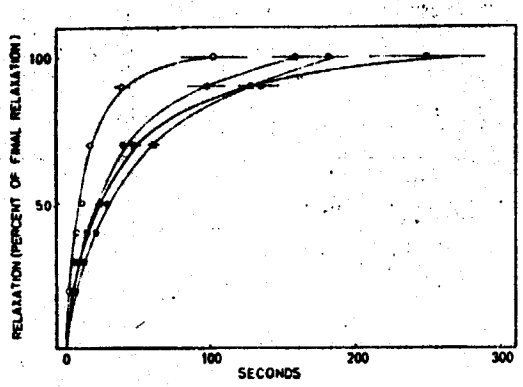

Fig. 8. Effect of calcium on the rate of the zinc + ATP- induced relaxation of glycerol-extracted fibre bundles.

Ordinate: relaxation expressed in percentage of total drop in tension.

Abscissa: time in sec from start of relaxation.

Magnesium (1 mM) consistently present. Contraction induced by 0.4 mM ATP in absence of calcium. Relaxation induced by 0.2 mM zinc + 0.4 mM ATP ± calcium:

(No calcium: ●;
0.1 mM calcium: ⊗;
1 mM calcium: ○;
10 mM calcium: ◐).

Number of experiments: 4. The standard error of the mean is indicated with a horizontal bar. The p-value for the difference between the control curve (without calcium) and the 10 mM calcium curve is < 0.01 at 70 % relaxation. The tension dropped to the initial tension during relaxation in all experiments.

5. *Influence of calcium on the relaxation induced by zinc and ATP.*

Since some of the relaxing factors described earlier, the Marsh-Bendall factor (MARSH 1952, BENDALL 1953, 1954, 1958 a) and EDTA (BOZLER 1954, WATANABE 1955, WATANABE and SLEATOR 1957, BENDALL 1958 b) are inhibited by even low concentrations of calcium, it is of interest to investigate whether calcium also inhibits the relaxation induced by zinc + ATP.

Magnesium in 1 mM concentration was consistently present. The fibre bundle was first contracted by 0.4 mM ATP in the absence of calcium for 2 min, and thereafter relaxed by 0.2 mM zinc + 0.4 mM ATP in the presence of 0.1 mM, 1 mM and 10 mM

calcium. Control experiments were performed with no calcium present in the bath during relaxation. Four contraction-relaxation cycles were carried out with the same fibre bundle, *i. e.* one cycle for each calcium concentration and one control cycle. Between each cycle the fibre bundle was washed with veronal buffer alone for 30 min. The order of the experiments in the series was varied. Fig. 8 summarizes the results on the rate of relaxation obtained with 4 fibre bundles. In all cases the tension returned to the initial level during relaxation regardless of the calcium concentration. Accordingly, at the concentrations studied calcium does not notably change the magnitude of the relaxation induced by zinc + ATP. On the other hand, the rate of the relaxation increases in the presence of 10 mM calcium, as shown in Fig. 8.

The effect of 10 mM calcium on the relaxation with zinc + ATP is entirely in accordance with the observation that calcium in that concentration itself induces a partial relaxation. As shown in an earlier study (EDMAN 1958), the relaxation obtained with 10 mM calcium is even greater in the absence of ATP than in the presence of 0.4 mM ATP. These effects of calcium on the zinc + ATP-induced relaxation must further mean that the mechanism acting in the relaxation with zinc is other than in the relaxation with the Marsh-Bendall factor and EDTA.

6. *Relaxation by ADP and inorganic polyphosphates in the presence and absence of zinc.*

It is known that ADP, inorganic pyrophosphate (PP) and tripolyphosphate (TPP) can induce relaxation of glycerol-extracted fibres that have been contracted with ATP (PORTZEHL 1952, WEBER and PORTZEHL 1952, 1954). It is therefore of interest to determine if the relaxation by these phosphate compounds is affected by the presence of zinc.

The procedure in the experiments was as follows. The bundles were first contracted by 0.4 mM ATP for about 2 min. The ATP was then exchanged for ADP, PP or TPP, either directly or after washing for 2 to 15 min with ATP-free buffer solution. When the fiber bundle had relaxed to equilibrium (4—6 min), the bath solution was exchanged for another containing, in addition to ADP, PP or TPP, 0.2 mM zinc. In some experiments two contraction-relaxation cycles were carried out instead on the same fibre bundle (with 10 min washing after the first relaxation), with the

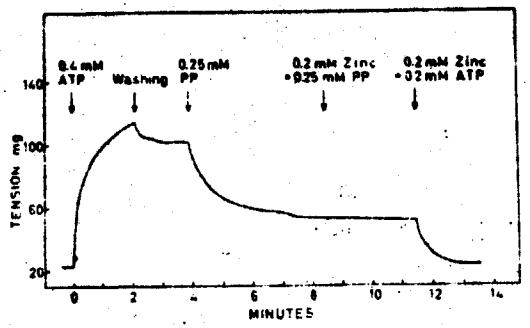

Fig. 9. Relaxing effect of 0.25 mM PP on glycerol-extracted fibre bundles in the absence and presence of 0.2 mM zinc. Washing after contraction performed with veronal buffer solution only. Note that zinc does not cause any decrease in tension in addition to that obtained with PP alone. Complete relaxation is obtained with 0.2 mM zinc + 0.2 mM ATP. Cross section of the fibre bundle: 117 × 168 μ.

relaxation induced in the first cycle in the absence of zinc and in the second cycle in the presence of zinc.

TPP in 0.5 mM concentration (2 exp.) and PP in 1 mM concentration (4 exp.) induces an almost total return of the tension to the initial level. It was possible with even as low as 0.25 mM concentrations of TPP (5 exp.) and PP (3 exp.) to demonstrate a partial relaxation in excess of the tension decrease of about 30 to 50 per cent that takes place as a result of removal of the ATP by washing in buffer solution for 2 to 15 min. ADP in concentrations of 0.25 mM (4 exp.), 1 mM (7 exp.) and 10 mM (3 exp.) produced no demonstrable relaxation in excess of that resulting from washing.[1]

The presence of 0.2 mM zinc does not change the reaction of the fibre bundle to ADP, PP and TPP in the concentrations studied. (The numbers of experiments were the same as stated for the experiments with the various concentrations of ADP, PP and TPP in the absence of zinc.) For purposes of comparison relaxation was finally induced with 0.2—0.4 mM *ATP* + 0.2 mM zinc in all experiments with ADP and in the experiments with 0.25 mM TPP and PP. The tension then dropped to the initial level. A typical example of the relaxation with 0.25 mM PP in the absence and presence of 0.2 mM zinc is given in Fig. 9.

[1] The preliminary finding of a relaxing effect of ADP mentioned in an earlier paper (Edman 1957) could not be verified.

Table III.

Comparison of the contraction-inducing effects of ATP, UTP and ITP on glycerol-extracted muscle fibre bundles in the presence of 1 mM magnesium.

Substance	Latency from immersion to start of contraction in sec	Isometric tension 3 min after the start in mg per 100 μ of fibre bundle circumference	Number of expts.
ATP 0.4 mM	0.3 ± 0.05	13.3 ± 0.98	10
UTP 0.4 mM	2.4 ± 0.6**	9.0 ± 0.45**	5
ITP 0.4 mM	19.3 ± 2.4***	3.5 ± 0.17***	5

Asterisks denote the degree of significance for the differences between the means obtained with ATP and those obtained with UTP and ITP.

*** = $P < 0.001$, ** = $0.001 < P < 0.01$, * = $0.01 < P < 0.05$.

7. *The effects of ITP and UTP in the presence and absence of zinc.*

It is possible to induce contraction in actomyosin systems with other nucleoside triphosphates than ATP, viz. acetyl-ATP, CTP, GTP, ITP and UTP (Bergkvist and Deutsch 1954, Portzehl 1954, Ranney 1955, Hasselbach 1956). In the following experiments the contraction-inducing effects of ATP, ITP and UTP on glycerol-extracted fibre bundles are compared under strictly isometric conditions. Furthermore, it is shown that relaxation may be obtained with zinc in the presence of UTP and ITP instead of ATP.

Table III shows the results obtained with 0.4 mM UTP and ITP as contraction-inducing substances, including for comparison the corresponding results with 0.4 mM ATP. A practically constant level was reached 3 min after the start of the contraction, both with ATP, UTP and ITP. In accordance with results of Hasselbach (1956), ITP gives the weakest isometric contraction and ATP the strongest, while UTP lies between. It is remarkable that the latency, *i. e.* the time from the application of the nucleotide until the start of contraction is considerably longer with UTP and, in particular, ITP than with ATP.

Application of zinc in a final concentration of 0.2 mM in the presence of 0.4 mM UTP induces total relaxation of a fibre bundle

that has been previously contracted with 0.4 mM UTP. The relaxation is complete within the course of one minute (without pretreatment with zinc). After 30 min washing with zinc-free buffer solution, a new contraction can be induced with 0.4 mM UTP.

Zinc in 0.2 mM concentration in the presence of 0.4 mM ITP also induces relaxation of a fibre bundle previously contracted with 0.4 mM ITP or 0.4 mM ATP, in the latter case followed by 15 min washing with ATP-free buffer solution. The tension returns to the initial level within only about 15—30 sec (without pretreatment with zinc). For the rate of relaxation after treatment with zinc, see later (Results, 8). A new contraction can be induced with 0.4 mM ITP or ATP after 30 min washing with zinc-free buffer solution.

Thus, under the conditions used here, isometric relaxation can be induced with zinc in the presence of UTP and ITP instead of ATP. This is in contrast to the findings obtained at relaxation with the Marsh-Bendall factor and EDTA. According to HASSELBACH (1956), who used a semi-isometric recording technique, ITP and UTP in concentrations up to 10 mM are unable to induce relaxation of contracted fibres in the presence of Marsh-Bendall factor and 10 mM magnesium. WATANABE and SLEATOR (1957) report that the combination 4 mM EDTA + 4 mM magnesium + 4.8 or 8.1 mM ITP stops the isotonic contraction of a glycerol-extracted fibre bundle induced by 8.1 mM ITP + 4 mM magnesium, but does not produce significant relaxation of the bundle as does 4 mM EDTA + 4 mM magnesium + 4 mM ATP. Results similar to those of WATANABE and SLEATOR were obtained by BENDALL (1958 b).

8. *Visco-elastic effects induced by zinc and ITP.*

The visco-elastic effects of zinc + ATP have been described earlier (EDMAN 1959). Because of the pronounced relaxation obtained with ITP it is of interest also to investigate the effect of zinc + ITP on the visco-elastic behaviour at and following quick stretch. For the performance of the experiments, see Methods III. Table IV summarizes the results of the visco-elasticity measurements performed with 13 fibre bundles. For comparison the results published earlier (EDMAN 1959) with 0.2 mM zinc + 0.4 mM ATP have also been included in the table. In the interpretation of the

Table IV.

Effects of zinc + ITP and zinc + ATP on the visco-elastic behaviour of resting glycerol-extracted muscle fibre bundles subjected to stretch.

Test medium	1	2	3	4	5	6	7
	Equilibrium tension of fibre bundle *before* stretch g/cm²	Stretch in % of initial fibre length	Initial tension increase at stretch g/cm² % stretch	Equilibrium tension increase after stretch g/cm² % stretch	Half time for tension decrease in sec	Number of expts.	Mean area of cross section cm². 10⁴.
Veronal buffer alone (1 mM Mg)	108 ± 3	0.83	506 ± 39	259 ± 36	4.1 ± 0.3	13	20.7 ± 0.52
Zn 0.2 mM + Mg 1 mM + ITP 0.4 mM	86 ± 5***	0.83	299 ± 9***	128 ± 4*** △△△	1.3 ± 0.1*** △△△	13	19.6 ± 0.36
Zn 0.2 mM + Mg 1 mM + ATP 0.4 mM	73 ± 7***	0.83	642 ± 33*	188 ± 12	6.3 ± 1.0*	13	20.0 ± 0.91

The degree of significance has been calculated for the differences between the means. Asterisks denote differences between tests (Zinc + ITP or zinc + ATP) and controls (veronal buffer alone); triangles denote differences between the tests.

*** $P < 0.001$. △ $0.01 < P < 0.05$.

Table V.

Visco-elastic parameters for the model used (see text) calculated from data of Table IV.

Test medium	C_1	C_2	ϱ
Veronal buffer alone (1 mM Mg)	1	1	1
Zn 0.2 mM + Mg 1 mM + ITP 0.4 mM	1.7	2.4	6.3
Zn 0.2 mM + Mg 1 mM + ATP 0.4 mM ...	0.79	2.0	0.74

results the same model was used as earlier (EDMAN 1959) in the analysis of the visco-elastic conditions, *i. e.* an elastic element of compliance C_1 in series with an elastic element of compliance C_2, that acts in parallel with a viscous element of fluidity ϱ and with a force F, which represents the active contractility. Table V shows the effect of the treatment on C_1, C_2 and ϱ computed from the mean values given in Table IV. As pointed out earlier (EDMAN 1959), no significant visco-elastic effect is obtained with 0.2 mM zinc + 0.4 mM ATP. The combination of 0.2 mM zinc + 0.4 mM ITP, on the other hand, gives increased compliance in both C_1 and C_2 and also increased fluidity (ϱ) of the viscous element.

It was demonstrated earlier (EDMAN 1959) that visco-elastic factors are not rate-limiting in the isometric relaxation process with 0.2 mM zinc + 0.4 mM ATP, and it was considered probable that the *isometric* relaxation of a glycerol-extracted fibre bundle induced by zinc + ATP is mainly the disappearance of a force probably not dependent upon visco-elastic factors. In accordance, the decrease in tension obtained with 0.2 mM zinc + 0.4 mM ITP is not more pronounced than that produced by 0.4 mM zinc + 0.4 mM ATP (see column 1, Table IV), although the visco-elastic effects are significantly greater with the zinc + ITP combination.

Fig. 10 illustrates the rate of relaxation after contraction and the rate of tension decrease after stretch with 0.2 mM zinc + 0.4 mM ITP and with 0.2 mM zinc + 0.4 mM ATP. (The stretch experiments with zinc + ITP are the same as those presented in Table IV; the stretch and relaxation experiments with zinc + ATP are the same as those published in Fig. 6, EDMAN 1959.) As is seen, the relaxation takes place at practically the same rate with the combinations zinc + ATP and zinc + ITP studied notwithstanding that the latter gives an appreciably more rapid

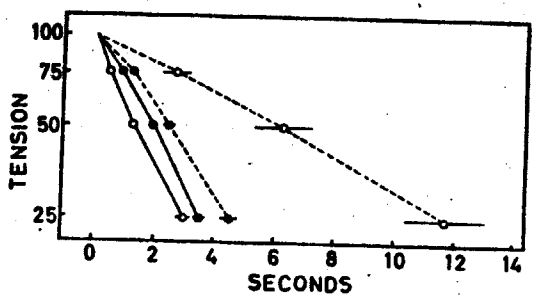

Fig. 10. Comparison between the decay of tension after stretch of resting glycerol-extracted muscle fibre bundles and the relaxation of isometrically contracted fibre bundles in the presence of 0.2 mM zinc + 0.4 mM ITP and of 0.2 mM zinc + 0.4 mM ATP.

Abscissa: time in sec after start of the relaxation and start of the tension decrease after stretch, respectively.

Ordinate: tension remaining in excess of the equilibrium tension, expressed in percentage of total tension decrease.

Number of experiments in respective groups: 13. The standard error of the mean is indicated with a horizontal bar.

o———o tension decrease after stretch, zinc + ITP
•———• relaxation after contraction, zinc + ITP
•- - - - - • relaxation after contraction, zinc + ATP
o- - - - - o tension decrease after stretch, zinc + ATP

decrease in tension after stretch. Since the fibre bundles were pretreated with zinc before the relaxation was started (see Methods III), limitation of the relaxation rate caused by diffusion of zinc into the bundles was avoided. However, it cannot be excluded that the rate of relaxation is limited instead by the diffusion of ITP and ATP into such zinc-loaded fibre bundles. Therefore, it is not possible to decide from these results whether the rate of relaxation for the individual contractile unit differs with 0.2 mM zinc + 0.4 mM ITP and 0.2 mM zinc + 0.4 mM ATP.

Summary.

1. The relaxing effect of 0.1 mM and 0.4 mM zinc on previously contracted, glycerol-extracted rabbit psoas fibre bundles was studied under isometric conditions in the presence of 1 mM magnesium and varied concentrations of ATP (0.0031—6.4 mM). No relaxation is obtained in the absence of ATP. A certain, partial

relaxation is obtained in the presence of 0.006 mM ATP and complete relaxation in the presence of 0.05—0.4 mM ATP. The relaxing effect decreases with higher ATP concentrations, probably due to a decrease in the free zinc ion concentration in the bath caused by complex formation between zinc and ATP.

2. The ATPase activity of homogenized, glycerol-extracted rabbit psoas is abolished by the combination 0.1 mM zinc + 1 mM magnesium + 0.2 mM ATP, which produces complete relaxation of isometrically contracted fibre bundles. The ATPase activity is retained with the combination 0.1 mM zinc + 1 mM magnesium + 2.6 mM ATP, which gives no relaxation.

3. Zinc and magnesium show mutually antagonistic action upon the contractility in glycerol-extracted fibre bundles. The relaxing effect of zinc is probably not due to a simple competition with magnesium.

4. The lowest zinc concentration found to give complete relaxation of a contracted fibre bundle is 0.0125 mM if magnesium is absent and 0.04—0.08 mM ATP is present.

5. Calcium in 10 mM concentration increases the rate of the relaxation induced by 0.2 mM zinc + 1 mM magnesium + 0.4 mM ATP. No significant influence of 1 mM and 0.1 mM calcium was demonstrated.

6. Inorganic pyrophosphate (PP) and tripolyphosphate (TPP) in concentrations of 0.25 mM and 1 mM induce relaxation of contracted fibre bundles. Adenosinediphosphate (ADP) in concentrations of 0.25 mM, 1 mM and 10 mM does not give any significant relaxation in excess of the drop in tension resulting from mere removal of ATP by washing. The presence of 0.2 mM zinc does not modify the action of ADP, PP and TPP.

7. Relaxation can be induced by 0.2 mM zinc in the presence of 1 mM magnesium and 0.4 mM inosinetriphosphate (ITP) or uridinetriphosphate (UTP) instead of ATP.

8. The effect of 0.2 mM zinc + 1 mM magnesium + 0.4 mM ITP on the visco-elastic behaviour of resting fibre bundles subjected to stretch was studied. There is an increase of the elastic compliance and also an increase in fluidity of the viscous resistance of the fibre bundle.

This investigation was supported by grants from the Medical Faculty of the University of Uppsala and the Magnus Bergvall Foundation. I wish to thank Professor Ernst Bárány for his advice and stimulating interest in this investigation. My thanks are also due Mrs Birgit Andersson and Mr. Ewert Lindberg for valuable technical assistance.

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Relaxation and Visco-Elastic Behaviour of Glycerol-extracted Muscle Fibre Bundles in the Presence of Zinc and ATP.

By

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Received 3 October 1958.

Abstract.

EDMAN, K. A. PAUL. Relaxation and visco-elastic behaviour of glycerol-extracted muscle fibre bundles in the presence of zinc and ATP. *Acta physiol. scand.* 1959. *45.* 164—180. — Zinc-induced relaxation in glycerol-extracted rabbit psoas fibre bundles was studied under strictly isotonic and isometric conditions and compared with the effects of zinc on the visco-elastic behaviour of resting fibre bundles subjected to quick stretch. 0.2 mM zinc + 1 mM magnesium + 0.4 mM ATP does not affect the visco-elastic properties but induces relaxation of isometrically and isotonically contracted fibre bundles. Isotonic relaxation is complete only when the previous shortening does not exceed 25 percent of initial fibre length; the relaxing effect is practically absent when the bundle has shortened about 75 percent of its initial length. 2 mM zinc + 1 mM magnesium + 5 mM ATP and 4 mM EDTA + 4 mM magnesium + 4 mM ATP, which markedly decrease the visco-elastic resistance in the fibre bundle produces a pronounced isotonic relaxation even after extreme shortening of the bundles. Thus, the isotonic relaxation of glycerol-extracted fibre bundles is dependent upon a decrease in the visco-elastic resistance. The isometric relaxation, on the other hand, does not need any pronounced visco-elastic change.

In an earlier publication (EDMAN 1958) certain effects of zinc on the isometric tension development of glycerol-extracted muscle fibre bundles were demonstrated. Among other effects it was shown that zinc (0.025—0.88 mM) in the presence of ATP[1] (0.4 mM) can reversibly relax isometrically contracted fibres. The effects of zinc are interesting considering that zinc is present physiologically in muscle tissue in concentrations as high as ~ 0.8 mmole/kg wet tissue. The present study extends the earlier work and treats the effects of zinc on the fibre bundles under strictly *isotonic* conditions. The work also includes recordings of the effect of zinc + ATP and EDTA + ATP upon the mechanical behaviour of resting fibre bundles subjected to stretch.

Methods.

The glycerol extraction and preparation of the fibre bundles from rabbit psoas were carried out as described earlier (EDMAN 1957). A KCl solution with pH 7.30 containing 10 mM diethylbarbituric acid (Veronal), 100 mM K and 1 mM Mg was used as medium after the glycerol extraction. Hereafter this solution will be called "veronal buffer". Ethylenediamine tetraacetate, disodium dihydrate, *pro analysi*, E. Merck, was used. For data on the quality of the other chemicals used, see EDMAN (1958).

In addition to the dibarium salt of ATP, Pabst lot no. 129 (EDMAN 1958), the disodium salt of ATP, "chromatographically pure", Pabst lot no. 109 A, was used. According to an analysis performed with the technique described earlier (EDMAN 1957), the amount of total nucleotide in 37.4 mg of the Na_2H_2ATP substance was 0.0581 mmole. The relative nucleotide concentrations were: ATP 96 %, ADP 2 %, AMP < 0.5 % and an unknown fraction, possibly uridine nucleotide, < 1 %. The solution was prepared by dissolving the Na_2H_2ATP salt in the veronal buffer and correcting the pH to 7.3 with KOH.

The water employed for washing and for preparation of solutions was double distilled in borosilicate glass. The pH determinations were done with a glass electrode. The experiments were performed at room

[1] The following abbreviations will be used: ADP, adenosinediphosphate; AMP, adenosinemonophosphate; ATP, adenosinetriphosphate; EDTA, ethylenediamine tetraacetate.

temperature, 20—22° C. If not otherwise stated the data on the concentrations refer to the total concentration in the bath.

The veronal buffer solution used as medium always contained 1 mM Mg (see above). Therefore, the Mg concentration will not be specially mentioned in the rest of the communication unless the concentration was different from usual.

Isotonic recording.

Two identical devices for isotonic recording with front-writing pens were used in parallel. Before use the pens were completely balanced in air. To achieve this it was necessary that the front-writing part be separately completely balanced. The tip of the writing part was a glass bead and was applied to the smoked drum by light pressure from a fine coil spring mounted around the axis of the writing part. (With conventional technique the tip is pressed against the paper by gravity which presupposes that the centre of gravity of the writing part does not fall on its axis.) The tempered and polished conical steel tips comprising the ends of the main axis were mounted in jewelled bearings. The kymograph paper was lightly sooted. To further reduce the friction the kymograph was kept faintly vibrating by an a.c.magnet. The pen was applied to the drum in a manner such that the rotation of the main axis was approximately equally distributed above and below the horizontal line during an ordinary contraction. Within the recording range used in the present investigation the tension of the bundle varied less than 1 mg. Shortening of the fibre bundle from 100 % to 75 % of the initial length (40 mm) corresponded to 19.1 mm on the kymogram. shortening from 75 % to 50 % of the initial length to 19.1 mm and shortening from 50 % to 25 % to 19.6 mm. The mean value (19.3 mm) will be used hereafter for 10 mm contraction of the fibre bundle regardless of the degree of contraction.

A loop of platinum wire (0.15 mm in diameter) with a weight of 7.3 mg was attached to the end of the fibre bundle that would become the lower end after mounting; a nylon thread (0.08 mm in diameter) was attached to the other end. The length of the fibre bundle between the attachments of the platinum loop and the nylon thread was 40.0 mm: for data on the thickness of the bundles, see later. During the experiments the fibre bundles were mounted vertically between a definite point on the pen and a platinum hook fused into a glass rod. The bath solution (40 ml) was kept in a cylindrical glass vessel. The exchange of the bath solution was carried out by exchanging the glass vessel for another containing the new solution. In this procedure the vessels were moved vertically, *i. e.* in the longitudinal direction of the fibre bundles. The bundles were not exposed to air for more than 10 sec. during the mounting to the pens and 4—5 sec. at most during the exchange of solutions.

The *shortening* experiments were carried out using 0.4 mM ATP and a load of 9 mg. Before the start of the experiments the fibre bundles lay in a Petri dish containing 60 ml ATP-free test solution

(0.01 mM zinc) or, respectively, control solution (veronal buffer only) for 1—2 hours. Cross section of the fibre bundles was 103—150 × 178—299 μ.

Relaxation was induced with 0.2 mM zinc + 0.4 mM ATP at a load varied between 59 and 78 mg in the different experiments (T_2 in Fig. 2). The fibre bundle was first contracted with 0.4 mM or 0.8 mM ATP at a load of 9 or 24 mg (T_1 in fig. 2). When the desired degree of shortening had been attained, the load was increased to T_2 and the ATP concentration was changed to 0.4 mM if a higher concentration of ATP had been used to induce the contraction. The relaxation was not started until a constant length had been reached under the new condition. From 4 to 8 contraction-relaxation cycles were carried out with each fibre bundle. During the different cycles the fibre bundles were allowed to shorten to varying degrees before relaxation was induced. The relaxation was recorded until constant length was attained, and the fibre bundle was subsequently washed with buffer solution for about 30 min. whereafter a new contraction was started.

In some experiments the relaxation was induced with 2 mM zinc + 5 mM ATP. The fibre bundle in these experiments was contracted by 0.8 mM ATP at a load of 23 mg. When the desired degree of shortening had been attained, the bundle was pretreated with 0.07 mM zinc alone for 2—3 min. at a load of 68 mg. The cross section of the fibre bundles used was: 94—168 × 206—262 μ.

Because of the complex formation between ATP and metals it is necessary to increase the total zinc concentration as well when the concentration of ATP is raised in order to keep the zinc ion concentration sufficiently high. Assuming a stability constant (log K) of 4.00 for the Mg-ATP complex (MARTELL and SCHWARZENBACH 1956: 4.00; RAAFLAUB 1956: 4.00; WALAAS 1957, 1958: 4.04; G. WEIZEL, pers. comm.: 4.04) and 4.76 for the zinc-ATP complex (G. WEIZEL, personal communication) the combination 0.2 mM zinc + 0.4 mM ATP corresponds to 0.071 mM Zn^{++} and 0.032 mM free ATP. The combination 2 mM zinc + 5 mM ATP corresponds to 0.017 mM Zn^{++} and 2.1 mM free ATP. The concentration of 2 mM total zinc was arrived at by experiment and is close to the lower limit giving complete isometric relaxation in the presence of 5 mM total ATP (EDMAN, unpublished).

Isometric recording.

The same technique was used as that described in a previous paper (EDMAN 1957).

Micro-elasticity measurements.

The principal aspects of the technique in measuring the elasticity have been described earlier (EDMAN 1957, page 236). The cross section of the fibre bundles used was 81—131 × 164—229 μ and the length 40 mm. A quick stretch was performed in 2 sec. The stretch comprised 0.83 % of the fibre length in the control experiments (veronal buffer alone) and in the experiments with zinc; in the experiments

with EDTA the fibre bundles were stretched by 2.5 % of their initial length because of the greater elastic compliance in these experiments. The force necessary to stretch the fibre bundle was recorded on the smoked drum. The recording was continued until equilibrium tension had been attained. In the control experiments and in the experiments with 0.2 mM zinc + 0.4 mM ATP the tension was always recorded during 5 min. after stretch.

The fibre bundles were given an initial tension of 22.2 mg while still in the veronal buffer. In the *control experiments* (véronal buffer alone) the stretch was carried out soon after the fibre bundle had been put into position in the recording device. In the experiments with *0.2 mM zinc + 0.4 mM ATP* an isometric contraction-relaxation cycle was produced before the visco-elasticity measurement. The fibre bundles in these experiments were contracted isometrically with 0.4 mM ATP for two min. and after that washed with buffer solution alone for two min. and for an additional 5-minutes with 0.2 mM zinc. Relaxation was then induced with 0.2 mM zinc + 0.4 mM ATP and the tension was recorded for 5 min. when it was certain that equilibrium had been attained. After the fibre bundle had been immersed in the solution of 0.2 mM zinc + 0.4 mM ATP for 60 min. the stretch was performed. In the experiments with *2 mM zinc + 5 mM ATP* and with *4 mM EDTA + 4 mM Mg + 4 mM ATP* the test solution was applied without previous contraction of the bundle. Immediately after the immersion in the test solution an increase in tension occurred initially and subsided completely in the experiments both with EDTA and with zinc. The stretch was carried out in these experiments after the fibre bundle had remained in the test solution for 10 min. when constant tension had certainly been reached.

The statistical significance of the results has been calculated using Fisher's t-test.

Results.

1. Effect of 0.01 mM zinc on isotonic contraction.

Fig. 1 shows the effect of 0.01 mM zinc on isotonic contraction of glycerol-extracted fibre bundles at 0.4 mM ATP concentration. Not unexpectedly the effect is similar to that described in isometric contraction (EDMAN 1958), *i. e.* an inhibition during the entire course of the shortening. This is clearly evident from Table I, in which the results obtained in this study on isotonic contraction are presented together with the earlier results obtained in studies on the effect of 0.01 mM zinc and 0.22 mM ATP on isometric contraction for the purpose of comparison. The initial absolute and relative rates of contraction in isotonic contraction are defined in analogy with the definition given for isometric recording (EDMAN

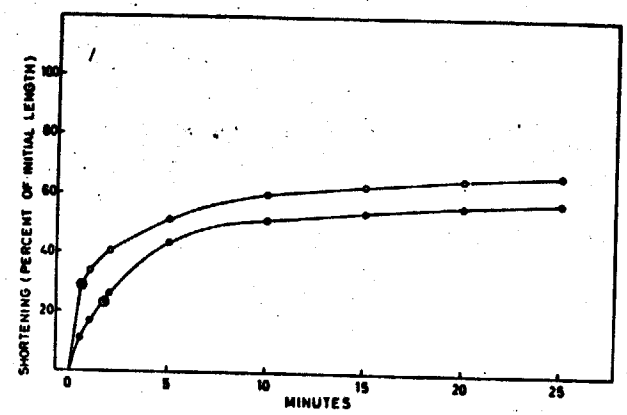

Fig. 1. Effect of 0.01 mM zinc on the isotonic contraction of glycerol-extracted fibre bundles. ATP concentration 0.4 mM. Load 9 mg. Cross section of the fibre bundles: 103—150 × 178—299 μ. Filled circles: test experiments, open circles: control experiments, both representing the mean shortening after different time intervals. 4 experiments in both groups. The average time for reaching 40 % of the final degree of shortening is indicated with ⊗ for the control curve and with ⊙ for the test curve. The standard error of the means does not exceed the size of the symbols.

1957, 1958). The initial *absolute* rate in an isotonic contraction is thus the mean shortening per unit of time during the time interval from 0 to 40 % of the magnitude of the final contraction. The initial *relative* contraction rate is the reciprocal of the time required to attain 40 % of the final level of the contraction. The

Table I.

Effect of 0.01 mM $ZnCl_2$ on the isotonic and isometric contraction of glycerol-extracted muscle fibre bundles in the presence of 1 mM magnesium.

Mode of contraction	Initial relative rate	Initial absolute rate	Final tension	Number of pairs of expts.
Isotonic	—66***	—71***	—14**	4
Isometric	—81***	—82***	— 9*	17

Isotonic contraction induced by 0.4 mM ATP, isometric contraction by 0.22 mM ATP. The effect is given as the difference between the mean of the tests and the mean of the controls in percentage of the controls.

*** = $P < 0.001$, ** = $0.001 < P < 0.01$, * = $0.01 < P < 0.05$.

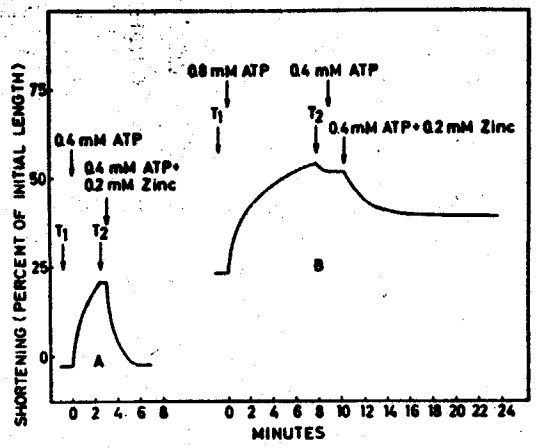

Fig. 2. Repeated isotonic relaxations with 0.2 mM zinc + 0.4 mM ATP in a glycerol-extracted fibre bundle at different degrees of shortening. Two contraction-relaxation cycles preceded the first cycle (A) shown in the figure. Between A and B three contraction-relaxation cycles were performed. After each relaxation the bundle was washed for 30 min. with zinc-free buffer solution. At T_1 the bundle was loaded with 24 mg, at T_2 with 72 mg. Cross section of the fibre bundle: 131 × 206 μ. Note the slower and smaller relaxation at the higher degree of shortening.

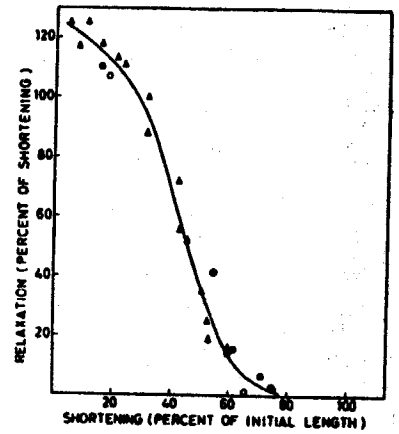

Fig. 3. Isotonic relaxation of glycerol-extracted fibre bundles with 0.2 mM zinc + 0.4 mM ATP in relation to the degree of shortening. Abscissa: degree of shortening when relaxation is induced, in percentage of the initial length of the fibre bundle. Ordinate: relaxation as percentage of the previous shortening. Cross section of the fibre bundles:

△ 131 × 206 μ. ▲ 94 × 197 μ.
○ 140 × 243 μ. ● 122 × 243 μ.

isotonic contraction is slow and is not entirely completed even after one hour (EDMAN 1953). The contraction level 25 minutes after the start was selected as an approximate value for the final level in this study.

2. Isotonic relaxation by zinc in the presence of ATP.

In one series of experiments isotonic relaxation from different degrees of shortening was induced with 0.2 mM zinc + 0.4 mM ATP. Several contraction-relaxation cycles were performed with each fibre bundle (see Methods). A typical example is presented in Fig. 2. The purpose of the examination was to study the relaxation ability at various degrees of deformation of the contractile protein. The lengthening has therefore been calculated in percentage of the actual total shortening consisting of the newly-induced shortening and the remainder of the previous contraction, if this had not been completely relaxed. The results are summarized in Fig. 3. It may be seen that complete relaxation was obtained, *i. e.* the fibre bundle stretched to at least its initial length, if it had not shortened by more than about 25 % of its initial length. At lower degrees of shortening the relaxed length even exceeded the initial length. When the shortening was continued to more than 25 % of the initial length, the capacity for relaxation decreased; when the fibre bundle shortened by 75 % of its initial length, no relaxation was obtained. The results are not markedly modified by the presence of a contraction remainder from an earlier contraction-relaxation cycle.

With 2 mM zinc + 5 mM ATP a pronounced relaxation can be induced (about 50—100 % of the shortening) at the load used here even if the fibre bundle has been allowed to shorten by about 60 % of its initial length as shown in Fig. 4.

In the presence of high concentrations of ATP (4—6 mM) the Marsh-Bendall factor (BENDALL 1953) and EDTA (WATANABE and SLEATOR 1957) are also able to produce a total or almost total isotonic relaxation at about 60 % shortening of the fibre

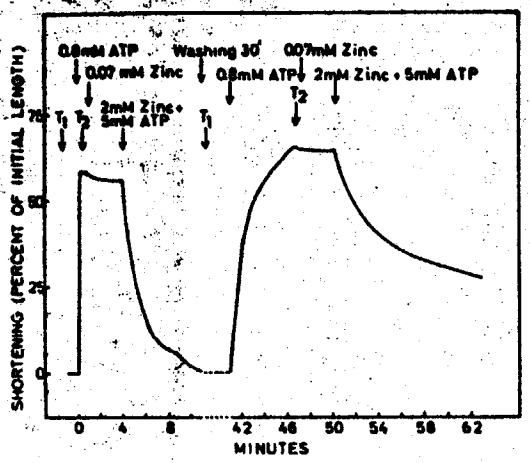

Fig. 4. Isotonic relaxation of a glycerol-extracted fibre bundle with 2 mM zinc + 5 mM ATP. At T_1 the bundle was loaded with 23 mg, at T_2 with 68 mg. Washing after the first relaxation performed with veronal buffer solution alone. Cross section of the fibre bundle: 108 × 224 μ.

bundle. The possibility of producing complete relaxation of a 60 % shortened fibre bundle with 4 mM EDTA + 4 mM Mg + 4 mM ATP as described in the report of WATANABE and SLEATOR (1957) has been verified under the experimental conditions used in the present study.

Since ATP has only a limited depth of penetration into glycerol-extracted fibres and fibre bundles (see Discussion, EDMAN 1957), the central parts will remain rigid and will consequently be passively deformed during isotonic contraction. In order to determine if the geometric aspects of the fibre bundle as a whole are changed thereby, the fibre bundle was subjected to direct microscopic inspection while in the vessel of bath solution during the course of isotonic contraction and relaxation in several experiments (magnification 6—40 ×). It was found that the fibre bundles retained parallelism of the visible fibres even under extreme shortening. No changes in the geometry of the fibre bundle, *e. g.* twisting or folding, which might explain why the capacity for relaxation with zinc + low ATP concentration diminishes with high-grade shortening could be demonstrated. The results are further discussed later.

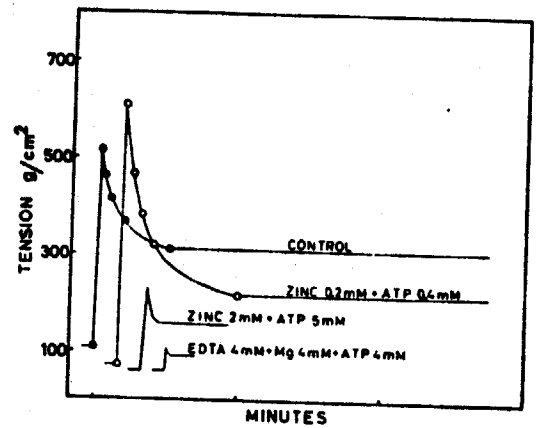

Fig. 5. The behaviour of glycerol-extracted fibre bundles at and following stretch. Stretch, comprising 0.83 % of the fibre length in the controls and in the experiments with zinc and 2.5 % of the fibre length in the experiments with EDTA, was achieved in 2 sec. The control curve (veronal buffer alone) and the curve for 0.2 mM zinc + 0.4 mM ATP are derived from the means of the experiments presented in Table II for the respective group. At the last symbols in these curves equilibrium tension has been reached (tension recorded 5 min). The curves for 2 mM zinc + 5 mM ATP and for EDTA are typical examples of experiments in these groups. The curves are arbitrarily displaced along the abscissa.

3. *Visco-elastic behaviour of glycerol-extracted muscle fibre bundles.*

When a fibre bundle is quickly stretched there is an initial steep rise in tension ("initial response") followed by a decrease until an equilibrium tension has been reached. The reciprocal of the initial rise in tension is an arbitrary measure of the elastic compliance[1] of the fibre bundle, while the subsequent decrease in tension is due to *visco*-elasticity. Table II presents quantitative data from the recordings and Fig. 5 shows the typical forms of the curves obtained.

The combination 0.2 mM zinc + 0.4 mM ATP does not significantly change the initial response to stretch and the subsequent

[1] The compliance calculated in this way is a "dynamic elasticity". PORTZEHL (1952), later quoted by WEBER and PORTZEHL (1954) and WEBER (1955, 1958), calculates the modulus of elasticity from the tension obtained 2 minutes after stretch, thereby obtaining a measure of the "static elasticity". In PORTZEHL's experiments the stretching comprised 5—10 % of the fibre length.

Table II.

Effects of zinc + ATP and EDTA + ATP on the visco-elastic behaviour of glycerol-extracted muscle fibre bundles at and following stretch.

Test medium	1 Equilibrium tension of fibre bundle *before* stretch g/cm²	2 Stretch in % of initial fibre length	3 Initial tension increase at stretch g/cm²/% stretch	4 Equilibrium tension increase after stretch g/cm²/% stretch	5 Half time for tension decrease in seconds	6 Number of expts.	7 Mean area of cross section cm² · 10⁴
Veronal buffer alone (1 mM Mg)	108 ± 3	0.83	506 ± 39	259 ± 36	4.1 ± 0.3	13	20.7 ± 0.52
Zinc 0.2 mM + Mg 1 mM + ATP 0.4 mM	73 ± 7***	0.83	642 ± 33*	188 ± 12	6.3 ± 1.0*	13	20.0 ± 0.91
Zinc 2 mM + Mg 1 mM + ATP 5 mM	76 ± 11*	0.83	187 ± 12***	52 ± 18***	~ 1[a]	6	19.8 ± 1.25
EDTA 4 mM + Mg 4 mM + ATP 4 mM	65 ± 11**	2.5	20 ± 2***	15 ± 2***	~ 0.5[a]	6	21.6 ± 0.35

The degree of significance has been calculated for the differences between the means of the tests (zinc or EDTA) and the means of the controls (veronal buffer alone). Asterisks: see Table I.

a. No statistical calculation has been performed but the values were quite outside the range of the controls

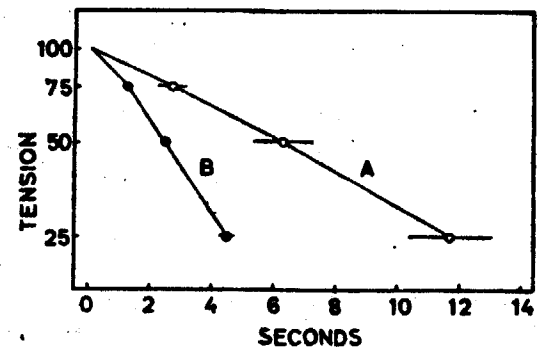

Fig. 6. Comparison between the decay of tension after stretch (curve A) of resting glycerol-extracted muscle fibre bundles and the relaxation (curve B) of isometrically contracted fibre bundles in the presence of 0.2 mM zinc + 0.4 mM ATP. Abscissa: time in sec. after the start of the tension decrease. Ordinate: tension remaining in excess of the equilibrium tension, expressed in percentage of total tension decrease. Relaxation and stretch experiments performed in succession on the same fibre bundle. Number of bundles: 13. The stretch experiments are the same as those shown in Table II for 0.2 mM zinc + 0.4 mM ATP.

The standard error of the means is indicated with a horizontal bar. Ratios between the times for tension decrease after stretch and relaxation:

At 75 %: 2.2 ± 0.35, $P < 0.01$;
at 50 %: 2.6 ± 0.39, $P < 0.01$;
at 25 %: 2.7 ± 0.36, $P < 0.001$.

decrease in tension. The combination 2 mM zinc + 5 mM ATP, on the other hand, increases the elastic compliance: the initial response to stretch is decreased 2.7 times. Furthermore, there is a more rapid decline in tension after the initial response, and, the equilibrium tension increase after stretch (= the equilibrium tension after stretch minus the initial tension before stretch) is 5 times smaller. With 4 mM EDTA + 4 mM Mg + 4 mM ATP the initial response to stretch is reduced 25 times and the equilibrium tension is attained almost instantaneously. It is conceivable that some yielding may already occur during stretch, especially in the case of EDTA + ATP, where the tension decrease is very rapid. This would cause an underestimation of the initial response to stretch.

In the experiments with 0.2 mM zinc + 0.4 mM ATP the relaxing effect and the effect on the visco-elastic behaviour at and following stretch were studied in the same fibre bundle. As is

evident from Fig. 6 the isometric relaxation of a contracted fibre bundle (curve B) is more rapid than the tension decrease after stretch of a resting bundle (curve A). This is in marked contrast to the situation in the living frog muscle fibre under isotonic conditions (BUCHTHAL, KAISER and ROSENFALCK 1951), where the passive adjustment of resting fibre length to a new load is much faster than relaxation after a contraction.

The time course of the tension decrease after stretch is almost linear when plotted on a semilogarithmic scale (Fig. 6, curve A). Hence, it is improbable that the loss in peak tension caused by the relative slowness of the stretching influences the estimate of the rate of tension decrease. The rate of relaxation (curve B) may be limited by the rate of diffusion of ATP into the fibre bundle. This will cause an initial delay in relaxation and an underestimation of the difference between the rate of tension decrease after stretch and the rate of relaxation. It is therefore probable that the differences between the courses of relaxation and tension decrease after stretch obtained with whole fibre bundles are really due to differences in the events within the single contractile units at relaxation and following stretch. The results are interpreted below.

Discussion.

1. *Contraction.*

Because of the passive resistance of the ATP-free central parts of the fibre bundle and of the individual fibres during shortening the degree of shortening will be determined not only by the contractility of the individual elements but also by the thickness of the peripheral, active layer.

The effects of 0.01 mM zinc on the ATP-induced contraction of glycerol-extracted fibre bundles are very similar in isotonic and isometric recording. The final level is less inhibited by zinc than the initial phase of the contraction. The explanation given for the isometric case (EDMAN 1958) is probably also applicable to the isotonic one.

2. *Relaxation.*

The visco-elastic properties of a muscle fibre bundle are complicated. A comprehensive treatment of these problems is given by BUCHTHAL *et al.* (1951) and REICHEL (1952). As a first approximation the passive properties of the fibre bundle can be described with the model in Fig. 7. An elastic element with compliance C_1 is in series with another elastic element with compliance C_2, which is shunted by a viscous element with fluidity ϱ symbolized by a dash pot. In the general case of a visco-elastic body a series dash pot is also present, but, since the fibre bundle can carry a load indefinitely, no such element can be present in our case. The active contractility can be represented by a force F, which acts in parallel with C_2 and ϱ. According to BUCHTHAL *et al.* (1951) it is certainly more adequate to use a system composed of several of the units described here coupled in series. However, the single unit may be used for the following simple considerations.

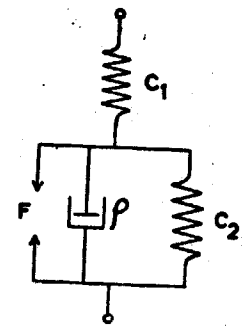

Fig. 7. Mechanical model of the glycerol-extracted muscle fibre bundle. C_1 and C_2 elastic elements, ϱ viscous element and F force (= active contractility).

Using this model, it follows that the initial tension increase after stretch is proportional to $1/C_1$, that the equilibrium tension increase is proportional $1/(C_1 + C_2)$, and, that the half-time of the system is proportional to $[1/(1/C_1 + 1/C_2)] \cdot 1/\varrho$.

From the measurements reported in Table II, it is consequently possible to calculate the changes in C_1, C_2 and ϱ caused by the various treatments. These are shown in Table III.

Taking the experimental uncertainties illustrated in Table II into account, it appears from Table III that 0.2 mM zinc + 0.4 mM ATP does not significantly affect the visco-elastic properties of the fibre bundle. With 2 mM zinc + 5 mM ATP the compliances

Table III.

Visco-elastic parameters for model shown in Fig. 7 calculated from data of Table II.

Test solution	C_1	C_2	ϱ
Veronal buffer alone (1 mM Mg)	1	1	1
Zinc 0.2 mM + Mg 1 mM + ATP 0.4 mM .	0.79	2.0	0.74
Zinc 2 mM + Mg 1 mM + ATP 5 mM	2.7	7.4	~ 17
EDTA 4 mM + Mg 4 mM + ATP 4 mM ..	25	8.4	~100

C_1 and C_2 both increase, as does the fluidity ϱ. Similar but even more pronounced effects are found with 4 mM EDTA + 4 mM Mg + 4 mM ATP.

The isometric tension of a fibre bundle can be lowered either by an unspecific increase in C_1 or by a real relaxation, *i. e.* by disappearance of the force F and return to rest length of the contractile elements.

The fact that reversible, isometric relaxation can be obtained with 0.2 mM zinc + 0.4 mM ATP without any significant change in C_1 makes it seem probable that the effect of the treatment with zinc + ATP is a real relaxation, according to the definition given above.

To what extent is a certain fluidity (ϱ) needed for relaxation? In a contraction that is strictly isometric even at the submicroscopic level, no fluidity would be necessary for relaxation, which would be a simple disappearance of a force. If, on the other hand, a series elastic component is present, there is also a shortening of the contractile unit, and it is probable that the disappearance of this shortening needs some fluidity. The need for fluidity should be greatest in relaxation from maximal isotonic shortening.

If isometric contraction of a fibre bundle includes shortening of the contractile units, the rate of relaxation could be dependent upon visco-elastic factors. However, if the visco-elastic factors were rate-determining, the isometric relaxation with 0.2 mM zinc + 0.4 mM ATP should follow the same time course as the tension decrease after stretch when the bundle has been equilibrated with the same solution. This is not the case as shown in Fig. 6. Moreover, no definite change in passive visco-elastic properties could be demonstrated with 0.2 mM zinc + 0.4 mM ATP. From these

facts it seems most probable that the reversible *isometric* relaxation produced by zinc + ATP is mainly a disappearance of a force.

The *isotonic* relaxation of a fibre bundle seems to be dependent upon the visco-elastic properties of the bundle. In extremely shortened fibre bundles no relaxation was obtained, at the load used, with 0.2 mM zinc + 0.4 mM ATP, but complete relaxation with the combinations 2 mM zinc + 5 mM ATP and 4 mM EDTA + 4 mM Mg + 4 mM ATP which markedly decrease the visco-elastic resistance, as shown in Fig. 5 and Table III. The role of the passively deformed, uncontracted parts of the fibre bundle during the isotonic relaxation is unclear. If the inactive parts have been plastically deformed during the shortening, it is conceivable that they could hamper the relaxation of the bundle. On the other hand, if they have been elastically deformed, they could be expected to facilitate the lengthening of the bundle during the relaxation.

Summary.

1. Zinc in 0.01 mM total bath concentration brings about partial inhibition of the isotonic contraction of glycerol-extracted rabbit psoas fibre bundles induced by 0.4 mM ATP in a manner similar to that found in isometric contraction.

2. Isotonically contracted fibre bundles loaded with about 250 g/cm² relax upon addition of 0.2 mM zinc + 1 mM Mg + 0.4 mM ATP. The relaxation is complete when the previous shortening does not exceed 25 % of initial fibre length; the relaxing effect decreases with an increase in the degree of previous shortening and is practically absent when the bundle has shortened about 75 % of its initial length. With 2 mM zinc + 1 mM Mg + 5 mM ATP it is possible to produce a pronounced relaxation even after extreme shortening as is also possible with 4 mM EDTA + 4 mM Mg + 4 mM ATP.

3. The effect of zinc + ATP and EDTA + ATP on the visco-elastic behaviour at and following stretch of resting fibre bundles was studied. No effect could be demonstrated with the combination 0.2 mM zinc + 1 mM Mg + 0.4 mM ATP, which is able to bring about a rapid isometric relaxation of contracted fibre bundles. The combination 2 mM zinc + 1 mM Mg + 5 mM ATP, on the other hand, increased the compliance of the elastic

components and, in addition, decreased the viscous resistance of the contractile unit. Similar, but more pronounced effects were obtained with the combination 4 mM EDTA + 4 mM Mg + 4 mM ATP.

4. In the discussion it is pointed out that the effect of zinc + ATP is a real relaxation, *i. e.* a disappearance of the force and a return to rest length of the contractile elements. Disappearance of the force, probably independent of changes in visco-elastic properties, seems to be the most important factor in *isometric* relaxation of glycerol-extracted muscle fibre bundles.

This investigation was supported by grants from the Medical Faculty of the University of Uppsala and the Magnus Bergvall Foundation.

I wish to thank Professor G. Weitzel for kindly supplying unpublished stability constants of metal-ATP complexes and Professor Ernst Bárány for his helpful criticism and stimulating interest in this investigation.

The technical assistance of Mrs. Birgit Andersson is gratefully acknowledged.

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Am. J. Physiol. 181:287-290 (1955)

Absorption, Deposition and Placental Transfer of Zinc[65] in the Rat[1]

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AMONG the functions ascribed to zinc is that of activation of the red blood cell enzyme carbonic anhydrase. Considering its essential nature in this capacity alone, zinc must be indispensable to the developing fetus. Guilbert (1) has reported that livers of young rats and fetal calves contain much more zinc than that of their dams and suggested that storage takes place in the young before birth by transmission across the placenta. Russoff and Gadum (2) found zinc among the trace elements detectable spectrographically in the new-born rat. Vallee, Fluharty and Gibson (3) noted that radiozinc was transmitted across the placenta of the dog. Studies confirming the transfer of zinc to milk in the cow and goat (4), in woman and in the mare (5) have been discussed.

This paper presents data on maternal absorption and deposition and the placental transfer of radiozinc as a function of fetal age, and discusses the quantitative transfer of zinc[65] to the milk of rats.

EXPERIMENTAL

The 44 adult nulliparous female rats used in the present investigation ranged in weight from 200-325 gm and were maintained on a stock diet. At definite stages of pregnancy, ranging from the 9th to the 21st day of the 22-day gestation period, single tracer doses of zinc[65] chloride[3] were administered orally or intraperitoneally to each of the rats. They were then placed in metabolism cages adapted for the quantitative separate collection of urine and feces (6).

At death, following balance periods of ½-96 hours, fetuses were removed, weighed and measured; these, as well as maternal blood, whole body and the urine and fecal collections, were analyzed for total and radiozinc content. In order to determine the pattern of zinc laydown in the adult rat, selected tissue samples were taken from a number of males and non-pregnant females, dosed orally or intraperitoneally, for radiozinc determination.

Tissues were analyzed for total zinc by means of the Leeds and Northrup Polarograph, following extraction of zinc by the dithizone procedure of Menzel and Jackson (7). Zinc[65] was measured with a dipping-type Geiger-Mueller counter tube, and for direct comparison, all data were calculated to the same basis of dose and body weight.

RESULTS AND DISCUSSION

Radiozinc Absorption and Excretion. In studies involving comparative transfer of elements from one metabolic pool to another, it is necessary to consider the behavior in terms of the percentage of the mineral actually available for transfer. The absorption value for the isotope, therefore, is essential for interpretation of the behavior pattern of that portion actually retained by the animal body.

It is significant that only about 5% of an oral tracer dose of radiozinc was retained by the adult rat. This value was not appreciably affected by gestation period, but did vary with time after dose administration. Fecal excretion of zinc[65] during the first 24 hours averaged 67.6% of the dose, 29.5% remaining in the gastrointestinal tract. Thus retention amounted to 2.9% of the zinc[65] administered. In 48 hours, 86.3% was excreted in the feces and 5.8% remained in the gastrointestinal tract, or 7.9% of the total amount of zinc[65] administered was retained. Urinary excretion was insignificant, averaging only 0.2% of the isotope administered. Sheline and co-workers (8) reported that mice excreted 50% of an intramuscular dose of zinc[65] in the feces and less than 5% in the urine after 7 days. Cattle receiving radiozinc by the oral route were reported by Feaster *et al.* (9) to excrete 70%

Received for publication November 29, 1954.

[1] Florida Agricultural Experiment Station Journal Series, No. 346. Published with the approval of the Director of the Florida Agricultural Experiment Station. This work was supported in part from funds granted by the Atomic Energy Commission, the National Institutes of Health and the Nutrition Foundation, Inc.

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[3] The zinc[65] chloride was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn., after allocation by the Isotope Division of the Atomic Energy Commission.

TABLE 1. DISTRIBUTION OF RETAINED LABELED ZINC[65] IN SELECTED TISSUES OF THE ADULT RAT*

Percentage of retained dose per 100 gm fresh tissue

Blood	G Muscle	Pancreas	Liver	Kidney	Spleen	Hide & Hair	Incisors	Femur
†	14.3	35.7	38.6	67.1	28.6	7.1	15.1	21.4

* Calculations based on 7% dose retention, and corrected to 100 gm body weight.

† Less than .04%.

in the feces and only 0.3% in the urine in 7-day balance studies.

It is pointed out that consideration is here given to the movement of approximately 5% of the administered dose and to the behavior of that radiozinc which was retained and made available to the maternal body for tissue deposition and/or transfer to the fetus and milk of rats.

Tissue Distribution of Zinc[65]. Table 1 presents a summary of the distribution of zinc[65] in selected tissues of rats killed 4 days after isotope administration. Zinc[65] values are expressed as percentage of the retained zinc[65] dose per 100 gm fresh weight; these values were calculated by dividing the percentage of the total isotope dose per 100 gm of fresh tissue by the percent of the total dose retained by the animal (7%) and multiplying the quotient by 100.

The kidney, liver and pancreas contained the highest levels of activity, and after 96 hours essentially all of the radiozinc had disappeared from the blood. Relatively little isotope accumulation was noted for muscle, hide and hair and bones. These findings are in agreement with the observation of Sheline *et al.* (8) and with the reports of other investigators (10, 11). The relatively high concentration of radiozinc in the pancreatic tissues may reflect the zinc content of the insulin produced by this organ.

Fetal Transfer of Radiozinc. Table 2 shows the placental transfer of zinc[65] in the rat as a function of fetal age at 24 and 48 hours after dose administration. Zinc[65] content was calculated for each litter and for the individual fetus on the basis of the percentage of the zinc[65] dose absorbed by the maternal animal, and reported as percentage of retained zinc[65]. It is shown that, for a given stage of gestation, the amount of isotope transferred to a litter was almost directly proportional to the number of fetuses present. This indicates that fetal demands may limit the transfer across the placenta. It is understood that a greater number of fetuses with a correspondingly larger number of placentas offers more surface area across which the transfer could take place (12), but there is also increased surface area with increased fetal age. This must be considered a factor in the observed increased zinc transfer with increased fetal age.

The percentage of the absorbed and retained dose of labeled zinc transferred to the litters over 24 and 48-hour periods after dose administration ranged from 1–60%, these extremes being observed at fetal ages of 11 and 22 days, respectively. The wide range in values for litters of a given age is in part a reflection of the variation in litter size—the amount of zinc[65] transferred, as noted above, being proportional to the number of fetuses in the litter. The high degree of permeability of the rat placenta to zinc is not surprising when consideration is given to the type of placenta involved. The placentas of rat, man, rabbit, and guinea pig are classified by Grosser (13) as the simple hemochorial type. The tissue layers interposed between maternal and fetal circulation systems of this group are wholly fetal and consist of chorionic epithelium and endothelium of fetal blood vessels. Flexner *et al.* (14) observed that placental permea-

TABLE 2. PERCENTAGE OF RETAINED ZINC[65] TRANSFERRED TO FETUSES OF RATS DURING GESTATION, 24 AND 48 HR. AFTER DOSE ADMINISTRATION

Days Gestation at Death	Per Litter		Per Fetus	
	Hours administration to death			
	24	48	24	48
	%	%	%	%
11		1.0^{10}		0.3
15	0^{1}	1.0^{1}	0	0.1
18	15.2^{1}		2.1	
19	15.8^{1}	6.3^{1}	1.7	0.6
20	52.4^{5}	14.6^{2}	7.2	2.0
	(18.3–70.0)†	(8.3–20.9)	(5.8–8.9)	(1.7–2.3)
21	40.8^{9}	21.8^{4}	4.0	2.9
	(26.5–55.0)	(5.6–45.7)	(2.4–5.6)	(1.4–5.1)
22	60.3^{8}	25.4^{7}	7.0	3.3
	(20.0–97.4)	(10.0–41.4)	(4.1–9.4)	(2.0–5.0)

* Superscript numeral represents number of litters included. † Range.

TABLE 3. TOTAL ZINC CONTENT OF THE RAT FETUS DURING DEVELOPMENT

Fetal Age, days	μg/Fetus	μg/gm
11	8.2[1*]	128.2
15	12.0[2]	55.3
	(6.3–17.7)†	(32.2–78.3)
18	32.4[4]	33.9
	(25.0–41.3)	(31.4–35.5)
19	44.5[2]	37.9
	(34.0–55.0)	(23.8–42.0)
20	66.9[1]	20.1
	(46.8–79.5)	(14.0–25.6)
21	79.1[1]	18.5
	(64.3–97.4)	(13.9–21.3)
22	101.9[1]	21.6
	(81.3–158.2)	(16.4–30.7)

* Superscript numeral represents number of litters included. † Range.

bility to sodium was directly related to the number of tissue layers between maternal and fetal circulations and that the members of the four placental types could be readily distinguished from one another by this difference in their permeability.

The data show that relatively little of the retained zinc[65] was transferred to the fetus prior to 18 days, but after this age there was a sharp increase in permeability of the placenta to zinc. Feaster and Hansard (15) noted a similar increase in the transfer of calcium[45] across the rat placenta at 18 days. Hellman *et al.* (16) reported a peak in permeability of the human placenta to water at about the 35th week and Flexner *et al.* (14) observed such a peak for sodium transfer at the 36th week; in both cases the decline which followed continued to term. In the present study, the 24-hour percentage retained-dose-per-fetus values indicate a peak in zinc[65] transfer at 20 days, corresponding to the 36-week stage in man.

The placental transfer, in terms of percentage retained zinc[65] per fetus, showed an increase from 0.12 at 15 days gestation to 3.3% at 22 days, or an increase of over 25 times in 7 days. With the rapid increase in fetal weight, however, a decrease in zinc[65] concentration was observed, denoting a decrease in the transfer of zinc relative to the transfer of other materials.

Total Zinc Content of Developing Rat Fetuses. Total zinc content of the fetuses, maternal blood, urine and feces was determined. Balance data indicated that little zinc was retained by rats. Fecal excretion averaged 3.8 mg/24-hr. period; urinary excretion averaged 0.6 mg/day or one-sixth intestinal excretion. Tribble and Scoular (17) reported that in women, urinary zinc excreted amounted to about one-fifth that in the feces.

Maternal blood concentration averaged 6.4 μg/ml, a value comparable to the 8.8-μg level found for man by Vallee and Gibson (18).

The total zinc content of the developing fetus is shown in table 3. While the zinc content of the fetus showed a steady increase with fetal development, the rate of increase in fetal weight exceeded the accumulation of zinc, resulting in a gradual decrease in zinc concentration per gram of fetus.

Mammary Transfer of Radiozinc. In a study of the quantity and rate of transfer of radiozinc in milk from the maternal rat, eight females were dosed intraperitoneally with tracer levels of zinc[65] chloride at time of parturition. Litters were standardized to eight pups, and pairs were selected at random for death at intervals of 24, 48, 72 and 96 hours after dose administration to the dam.

Calculation of radiozinc values on the basis of the percentage retained zinc[65] dose per pup showed that mammary transfer to the pup through the maternal milk amounted to 21% at 24 hours, 37% at 48 hours, 45% at 72 hours, and at 96 hours after dosing, 57% of that zinc[65] available from the body of the dam had passed through the milk to the suckling pup. These studies would indicate that—while the absorption of zinc from the gastrointestinal tract of rats following oral administration limits the supply available to the animal body—zinc reaching the blood from the maternal body is readily transferred to the milk.

SUMMARY

Data are presented showing that absorption of zinc[65] from the gastrointestinal tract of rats is poor, but that there is relatively free movement across the placenta to the fetuses at all stages of gestation of that radiozinc absorbed and retained by the dam. This retained zinc appeared to be available for mammary transfer; more than half of the retained portion of the dose was transferred through the milk to the young during a 96-hour period.

Radiozinc concentration paralleled total zinc values in maternal tissues, with the kidney showing the highest concentration, followed in order by the liver, pancreas and femur. Transfer of total zinc to the fetuses paralleled radiozinc transfer; fetal content of both forms of this element increased throughout the gestation period, reaching a peak at term.

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Fed. Proc. 19:643-7 (1960)

Nutritional interactions of zinc and calcium

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During the past five years a reawakened interest in the role of zinc in animal nutrition has resulted in considerable clarification of the enzymic roles of this element (24) and of practical conditions modifying the response of swine and of poultry to diets of moderate zinc content. Two major factors which appear to affect zinc requirements are the source of dietary protein and the level of dietary calcium. It is the purpose of this review to bring together the evidence concerning the calcium-zinc interrelationship and to evaluate the several proposals that have been made to explain the mechanism of this interrelationship.

SWINE STUDIES SHOWING AN EFFECT OF CALCIUM ON SWINE PARAKERATOSIS

The initial conclusive report showing a practical need for zinc supplements to swine rations (22), and showing a deleterious effect of calcium supplements which could be overcome by zinc, has been followed by many corroborative papers. Tucker and Salmon (22) found that hogs fed practical diets containing either hydraulic or solvent process peanut meals developed severe dermatitis and impairment of feed intake and weight gain. These diets contained bonemeal and salt as sole mineral supplements and contained 34 to 44 ppm of zinc. Addition of 2% bonemeal or 1.5% $CaCO_3$ increased the symptoms, but they could be alleviated dramatically by a 0.02% supplement of $ZnCO_3$.

In view of the practical implications of these findings and the surprise that a ration containing 7–10 times the concentration of zinc reportedly required by the rat (8), it is understandable that intensive investigations in this area of nutrition were instituted quickly. In table 1 are found growth data and certain specifications of experiments reported in the literature showing that increments of calcium are inhibitory to the weight gain of young pigs and that this inhibition may be lessened by zinc supplementation. These experiments were not designed to answer questions relative to the effect of calcium on zinc requirement. All that may be said with certainty is that growth depression accompanying high calcium diets is decreased by zinc supplementation of diets already deficient in zinc. One might as logically infer that the unfavorable effect of excess calcium is magnified in the presence of a zinc deficiency.

Retarded growth is only the first of the symptoms of zinc deficiency. Incidence of skin lesions, of the type termed parakeratosis, is a more specific symptom, but one more difficult to express quantitatively. In the report of Lewis *et al.* (10), the appearance of skin lesions closely paralleled the depression of weight gain. It is significant from the standpoint of interpreting other experiments that in Lewis' work, animals receiving 28 ppm zinc and 0.8% calcium gained well and showed a minimum of parakeratosis for eleven weeks, while those receiving 28 ppm zinc and 1.2% calcium showed parakeratotic symptoms and depressed gain after the second week. During weeks 12–20 the 0.8% calcium group showed both symptoms of zinc deficiency, thus illustrating the advantage of relatively long-term experimentation.

Luecke *et al.* (13) also report a close correspondence between parakeratosis and depressed weight gains. All pigs receiving 1.5% calcium and 31 ppm zinc showed dermatosis (7 severe and 3 mild), while only 3 of 10 pigs receiving .98% calcium and 29 ppm zinc showed dermatosis (2 severe and 1 mild). Of the remaining 20 pigs on their experiment, only one showed dermatosis, and this was a mild case.

An exception to the correlation between weight gain and dermatosis is seen in the report of Lewis *et al.* (11) in which they found that addition of NaH_2PO_4 to provide 0.8% P in the diet, rather than the basal level of .47%, had no effect on weight gains but decreased the parakeratosis to essentially the same low level as the basal + zinc group. It is in this paper that the suggestion is first made that calcium may interfere with zinc absorption. The data of Luecke *et al.* (12) also show a good correlation between weight gain and appearance of dermatosis, both being similarly affected by levels of calcium and of zinc in the diet.

Among other criteria of effects of varying levels of zinc and calcium in the diet of growing swine are tissue levels of zinc, blood alkaline phosphatase levels, and endogenous fecal zinc excretion. Hoekstra *et al.* (7) and Lewis *et al.* (10) have reported changes in tissue zinc levels as a result of increasing calcium content of the ration. Hoekstra *et al.* report that addition of bonemeal to a ration containing 80 ppm zinc lowers zinc content of liver and of kidney cortex, but not of spleen or pancreas. Bonemeal addition to a ration containing 35 ppm zinc does not affect the zinc content of any of these tissues. In contrast,

Lewis *et al.* report that additions of calcium to rations of 35 ppm zinc content lowered the zinc concentration in liver, kidney cortex, hair, pancreas and bone. The changes observed were of the order of reductions to 65–69% of values in liver, hair and bone of the 'low-calcium' animals. The physiological significance of these changes is obscure but may be related to differences in total feed

TABLE 1. *Summary of Growth Data Showing Stimulatory Effect of Zinc on High-Calcium Diets Fed to Swine*

	Ca, %	P, %	Zn, ppm	Av. gain lb./day	Remarks	Ref.
Corn-soybean meal	.98	.7	35	.91	8-week period	(13)
	.98	.7	55	1.25	10 pigs per lot	
	1.5	.8	35	.54		
	1.5	.8	55	1.06		
Corn-soybean meal	.82	.5	35	.39	12-week period	(11)
	1.2	.8	35	.09	7–8 pigs per lot	
	1.4	.8	35	.07		
	1.4	.5	35	.10		
	.82	.8	35	.35		
	.82	.5	85	.73		
	1.40	.8	85	.89		
Corn-cotton-seed meal	.48		32	.79	6-week period	(21)
	.48		44	1.10	8 pigs per lot	
	.67		32	.48		
	.67		44	1.05		
	.67		80	1.19		
	1.03		32	.12		
	1.03		44	.79		
	1.03		80	1.19		
Semipurified Isolated soy Protein	.68		28	1.02	9-week period	(3)
	1.06		28	.83	6 pigs per lot	
	.68		128	1.13		
	1.06		128	1.10		
Corn-soybean	.5	.5	28	1.14	20-week period	(10)
	.8	.5	28	.8	6 pigs per group	
	1.2	.9	28	.1	at 12 weeks only	
	.8	.5	128	1.20	1.2% Ca group retarded	
Corn-soybean meal	.65	.5	45	1.22	9-week period	(12)
	.65	.5	95	1.30	10 pigs per group	
	1.25	.9	40	.75		
	.5	.6	32	.59	8-week period	
	.5	.6	82	1.23	10 pigs per group	
	1.2	.6	29	.21		
	1.2	.6	79	1.16		
	1.9	.6	31	.17		
	1.9	.6	81	.98		
Corn-soybean meal	.64	.54	30	.17	6-week period	(17)
	1.19	.93	33	.02	Controlled feed intake	
	1.23	.96	94	.29		

(and hence zinc) intake rather than to a specific effect of calcium on zinc level in the tissues. The authors state, "Excessive calcium probably reduced the zinc content of these organs by hindering zinc absorption from the intestinal tract," and, "It is likely that none of the body components analyzed to date represent the site at which zinc exerts its beneficial effect on parakeratotic swine. Perhaps the point of action is in the gastrointestinal tract rather than in the body proper."

Luecke *et al.* (12, 14) and Newland *et al.* (17) have shown parakeratosis to be accompanied by low blood serum alkaline phosphatase. There is a trend in their data for calcium to decrease blood serum alkaline phosphatase, while zinc raises it markedly.

A definite attempt to account for the observed relationship between calcium and zinc is that of Newland *et al.* In a carefully controlled experiment involving individually fed weanling pigs, an attempt was made to measure endogenous fecal zinc excretion of pigs fed *1*) 30 ppm zinc with .64% Ca and .54% P, *2*) 33 ppm Zn with 1.19% Ca and .93% P, or *3*) 94 ppm Zn with 1.23% Ca and .96% P. Equal feeding was an integral portion of the experimental design. When the pigs had been on these diets 8 days they were given a single injection of Zn^{65}. Collection of feces was made during days 16 through 21, during which time the same diets were fed. At the 21st day the animals were slaughtered and tissues and excreta examined for total zinc and Zn^{65}. Total fecal zinc excretion amounted to 110, 93 and 93% of the intake, respectively, for the three groups of pigs. Total zinc concentrations of blood, liver and kidney were not altered by the treatments, but specific activity of feces and of liver was higher in *group 2*, receiving high calcium and low zinc, than in the other two groups. The percentage of fecal zinc of endogenous origin was calculated to be 6.4, 10.7 and 4.4, respectively. These figures, however, like those for specific activity of fecal zinc, need careful interpretation, since the zinc intake was 2–4 times greater in *group 3* than in the other groups. This fact alone would lower the specific activity of tissue and of fecal zinc for *group 3*, aside from variation in calcium intake. Further, it is not clear how the facts of higher specific activity (and total activity) in both feces and tissues 13 days after a single injection led to a conclusion that a 'higher rate of zinc metabolism' exists. Actually, the greater specific activity of fecal zinc from the high calcium-low zinc fed pigs is in large part accounted for by the lower total zinc concentration in the feces of these animals, a reflection of more complete absorption of dietary zinc or of a less complete apparent absorption of other dietary nutrients in the presence of high calcium.

SWINE STUDIES NOT SHOWING RELATIONSHIP BETWEEN CALCIUM, ZINC AND PARAKERATOSIS

A number of experiments with swine have not demonstrated a significant degree of interaction between calcium and zinc in the development of symptoms of parakeratosis. Results of these experiments are summarized in table 2. One of these was conducted with a casein-glucose semipurified basal diet containing 0.6% calcium and 9 ppm zinc (1). In this meticulous experiment the addition of $CaCO_3$ to provide a total of 1.5% calcium did not affect weight gain over a 6-week period. Pigs started to fail in feed intake during the second week. skin lesions appeared rapidly during the third week. Onset of symptoms and their eventual severity were not affected by calcium level.

Bellis and Philp (2) employed a barley-fish meal-peanut meal ration in their experiment. Calcium fed at

levels from .75 to 1.55% failed to affect weight gains. In diets containing 1.55% calcium, increasing phosphorus from 0.7 to 1.30% depressed weight gain and decreased the incidence of skin lesions. Calcium levels from .95 to 1.32% all were accompanied by about 50-60% incidence of mild parakeratosis, whereas only 32% (13 out of 40) of the pigs fed 0.75% calcium developed even a mild parakeratosis. Incidence of skin lesions in pigs fed the 1.55% calcium rations was somewhat higher than in those fed lower levels of calcium,

TABLE 2. *Summary of Growth Data from Swine Feeding Experiments in which Calcium-Zinc Interrelationship is not Apparent*

Ration	Ca %	P %	Zn ppm	Av. gain lb./day	Remarks	Ref.
Casein-glucose	.6	.75	9	.21	6-week period	(1)
	1.5	.71	10	.23	7 pigs per group	
	.6	.73	104	.67		
Barley-fish meal	1.32	.7	20	.80	5-week period	(2)
	1.15	.7	20	.77	40 pigs per group	
	.95	.7	20	.87		
	.75	.7	20	.74		
	1.55	.7	20	.77	4-week period	
	1.55	1.0	20	.63	34 pigs per group	
	1.55	1.3	20	.63		
Corn-oats-barley-soybean meal-fish meal	.6	.6	33	.85	11-week period	(5)
	.6	.6	50	1.17	10 pigs per group	
	.6	.6	100	1.00		
	1.0	.6	38	.84		
	1.0	.6	50	1.03		
	1.0	.6	100	1.10		
	1.8	.6	32	.84		
	1.8	.6	50	.93		
	1.8	.6	100	.85		
	3.0	.6	33	.53		
	3.0	.6	50	.67		
	3.0	.6	100	.66		
Starch-oats-soybean meal-fish meal	.4	.46	34	.72	8 pigs each treatment in 4 successive 4-week periods	(25)
	.86	.46	34	.72		
	.4	.46	140	.74		
	.86	.46	140	.67		
Corn-soybean	.3		30	1.23	16-week period	(6)
	.3		90	1.48	10 pigs per group	
	.5		30	.89		
	.5		90	1.46		
	.7		30	.73		
	.7		90	1.24		

and some pigs (12 of 34) developed severe lesions on the 0.7% phosphorus diet. The authors state the opinion that the action of calcium is not necessarily a simple chemical one, that the answer may be found at the level of cell nutrition.

Heigener *et al.* (5), in a well-planned experiment, found that increasing dietary calcium from 0.60 to 3.02% decreased growth, and that supplements of zinc above the 33 ppm basal level increased growth proportionately at each calcium level. Their data also show that maximum growth at any calcium level was attained with 50 ppm zinc, and that zinc supplementation alleviated skin lesions but that 100 ppm was no more effective in this respect than 50 ppm.

The study by Hoefer *et al.* (6) shows that on low zinc rations, an increase of dietary calcium concentration from 0.3 to 0.7% decreases weight gain and increases severity of parakeratosis. Sixty ppm Zn added to these rations eliminated the skin syndrome and increased weight gain, but animals still gained less on the highest calcium level than on the two lower levels.

Studies of zinc balance and excretion under different conditions of calcium nutriture in swine have been conducted in an attempt to reveal a relationship that would explain aggravation of symptoms of zinc deficiency as a result of increasing calcium intake.

The data of Beardsley (1) show that calcium supplementation of a very low zinc diet (9 ppm) had no effect on zinc absorption, which averaged 68% of the intake. The urinary excretion of zinc was increased, however, by pigs fed 1.5% calcium, so that the retention of apparently absorbed zinc amounted to 91 and 76%, respectively, for pigs fed low- and high-calcium diets. Since rate of growth and tissue concentration of zinc were not lowered by calcium supplementation, the significance of the greater urinary excretion is not clear.

The detailed balance study of Whiting and Bezeau (25) failed to show that calcium has an effect on absorption of zinc or on retention of absorbed zinc. On the basal, 34 ppm zinc diet, 21% of the zinc was apparently absorbed, irrespective of calcium level, and retention of absorbed zinc equalled 31 and 22% for low- and high-calcium diets. Thus, qualitatively the data are in good agreement with those of Beardsley (1), although the degree of zinc deficiency was much greater in the latter's experiment.

The following experiment, conducted by M. K. Lloyd and M. C. Bell (personal communication), demonstrates a beneficial effect of high calcium on absorption and retention of zinc from swine diets containing a moderate excess of this element. Eight Yorkshire barrows averaging 134 lb. were divided into 2 groups of 4 pigs each and placed on rations containing 0.5% and 1.2% calcium. They were fed a ration of corn, soybean meal, meat and bonemeal, alfalfa meal and mineral and vitamin mix. The trace mineral mix contained an excess of zinc to supply about 140 ppm of zinc in each ration. An average of 29 and 59% of orally administered Zn^{65} was retained on the low Ca and high Ca rations, respectively. Stable Zn retention on these rations was 31 and 45% for low Ca and high Ca, respectively. Intraperitoneal injection of Zn^{65} gave a much higher retention of Zn^{65}, with 93 and 95% retained. It was noted that calcium supplementation resulted in decreased phosphorus balance and decreased urinary phosphate excretion. In the work of Beardsley (1) calcium supplementation did not affect phosphorus balance, but markedly increased fecal phosphate excretion while decreasing urinary phosphate output (Forbes, unpublished data).

STUDIES OF CALCIUM-ZINC INTERACTION IN POULTRY NUTRITION

The published experiments concerning the effect of calcium on zinc deficiency in poultry present a diversity of results, as do those for swine. The first such experiments are those of Young *et al.* (26) in which the effect on zinc requirement of increasing Ca from 1.2 to 1.8%, with 0.6% phosphorus, was tested in 10-day experiments with chicks. The basal diet was a semipurified isolated soy protein-glucose diet containing 15 ppm zinc. At both calcium levels, 40 ppm-added zinc were required for optimum weight gain. Chicks on the higher calcium level gained as well as those on the lower, and consumed less feed. Results of a similar nature are presented by Pensack *et al.* (19), employing casein-glucose diets with 6, 20 and 40 ppm zinc and 1.0 and 2.0% calcium in a 4-week test. Twenty parts per million of zinc in diets containing either 1.0 or 2.0% calcium were sufficient for maximum weight gain. Zinc analysis of tissues from these chicks (Bogdenoff *et al.*, personal communication) showed that in ash of liver, bone and feathers the zinc content was decreased by raising dietary calcium, increased by raising dietary zinc, and increased further by both additions. Muscle ash zinc was decreased by calcium; zinc had no effect. Whole blood ash zinc was increased by calcium and by zinc, but effects were not additive.

Investigations leading to the conclusion that zinc utilization by the chick is affected by dietary calcium include those of Morrison and Sarett (16), O'Dell *et al.* (18) and Turk *et al.* (23). The first cited authors employed an isolated soy protein-sucrose basal diet containing 30 ppm zinc and 1.2% calcium. Supplements of 0, 5 and 25 ppm zinc were made, and at each zinc level, 0.5 and 1.0% calcium were added. Weight gain after 14 days on experiment indicated that the depressing effect of each level of calcium supplementation was eliminated by the supplement of 25 ppm zinc. O'Dell *et al.*, using a washed soy protein-glucose basal diet containing 1.1% calcium, found that raising calcium to 1.6 depressed growth and doubled incidence of hyperkeratosis, and that a further increase to 2.1% calcium was no more detrimental. Addition of 63 ppm zinc more than doubled the weight gain above that obtained on the basal diet and eliminated incidence of hyperkeratosis. No effect of calcium was observed on any of the zinc-supplemented diets.

Turk *et al.* (23) reported that chicks hatched from hens fed a 9-ppm Zn soybean meal diet were zinc-deficient. The deficiency was aggravated by raising calcium content of the hens' ration from 2.23 to 4.0%, and hatchability of eggs was decreased. Addition of 50 ppm zinc eliminated signs of zinc deficiency.

EFFECT OF CALCIUM ON ZINC REQUIREMENT AND EXCRETION OF THE RAT

Forbes and Yohe (4), employing casein-glucose and isolated soy protein-glucose diets fed to weanling male albino rats, have investigated the effects of calcium supplementation on weight gain and on zinc balance. In their first experiment 24 rats were fed for 6 weeks 0.8 and 1.8% calcium at zinc levels of 7, 18, 31 and 52 ppm in casein-glucose diets. Feed intake was equalized except for the animals on the basal level of zinc. Collection of urine and feces was made during the second and sixth weeks. Eighteen ppm of zinc were adequate for maximum weight gain at both calcium levels. At each zinc level weight gain was less by animals receiving 1.6% than by those receiving .8% calcium. Although the difference between gains due to calcium was greatest on the 7 ppm zinc level, statistical analysis did not show a significant interaction between calcium and zinc. Balance data showed no influence of calcium on zinc excretion. Of the total zinc intake, 6% appeared in urine, and 43% appeared in feces. The total zinc retention was, thus, 51% as an average of all levels of zinc fed. In view of finding a higher zinc requirement on the part of rats fed a soy protein diet, and the fact that investigators in swine nutrition had observed a detrimental effect of calcium on parakeratosis, a further experiment was conducted using isolated soybean protein-glucose diets. The results of the second experiment were qualitatively similar to those of the first. Calcium depressed growth at all levels of zinc. The percentage of zinc consumed appearing in the feces amounted to 54%, there being no consistent effect of calcium on fecal zinc.

TABLE 3. *Effect of Dietary Calcium on Tissue Zinc Concentration*

Tissue	% change in tissue zinc conc. accompanying higher dietary calcium	References
Pig kidney	+11	(1) (17)
Pig kidney cortex	−12	(7) (10)
Pig liver	−17	(1) (17)
Pig bone	−19	(1) (10)
Pig blood	−10	(1) (10) (17)
Pig hair	−33	(10)
Rat liver	0	(4)
Rat carcass	0	(4)
Chick liver	−15*, +18†	Bogdonoff (personal communication)
Chick muscle	−41, −38	
Chick blood	+22, −10	
Chick feathers	−36, +44	
Chick bone	−25, +13	

* Data from animals fed 6 ppm Zn.
† Data from animals fed 40 ppm Zn.

DISCUSSION

As one reviews the literature dealing with the effect of calcium on zinc utilization, several things become apparent. First, one must conclude in the face of the diverse evidence that under some, but not all, conditions there does exist a relationship between calcium intake and the status of zinc nutriture of swine and of poultry. Delineation of these conditions is requisite to an

understanding of the relationship. Second, although it is apparent that, in the presence of suboptimum zinc nutriture, increasing the calcium intake may magnify the skin lesions and growth depression, it has not been demonstrated that the requirement for zinc has been altered. Third, of the various proposals that have been made in attempts to explain the apparent decrease in zinc utilization accompanying an increase in calcium intake, none of them that have been tested have provided the answer. Fourth, although the practical solution to the problem of parakeratosis is obvious, there remain intriguing problems concerned with zinc metabolism as affected by calcium/zinc ratios and by the relationship of other dietary and environmental factors to the zinc supply.

In attempting to explain the observed effect of calcium on zinc utilization it seems logical to consider the possible sites at which calcium may interfere. For example, is it possible that increasing calcium concentrations in the diet may promote a chemical reaction in the diet that would render the zinc less absorbable? Or, is the effect of calcium to produce conditions in the intestinal tract which are less favorable for zinc absorption? While neither of these hypotheses has been examined separately, and this would be difficult, those studies which have been made of zinc absorption have not revealed a depression in apparent or true absorption of zinc as a consequence of increasing the calcium level of the diet. Two studies (9, 20) of the effect of calcium on zinc solubility in aqueous solution have been conducted. These do show calcium to decrease zinc solubility, but the conditions employed have little in common with those present in the intestinal tract. Zinc excretion via the kidney has, in the two balance studies with swine, been shown to be increased in animals receiving high calcium diets. The amounts of zinc excreted in this manner are small but may be sufficient to account for the small trend toward lower levels of zinc in tissues of animals fed high calcium diets, as shown in table 3.

The results of tissue analysis for zinc as affected by increased concentration of dietary calcium vary between investigators, between species and among tissues. This may be accounted for either by variation between animals, between other dietary or environmental factors, or in the zinc analysis itself. A summary of available data on tissues showing the greatest differences is shown in table 3. In general, the differences observed in zinc content of these tissues were shown by the investigators cited to be statistically significant. Other tissues analyzed by these investigators have revealed little or no change in zinc concentration due to change of calcium intake. Recently Miller *et al.* (15) have reported nonsignificant changes in zinc concentration in swine liver and kidney as a result of increasing dietary calcium from 0.6 to 1.25%. It thus appears that the result of tissue analysis for zinc do not account for the increased severity of deficiency symptoms occasioned by increased dietary calcium concentration.

CONCLUSIONS

It seems most probable, on the basis of present evidence, that interference in zinc function by calcium occurs at the cellular level. Although a variety of enzymes are known to contain zinc, and other reactions are catalyzed by zinc, these activities do not appear to be depressed in tissues of zinc-deficient animals, and furthermore, the skin lesions of zinc deficiency cannot yet be explained on the basis of known enzymic or catalytic functions of zinc. Until we can describe the biochemical lesion(s) of zinc deficiency we will not be able to explain the effect of calcium on the visible syndrome of this deficiency.

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From: Newer Methods in Nutritional Biochemistry
Vol. 3. 1967.

Chapter 7

Studies of Zinc Metabolism

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I. Introduction

The techniques employed in studies of zinc metabolism range from those of animal husbandry to those employing the most modern methods of enzyme kinetics and studies of "reaction sites." Since much of this material has been reviewed recently (1, 2), it is the purpose of the present chapter to collate as far as possible developments in this area since 1960, referring to earlier work when necessary to give proper perspective to current views of the problem of zinc metabolism and nutriture in animals.

Current interest in the metabolic role of zinc stems from the work of Tucker and Salmon (3) who demonstrated for the first time the practical necessity for zinc supplementation of diets for swine raised by usual husbandry procedures, from investigations such as those of Nason *et al.* (4) and of Keilin and Mann (5) and Vallee (6) demonstrating the nature of the enzymatic role of this trace element, and from clinical evidence of Prasad and co-workers showing an abnormality of zinc metabolism in humans (7–9). As Underwood has pointed out (10), the biochemical lesions responsible for the clinical syndrome of zinc deficiency are as yet unknown despite the known roles of zinc in a

number of enzymes and enzyme-catalyzed reactions. Thus the search must go on.

II. Methods of Analysis

A wide variety of techniques has been employed in quantitative analysis for zinc in biological materials. In most instances some means of separation of zinc from interfering materials must precede the actual determination. Whatever methods are used, scrupulous care to avoid contamination is required since zinc is so common an element in the environment. This aspect of the problem has been treated in detail by Thiers (11). Of particular interest with respect to zinc is Thiers' warning that care must be taken in dry ashing procedures to prevent the sample from becoming heated above 450°C in order to avoid loss through volatilization. In spite of this, Thiers favors controlled dry ashing over wet ashing because of the contamination hazards inherent in the latter procedure. A survey of methods available for determination of zinc in biological materials was published by Malmstrom (12) in 1954, and specifics of the widely used dithizone procedure are given. A more recent and detailed study of the separations prior to color development with dithizone is that of Margerum and Santacana (13). In their hands the use of diethanoldithiocarbamate according to the procedure of Butts *et al.* (14) gave the most satisfactory separations of zinc. We have employed this procedure as well as that of Vallee and Gibson (15) using the mixed colorimetric method for the determination of the zinc dithizonate. Both methods were satisfactory, but the procedure of Butts was simpler in terms of manipulations and in our opinion is the method of choice among the dithizone procedures. Reference to other previously reviewed methods of zinc detection may be found in the article by Vallee (1).

The advent of atomic absorption spectroscopy as a practical means of trace-element analysis has been reviewed by Robinson (16), and this principle has been rapidly applied to the determination of zinc in biological materials. In view of the sensitivity, accuracy, freedom from interference, and simplicity of operation this is now the method of choice. Application of the method to biological fluids is discussed by Fuwa *et al.* (17) who used a hydrogen-air flame. We have used an acetylene-air flame in making measurements of zinc content of animal tissues, excreta, and feedstuffs. Sample preparation involves ashing with appropriate care to prevent contamination and then dilution of the sample with HCl to make a final concentration of 0.36 *N* HCl. A more general treatment of the applications of this method is that of Allen (18). Recent applications of emission spectroscopy (19) and neutron-activation analysis (20) have also been published.

III. Zinc in Tissues

The quantitative and qualitative aspects of the zinc content of animal tissues have been treated in detail in previous reviews (1, 2). The zinc concentration in most of the soft tissues of the body approximates 25 ppm on the fresh basis and, except in the liver, is not appreciably changed by alteration of zinc intake (21–24). Considerably higher (100–400 ppm) concentrations of zinc are found in bone, hair and wool, and portions of the prostate and the eye. Human prostatic fluid contains 10 times the concentration of zinc as is found in the whole gland (25), a finding in agreement with histochemical demonstration of zinc concentration in the glandular epithelium. In the young, growing animal the zinc concentration in bone ash is a sensitive reflection of zinc absorption, especially at suboptimal intake levels (26–29). It is apparent that zinc accumulates in cartilage at sites of calcification and, once deposited in the calcified tissue, is firmly bound (30–32). Analysis of human autopsy material did not reveal age changes in bone ash zinc (33), nor did an experiment in which rats were fed a constant diet over more than a year. It was found that the shaft of the rat femur had a lower zinc concentration (330 ppm of ash) than the head of the femur (430 ppm).

The major portion of the zinc in whole blood is in the erythrocytes where it occurs mainly as a constituent of carbonic anhydrase. Species differences are apparent in blood zinc, but the relative distribution among blood components is similar. More data are available for man than for other species, and average values indicate 7–8, 1.1–1.3, and 12–18 μg of zinc per milliliter of whole blood, plasma, and erythrocytes, respectively. According to Underwood (2) leukocytes contain 3% of whole blood zinc, but each leukocyte contains 25 times as much zinc as each erythrocyte. Plasma zinc is in part protein bound (34, 35).

The occurrence of zinc in skin and other epidermal structures has been of interest because of its concentration in hair, wool, and nails (100–200 ppm) and because of the marked histological aberration in the skin of zinc-deficient animals. Actually, the skin itself does not contain remarkable concentrations of zinc [20–60 ppm of dry tissue (36, 37)]. The epidermis does, however, contain 3 times the concentration of zinc that the corium does (25 vs. 75 ppm). Recent investigations (38) indicate that the transitional zone of human epidermis concentrates zinc, and evidence points to protein-bound histidine as the binding agent. This viewpoint is supported by the similarity in distribution of histidine and of zinc in epidermal structures, by the disappearance of histochemically detectable histidine following zinc uptake, and by the absence of other structures that might have zinc-binding capabilities.

Only fragmentary data are available on the zinc content of skeletal muscle. Large variation in zinc content of individual muscles of swine has been reported recently (39). Porcine trapezius muscle had 3 times the concentration of zinc and of myoglobin that was present in longissumus dorsi. Interestingly, it also had a higher lactic dehydrogenase activity. The relationship between these findings and observed differences in post-mortem changes is not clear.

The previously reported intracellular distribution of zinc in rat liver (40) has been confirmed (41, 42). With the data reported in terms of micrograms of zinc per milligram of nitrogen, the following distribution was found: nuclei, 0.77; mitochondria, 0.42; microsomes, 0.65; supernatant, 2.0; and reconstituted whole liver, 1.05. These findings are consistent with the view (40) that the majority of the known zinc-containing enzymes are in the supernatant fraction of the liver cell. On a percentage basis 18–28, 7–9, 11–16, and 54–58% of the total liver zinc is present, respectively, in nuclei, mitochondria, microsomes, and supernatant. The intracellular distribution of a single dose of ^{65}Zn in mouse liver is reported not to change within the time limits of 1 hour and 7 days (42).

The unusual concentration of zinc in certain portions of the eye has attracted much attention and has been carefully documented (1, 2). The choroid of carnivora and particularly the tapetum lucidum contains higher concentrations of zinc than any other animal organ; this amounts in some species to as much as 13% of the dry tissue. A significant amount of the zinc seems to be bound in a zinc-cysteine complex, but its function is not known. The lower but significant amounts of zinc in the retina may be incorporated in part with retinene reductase which catalyzes the interconversion of vitamin A alcohol and aldehyde and may point to a vitamin A–zinc interrelationship. Another reason to investigate such a relationship is the general similarity of skin lesions in deficiencies of vitamin A and of zinc.

Previously reported investigations have indicated that species variation in zinc content of milk is not large and that "mature" milk may be expected to contain 37.5 ppm whereas colostrum may contain 4 times this amount. In recent investigations mature cow's milk was found to contain 4.1 ppm (43) and sow's milk 7.3 ppm (44). Miller *et al.* (43) found supplementation of the cow's ration with 500, 1000, and 2000 ppm of zinc resulted in milk containing, respectively, 6.7, 8.0, and 8.4 ppm of zinc. Stevenson and Earle (44) also found an influence of dietary zinc on sow-milk zinc: the addition of 100 ppm of zinc to the ration raised the milk zinc from 7.3 to 10.4 ppm. Sow colostrum, however, was not affected by the zinc supplement and contained about 23 ppm of zinc, 2–3 times the value in the mature milk.

An area of great interest in zinc metabolism is related to the possible role of the large amounts of zinc found in portions of the male reproductive tract and its secretions. The prostate gland in particular concentrates zinc, although there are wide variations between species in the distribution of this element in the gland. In the rat, for example, the dorsal prostate may contain 180 ppm of zinc on the fresh basis whereas the ventral prostate contains less than one-tenth of this amount (45). On the other hand, in the human prostate no correlation could be found between zinc content and histologic features of the gland, although there was a 20-fold difference in zinc content of different areas of a given gland (46) and a general increase in zinc content as samples were taken further from the bladder. One gland in this series contained a localized carcinoma, and this area contained about one-third as much zinc as did unaffected areas of the same gland. This is roughly comparable to the earlier report (47) that five cancerous human prostates contained 190 ppm zinc on the dry basis whereas thirty-one normal prostates contained 682 ppm. As yet no functional role has been found for these significant concentrations of zinc. Although there is a general correlation between zinc content and carbonic anhydrase activity of prostatic tissue, the latter accounts for only a small portion of the total zinc present (48), and there is no correlation between zinc content and the activity of either acid or alkaline phosphatase. It is interesting that the necrotizing effect of dithizone on the prostate of the rat and the dog is correlated with formation of a fine intracellular deposit of zinc dithizonate (49, 50). This indicates that a large portion of the zinc present is not so firmly bound to protein that it is unavailable for chelation.

The high zinc content of testes and of spermatazoa has been well documented (1). Recent investigations have shown that sperm obtained from vas deferens and epididymis contain as much zinc as those from the total ejaculate (51), which shows that they are not dependent on the zinc-rich prostatic fluid for their zinc content. The role of zinc in normal sperm function remains obscure since zinc deficiency produces testicular degeneration and aspermia, as well as markedly reducing the zinc concentration in the total male reproductive tract.

The zinc content of the pancreas and its endocrine and exocrine secretions has been of much interest since the finding that crystalline zinc-insulin has certain advantages in treatment of diabetes over metal-free insulin. This matter has been thoughtfully discussed by Vallee (1) who raises pertinent questions about some of the analytical methods used to detect zinc in the whole pancreas and in the insulin-secreting β-cells of the islands of Langerhans. Whatever the facts of this portion of the story may be, it is clear that zinc-free insulin (and glucagen) are fully

active biologically, and the ultimate test of the necessity for zinc in their activity has yet to be devised.

IV. Biochemically Recognized Functions

Specific information on the manner in which zinc is involved in metabolic reactions in the animal body dates from the original discovery that zinc is a constituent of bovine erythrocyte carbonic anhydrase (52). Since that time a considerable body of research has appeared, revealing the roles in which zinc may participate in metabolism either as an activator of enzyme systems, forming a metal-enzyme complex, or, more specifically, as a constituent of enzymes, forming metalloenzymes. While it is interesting to study the former situation—the activating effects of metals on specific enzyme systems *in vitro*—the results of such investigations tell little about the *in vivo* relationships of the metal and indeed, more often than not, merely demonstrate that the metal requirements for this sort of activation are nonspecific. It is typical of the metal-enzyme complex that it is readily and reversibly inactivated by removal of the metal from the system and that a number of different metals may activate a specific reaction. The case of the metalloenzymes is more specific; in such compounds the metal is firmly bound to the enzyme. Removal of the metal irreversibly inactivates the enzyme, and in such enzymes, purified to the point of physicochemical homogeneity, the ratio of metal to enzyme becomes constant, and stoichiometry can be shown to exist between moles of metal and moles of coenzyme or prosthetic group involved in enzyme action. These examples represent classified extremes which are bridged by a continuous spectrum of activities of the metal-enzyme complexes on the one hand and metalloenzymes on the other. These views have been discussed (53), and the application of these concepts to the study of zinc metabolism has been expertly reviewed (1, 53, 54). Tables I and II list the known zinc metalloenzymes and the metal-enzyme complexes activated by zinc as cited in the review by Vallee (1) who has been responsible for the major portion of the work done on zinc metalloenzymes.

In addition to the data of Tables I and II, more recent investigations have shown that D-glyceraldehyde-3-phosphate dehydrogenase, isolated from bovine and crayfish muscle and bakers yeast, is a zinc metalloenzyme with 2 moles of zinc per mole of enzyme (55), apparently bound by means of cysteine and histidine (56). Malic dehydrogenase has also been reported to contain zinc in a 1:1 mole ratio with the enzyme (57) and to possess the qualities of a metalloenzyme.

The role of zinc in regulating the activity of alkaline phosphatase has been of continued interest since some preparations of this enzyme

TABLE I
ZINC METALLOENZYMES OF KNOWN METAL CONTENT

Enzyme	Abbreviation	Molecular weight	Amount of metal (%)	Ratio of metal to protein (gm atom/mole)	Ratio of coenzyme[a] to protein (mole/mole)	Ratio of metal to coenzyme[a] (gm atom/mole)	Empirical formula
Carbonic anhydrase (bovine erythrocytes)	CAD	30,000	0.2–0.3	1 (? 2)	—	—	$[(CAD)Zn_{1-2}]$
Carboxypeptidase (bovine pancreas)	CPD	34,300	0.18	1	—	—	$[(CPD)Zn]$
Alcohol dehydrogenase (yeast)	YADH	150,000	0.18	4	4 (NAD)	1 Zn/NAD	$[(YADH)Zn_4](NAD)_4$
Alcohol dehydrogenase (equine liver)	LADH	73,000 (84,000)	0.18	2	2 (NAD)	1 Zn/NAD	$[(LADH)Zn_2](NAD)_2$
Glutamic dehydrogenase (bovine liver)	GDH	1,000,000	0.02–0.03	2–4	2–4 (NAD)	1 Zn/NAD	$[(GDH)Zn_{2-4}](NAD)_{2-4}$
Lactic dehydrogenase (rabbit skeletal muscle)	SLDH	?	0.07	—	—	—	$[(SLDH)Zn_7](NAD)_7$
Alkaline phosphatase (porcine kidney)	—	?	0.15	—	—	—	—
Alkaline phosphatase (*Escherichia coli*)	—	80,000	0.17	2.1	—	—	—

[a] NAD, previously known as DPN.

have been reported to constitute zinc metalloenzymes and also to exhibit properties of metal-enzyme complexes. Reports of animal feeding studies have shown that low levels of serum alkaline phosphatase accompany zinc deficiency (58–61), although lack of such correlation has also been reported (62, 63). The possible role of reduced feed intake in contributing to this effect has not been extensively investigated, and in fact has ordinarily not been considered. An exception to this is the report by Starcher and Kratzer (64) who investigated effects of zinc deficiency on bone alkaline phosphatase in turkey poults. In this experiment it was shown that restriction of intake by zinc-adequate birds to that

TABLE II
METAL-ENZYME COMPLEXES ACTIVATED BY ZINC[a]

Enzyme	Activating metals
Glycylglycine dipeptidase	Zn^{2+}
Arginase	Zn^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}
Dehydropeptidase	Zn^{2+}
Alanyl- and leucylglycine dipeptidase	Zn^{2+}, Pb^{2+}, Cu^{2+}, Mn^{2+}, Sn^{2+}, Cd^{2+}, Hg^{2+}
Tripeptidase	Zn^{2+}, Co^{2+}
Glycyl-L-leucine dipeptidase	Zn^{2+}, Mn^{2+}
Carnosinase	Zn^{2+}, Mn^{2+}
Aminopeptidase	Zn^{2+}, Co^{2+}, Mn^{2+}
Histidine deaminase	Zn^{2+}, Hg^{2+}, Cd^{2+}
Alkaline phosphatase	Zn^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Ca^{2+}
Lecithinase	Zn^{2+}, Cu^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}
Enolase	Zn^{2+}, Mg^{2+}, Mn^{2+}
Yeast and *Clostridium* aldolase	Zn^{2+}, Fe^{2+}, Co^{2+}
Oxalacetic decarboxylase	Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Pb^{2+}, Ni^{2+}, Fe^{2+}, Mg^{2+}, Ba^{2+}, Cu^{2+}

[a] Other metals which increase the observed activity are also listed.

of severely zinc-deficient birds lowered bone alkaline phosphatase below that of the *ad libitum* positive controls, but the values were higher than those of the zinc-deficient birds. Thus it appears that both feed intake and zinc status play a role in activity of bone alkaline phosphatase and might well do so also for other phosphatases.

Since the original discovery that carboxypeptidase from bovine pancreas is a zinc metalloenzyme (65), Vallee and his associates have conducted a long series of meticulous experiments whose objective has been to learn more about the nature of the active binding site in this enzyme and others. These findings have been summarized (66, 67) in detail, and the reader is referred to them for study of the application of pres-

ent-day techniques in biochemistry to the problems of enzyme function and specificity. With reference to alcohol dehydrogenase Vallee states (66) that, "The available data suggest that the metal is bound as a dimercaptide between two adjacent subunits of the molecule while it maintains the molecular configuration which is crucial to the binding of DPN [NAD[1]] by a three-point attachment: one each of two adjacent subunits and the third to zinc." With reference to carboxypeptidase he summarizes that, "The catalytically active zinc atom is bound to the sulphur atom of the sole cysteine residue and to the N-terminal asparagine residue in the single peptide chain of γ- and δ-carboxypeptidase. Replacement of zinc by other metal atoms results in enzymatically active metallocarboxypeptidases which differ from the native enzyme both in specific activity and in substrate specificity." These concise statements illustrate the current status and future direction of progress of our understanding of the ultimate mechanism of function of many trace metals in general and of zinc in particular.

In addition to following enzymatic activity and correlating this with zinc content, other metalloproteins may be investigated without regard to their biological activity. Identification of metals in stoichiometric amount in highly purified proteins has revealed the presence of a number of metals including copper, cadmium, and zinc (68, 69). This approach, though laborious, holds promise of expanding our knowledge of metabolic roles of zinc and other trace elements as we become more familiar with the function of these newly identified metalloproteins.

A wide variety of metals including zinc has been found in ribonucleic acids from diverse sources (70), and it is suggested that they play a role in maintaining configuration of the ribonucleic acid (RNA) molecule and thus have a functional relationship to protein synthesis and the transmission of genetic information. In *Rhizopus nigricans* cultures, addition of zinc to deficient cultures results in an immediate increase in RNA content, followed by an increase in protein content (71); these results verify the importance of zinc in RNA metabolism.

V. Accumulation and Turnover of Zinc-65 in Animal Tissues

Employment of ^{65}Zn as a tool to study the metabolism of zinc in animal bodies and tissues has been a fruitful venture for investigators interested in rates of deposition and removal, sites of concentration, and various factors affecting these parameters of zinc utilization.

Representative of early studies on uptake and removal is the report by Gilbert and Taylor (32) in which it was shown that, after intravenous

[1] NAD (nicotinamide-adenine dinucleotide), also known as DPN (diphosphopyridine dinucleotide).

administration to rats of a single dose of ^{65}Zn, 11–15% of the dose was present in the skeleton after 3 days and was still present when the animals were sacrificed after 259 days. The ^{65}Zn deposited in hair amounted to a maximum of 12% of the dose at 9 days and then decreased slowly. In other tissues the evidence indicated equilibration with the dose at about 3 days with an exponential decrease thereafter. Of the total zinc administered, 73% was excreted by way of the intestine, indicating the importance of this pathway, since 12% was retained in the bone and 9% appeared in the hair and was probably lost by shedding. Another early report in this field (72) showed that 18% of the dose was in liver of rats 24 hours after an injection of ^{65}Zn and that 2.6% was in pancreas of normal rats, but only 0.3% in pancreas of alloxan-diabetic rats. Rubini *et al.* (73) in a study of absorption, deposition, and excretion of ^{65}Zn in mice, rats, and dogs found that injected ^{65}Zn accumulated first in pancreas, liver, and spleen. Subsequently a large portion was transferred to bone. Similar findings covering the preferred sites of initial deposition were reported by McKenney *et al.* (74) using pregnant ewes as experimental subjects. They also reported a limited transmission of ^{65}Zn across the placenta, the fetuses containing 0.1–0.5 times the concentration of ^{65}Zn found in comparable tissues of the ewes. Other evidence for transfer of ^{65}Zn across the placenta has been obtained with the rabbit (75) and the mouse (76); in the latter instance the presence of ^{65}Zn in the milk was suggested since mice previously unexposed to ^{65}Zn accumulated this element while suckling mothers which had been injected with ^{65}Zn. Stand *et al.* (77) have also cited pancreas and liver as two of the sites of early accumulation of ^{65}Zn in mice. They also found that chelated ^{65}Zn was more rapidly excreted and accumulated to a lesser degree in the body than was ionic zinc. In the human, Rosoff *et al.* (78) found liver, kidney, spleen, and pancreas to be preferred sites of concentration of a single intravenous dose of ^{65}Zn.

The effect of status of zinc nutriture on uptake of ^{65}Zn by tissues of chickens has been recently reported by two groups of investigators. Zeigler *et al.* (79) demonstrated that zinc deficiency altered total zinc concentration only of bone, liver, and duodenum but that all tissues in zinc-deficient chicks accumulated higher concentrations of intramuscularly administered ^{65}Zn than did those of normal chicks. It was also observed that ^{65}Zn is rapidly removed from blood plasma as it accumulates in other tissues. The data of Turk (80), obtained with laying hens, are in good agreement with those obtained with the chick. There was little ^{65}Zn incorporated in the egg shell.

The rapid removal of zinc from the blood has been verified by Dennes *et al.* (81), who employed rabbits and made more detailed studies on

the binding of zinc to plasma proteins. They found, after injection of a ^{65}Zn glycine complex, that 90% of the intravenously injected zinc left the bloodstream within 3 hours. During the period of 3–24 hours after injection essentially all of the zinc in the plasma was bound, about half of it to α-globulin. They also found that leukocytes concentrated zinc 30–70-fold more than did erythrocytes, and that, whereas there was erythrocyte-plasma exchange of ^{65}Zn, the leukocytes retained their ^{65}Zn and did not exchange it back to plasma. They thus consider red cell zinc as a source of mobilizable or "available" zinc in the animal body. The exchange of ^{65}Zn between red cells and plasma has been verified by Herrick *et al.* (82) with rat red cells and plasma by means of *in vitro* incubation systems.

The occurrence of high concentrations of zinc in the male reproductive system has led to studies of ^{65}Zn movement in these organs. Gunn *et al.* (83) have demonstrated in the rat that the dorsolateral prostate concentrates a single dose of ^{65}Zn 15–25 times as much as other tissues, and that this accumulation is maximum at 1–2 days and rapidly reduces by 4 days to a fairly constant value. They have also reported (84) on the movement of ^{65}Zn in testes and epididymes, finding maximum concentration in the testes at 5 days, with a fall thereafter. As ^{65}Zn decreases in testes it accumulates in epididymes. They have reported that destruction of testicular tubules by cadmium results in a maximum ^{65}Zn uptake by testes only 30% of normal. They thus suggest that a portion of the zinc in testes moves out with spermatazoa and that another portion is associated with the nontubular elements and leaves the testes by plasma exchange. Other recent investigations of the distribution of ^{65}Zn in the prostate may be consulted for further details (85–88).

It has been shown (89) that hepatic injury by CCl_4 does not influence accumulation of ^{65}Zn by the liver or other organs, although it does decrease deposition of ^{54}Mn in the liver. It is suggested that this difference in behavior is the result of a difference in intracellular distribution. Manganese is normally concentrated in mitochondria and microsomes, which were damaged by CCl_4, whereas zinc is mainly in nuclei and cell walls, which were little affected by CCl_4.

Whole body counting of ^{65}Zn has been employed in studies of the overall turnover of zinc. Cotzias *et al.* (90) administered ^{65}Zn by injection to mice and followed the retention of the element for 10–12 days as influenced by the administration of zinc citrate or sodium citrate. They showed that the increased zinc load increased the rate of removal of the injected ^{65}Zn but sodium did not. It was further noted that the loss of zinc from the body was via the feces. The same technique has also been used (91, 92) to study the effects of a number of dietary variables on zinc absorption and turnover. These studies have shown that increasing the dietary zinc lowers ^{65}Zn absorption and increases turnover, as does increased dietary calcium. Purified diets allowed better ^{65}Zn absorption than did natural (phytate-containing) diets of similar calcium, phosphorus, and zinc content.

VI. Zinc–Hormone Interrelations

Previously reviewed investigations have indicated that gonadotropins and testosterone increase not only the size but the zinc content of and rate of ^{65}Zn uptake by the rat prostate (93, 94), and that androgen and estrogen variously affect zinc uptake by the prostate, depending on the presence or absence of functional testes (95). More recent investigations (96) have shown that gonadotropins and testosterone will stimulate growth in size of male accessory sex organs of zinc-deficient rats but will not prevent the lowered zinc content of these organs or the testicular atrophy that accompanies zinc deficiency. Thus, the atrophy is probably specifically due to the lack of sufficient zinc to maintain spermatogenesis and the survival of the germinal epithelium. The reduction in size of the accessory organs is probably a result of a depressed gonadotropin output. In a study differentiating the effects of the follicle-stimulating hormone (FSH) and interstitial-cell-stimulating hormone (ICSH) components of pituitary gonadotropin (97) it was found that ICSH would prevent the fall of ^{65}Zn uptake by testes in hypophysectomized rats; FSH was much less effective, and growth hormone and prolactin were ineffective. The same investigators (98) had previously suggested that ^{65}Zn uptake by the dorsolateral prostate of hypophysectomized rats might be used as a specific bioassay for ICSH.

Further evidence of sex hormone influence on zinc metabolism has been reported, with chickens used as subjects (99). Six-week-old chicks were either normal, testosterone treated, or given dienestrol diacetate, and retention of intravenous ^{65}Zn uptake at 24 hours was determined. Testosterone given to females decreased femur ^{65}Zn uptake, but this was not true of males. The untreated males had lower ^{65}Zn uptake than untreated females. Dienestrol diacetate had the opposite effect; it increased femur zinc uptake of males but not that of females.

Adrenalectomy also lowers zinc content and ^{65}Zn uptake of testes and the dorsolateral prostate of the rat. This effect can be reversed by cortisone given to adrenalectomized animals (100) and by adrenocorticotropic hormone (ACTH) given to hypophysectomized animals (101); these results point to a relationship between adrenal activity and zinc metabolism.

The relationship of zinc to pancreas function has been well reviewed

(1) and need not be detailed here except to point out that a control of zinc over endogenous insulin function or a control of insulin on zinc metabolism has yet to be found.

VII. The Zinc-Deficiency Syndrome

The physiological changes observed in zinc-deficient animals have been described many times since the initial experimental production of this condition in the rat by Todd *et al.* (102) who noted particularly a reduced growth rate and the appearance of alopecia. The initial histological studies of zinc deficiency were made on the rat by Follis *et al.* (103) who noted the hyperkeratinization of the skin and esophagus. Since these early investigations, zinc deficiency has been described further in rats (104–106), mice (107, 108), pigs (59, 109–112), dogs (113), chickens (63, 114, 115–119), turkeys (120, 121), pheasant (122), quail (28, 34), cattle (123–125), sheep (126), goats (127), and man (7).

So far as animals other than man are concerned, young of all species exhibit a sharp decrease in feed intake and weight gain when placed on zinc-deficient diets. This is noticeable within 1–2 weeks after initiating the low-zinc regimen. The development of parakeratotic skin lesions is also common to these animals, although there seem to be some species differences in relative severity and distribution of the lesions. In the pig in particular the lesions thicken and crack, and the fissures thus developed provide ready access for secondary infection. Hair follicles disappear in the affected areas so that alopecia develops. Much of the hair that does remain is coarse, oily, and matted, perhaps as a result of continued activity of the sebaceous glands which do not atrophy as do the hair follicles. In poultry the feather development is poor. Skeletal development is retarded, and particularly in poultry the leg bones are not only shorter but also are thicker than normal. Decreased hatchability of eggs and production of deformed embryos in poultry are also reported. In male animals there is retarded development of the sexual organs and a lack of spermatogenesis due to testicular atrophy involving loss of function of the germinal epithelium. Except for testicular atrophy, these symptoms are readily reversible by addition of zinc to the diet.

The time sequence of development of and recovery from zinc deficiency is well described by Beardsley (109). A group of 2-week-old pigs, placed on a zinc-deficient purified diet (10 ppm of zinc), exhibited, after 14 days, a decreased feed intake and rate of gain relative to pigs on the same diet with supplemental zinc (100 ppm). The first skin lesions appeared at 21 days in the form of reddish, rough patches on the scrotum. The spread of the dermatitis was rapid, and within 1 week nearly all

areas of the body were involved, with greatest severity occurring on the legs, thighs, and ventral surfaces of the body and head. The reddish areas soon became encrusted and cracked. After 6 weeks on the deficient diet some of the animals were given a zinc supplement. A marked increase in appetite was noticeable in 2 days, and weight gain was stimulated correspondingly. The skin lesions had almost completely healed in 2 weeks, and the heavy crusts had sloughed off and were replaced by new, pliable skin.

Zinc deficiency in man has not been produced experimentally but apparently occurs in conjunction with parasitic infestation or dietary abnormalities such as geophagia which may reduce zinc absorption. Dwarfism and hypogonadism occur in many of these cases together with low blood and urine zinc as well as plasma ^{65}Zn disappearance curves suggestive of zinc deficiency (8, 9). Hair zinc has also been shown to be low in these individuals and to increase when oral zinc sulfate was administered (128).

In spite of the extensive knowledge concerning zinc-enzyme relationships, the metabolic abnormalities leading to the production of the clinically observable deficiency symptoms are as yet unknown. As previously cited, total zinc content of most tissues is not materially affected by zinc deficiency. Also changes in enzyme activity have not been revealed consistently in zinc-deficient animals except perhaps in studies showing a decrease in serum alkaline phosphatases, but these results are complicated by the coexistent decrease in feed intake (see Section IV). In any event, a relationship between alkaline phosphatase and maintenance, for example, of normal epithelia has not been demonstrated, and further search for the specific metabolic lesions in zinc-deficient animals is indicated.

VIII. Zinc Requirements and Dietary Factors Influencing Utilization

The statement (129) that a zinc intake of 40 μg daily by young rats "entirely prohibited the production of a zinc deficiency" was the first expression of a quantitative zinc requirement. Liberally interpreted, the above figure implies a required zinc concentration in the diet of 4 ppm or less and was for many years the basis on which zinc was relegated by nutritionists to an unimportant role in practical animal nutrition.

In more recent investigations with the rat (108), pig (130), and chick (118, 131, 132) employing optimum conditions for zinc utilization and control of contamination from nonfeed zinc sources it is apparent that 8–10 ppm of absorbable zinc or 10–12 ppm of dietary zinc are

minimum values consistent with optimum weight gain of young animals and freedom from symptoms of zinc deficiency. The fact that such levels are not sufficient in many practical situations has been clearly demonstrated and well summarized (2) with the conclusion that, particularly in the presence of plant proteins, a level of 40–60 ppm of zinc constitutes a more realistic dietary allowance figure. The difference between these two sets of figures lies largely in the effects of chelating agents such as phytic acid on absorbability of dietary zinc, although it seems likely that other interfering materials may be present in specific feedstuffs such as sesame meal (133–135).

The absorption of zinc into the body occurs mainly through the walls of the small intestine, but few studies have been conducted on the mechanism or sites of absorption. Zinc has the reputation of being poorly absorbed from usual diets (2) but when fed at low levels in purified diets may be absorbed at a rate exceeding 80% of that fed (104, 136). Increasing the level of zinc (104) decreases the percent of absorption, although the absolute amount absorbed increases. There is no evidence of active absorption or of an intestinal block to absorption, although evidence has been cited for a homeostatic feedback mechanism regulating zinc absorption and excretion (137). The major pathway of zinc excretion from the body is via the feces; primarily, zinc enters the intestinal lumen via pancreatic secretions and very little by way of the bile (138). A recent study of the sites of absorption of zinc in cattle (139), employing ^{144}Ce and Cr_2O_3 as unabsorbed markers and determining the ratio of ^{65}Zn to marker, showed that 35% of the daily administered ^{65}Zn was absorbed in the abomasum. Secretion of zinc into the first sixth of the small intestine raised its concentration markedly, but in subsequent sections net absorption occurred although there was no absorption through the cecum, colon, or rectum. Net absorption of ^{65}Zn by baby calves, older calves, and mature cows was 55, 20, and 12%, respectively.

Under normal circumstances zinc excretion by the urine is a small fraction of the intake and is uninfluenced by the amount of zinc absorbed (104, 139). However, chelating agents administered other than by way of the gastrointestinal tract may markedly increase urinary zinc excretion (140, 141).

Since the original publication by Tucker and Salmon (3) implicated high calcium content of diets as a factor in production of zinc deficiency, many investigations have been conducted in order to verify the finding and to define the problem more clearly. The situation as it existed up to 1960 was reviewed (142) with the conclusion that the interference of calcium with zinc function was at the cellular level, rather than at the intestinal level. Subsequent work has contributed much toward clarifying the situation, and it now seems certain that the major effect of calcium is indeed to depress the absorption of dietary zinc through the intestinal wall (143), although in the presence of adequate zinc, calcium sometimes increases zinc utilization (144, 145). The breakthrough in this problem stems from the finding of O'Dell and Savage (146) that phytic acid, present in many plant protein sources, would reduce weight gain and produce zinc deficiency in chicks, and that this could be prevented by increasing the zinc content of the diet. It was hypothesized that a zinc-protein-phytic acid complex was formed which rendered zinc unavailable for absorption. The original finding of O'Dell has been confirmed for the chick (59, 147–149), the rat (27), and the pig (59).

Specific evidence that phytic acid interferes with zinc absorption has been provided by balance studies with the rat (27) and the chick (147). These studies with purified diets have also revealed that the effects of calcium and of phytic acid on zinc utilization are synergistic. In other words, in the absence of phytic acid calcium has only a slight, if any, effect on zinc utilization, and, conversely, at low levels of dietary calcium the phytic acid effect is minimal. Again, these facts have been demonstrated in terms of weight gain (59, 149, 150) and zinc absorption (27). Studies of solubility of phytate salts in aqueous solutions (150, 151) have shown that the mixed salts of calcium and zinc are much less soluble than either single salt, which appears to explain the above *in vivo* findings. These findings may also explain the reduction in parakeratosis in swine fed autoclaved soybean meal as compared to unautoclaved meal (152, 153) since phytic acid may be destroyed by autoclaving. It has also been shown that dietary protein is not a requisite for phytic acid interference with zinc utilization (149). In this study phytic acid interfered with zinc utilization by chicks, as measured by weight gain and concentration of zinc in bone ash, whether the diet contained casein or free amino acids as the sole nitrogen source.

Another important facet to our understanding of zinc utilization was initially revealed by the publication of Kratzer *et al.* (152) who showed that zinc utilization was improved by the addition of 200 ppm ethylenediaminetetraacetic acid (EDTA) to soybean protein diets fed to turkey poults. The beneficial effects of EDTA have been verified in experiments with chicks (133, 150, 154–156), pigs (130, 157, 158), and rats (26) in which it has also been demonstrated that the decreased utilization of zinc accompanying addition of phytic acid to diets can be overcome by addition of EDTA. In an extensive screening process, Vohra and Kratzer (159) investigated the growth-promoting ability of twenty-three chelating compounds added to zinc-deficient soybean protein diets for turkey poults. Stability constants of the chelators for zinc ranged from

5.3 to 18.8. It was found that effective chelators possessed stability constants for zinc between 13 and 17.

Although the mechanism is not clear, it appears that the chelating ability of EDTA in some way either destroys or prevents the formation of calcium-zinc-phytate complexes, yet permits the zinc to be readily absorbed. Evidence for the absorption of the EDTA portion of the chelate indicates that orally administered EDTA is only slightly absorbed (160, 161) and that ^{65}Zn absorbed from EDTA-^{14}C-^{65}Zn is a greater percent of the dose than is the ^{14}C (162).

In a series of experiments with weanling pigs (163) it was reported that replacement of corn by glucose in a corn-soybean meal diet apparently increased the zinc requirement, and there seemed to be differences in the availability of the zinc in corn from different sources. Although it may have no bearing on this problem, there is preliminary evidence (164, 165) that, particularly in the presence of phytic acid, zinc utilization by rats is better when starch rather than glucose is the dietary carbohydrate. The mechanisms of these effects remain obscure.

As the evidence above indicates, studies of zinc utilization must take into account a number of factors other than the zinc content of the diet. In addition to interactions between zinc and organic dietary constituents, the levels of inorganic compounds must also be considered. This viewpoint has been emphasized by Underwood (2) and by Hoekstra (166) not only with respect to zinc but to mineral utilization in general. In addition to the calcium-zinc relationship already discussed, interactions of zinc with copper, molybdenum, iron, cadmium, and phosphorus have been investigated.

The partial alleviation of zinc deficiency of swine by increasing the dietary level of phosphorus (112, 167) might be a result of a more favorable Ca:P ratio, but in a balance study with rats fed either zinc-deficient or adequate diets, zinc absorption was not influenced by addition of phosphorus to change the calcium-phosphorous ratio from 1:1 to 1:2 or from 2:1 to 1:1 (136).

An unresolved controversy involves the effects of copper in preventing or alleviating zinc deficiency in swine. A positive effect has been reported in a series of three experiments (58, 168, 169) from one laboratory, but lack of this effect has also been reported (166, 170, 171) from two laboratories, and copper has been shown to reduce the growth rate of zinc-deficient chicks (172). A combined copper-zinc deficiency reported in cattle was not relieved by copper alone (173).

Cadmium appears to exert a definite antagonistic effect on zinc. Although cadmium loading of tissues does not affect zinc absorption (137), it does depress excretion of zinc via the intestine (174). Cadmium injury

to rat testes can be prevented by simultaneous administration of zinc (175), and more recent studies have shown (176) that cadmium depresses ^{65}Zn uptake by testes and the dorsolateral prostate (a function under ICSH or androgen control) but not that of the ventral prostate (whose ^{65}Zn uptake is not under ICSH control). Cadmium-induced testicular injury is the result of injury to the vascular supply (177), and both the seminiferous tubules and interstitial tissue undergo extensive necrosis. Eventually the latter regenerates and interstitial-cell tumors develop (178). The vascular injury, necrosis, and tumor development may be prevented by zinc (179). The above studies have all been conducted on rats and mice. The same general type of injury together with skin lesions reminiscent of zinc deficiency have been produced by cadmium administered to dairy calves (180) and to turkey poults (181) and chicks (182). In the latter cases evidence is clear that increasing the zinc intake is protective against the cadmium toxicity. Further aspects of zinc interactions with other elements will be considered under the heading of zinc toxicity.

IX. Clinical Aspects

Studies of zinc metabolism in clinical disease states have revealed certain aberrations of as yet unknown significance. It seems clear, for example, that chronic liver disease results in decreased zinc levels in the liver (1, 183), serum, and leukocytes (184, 185) as well as in an increased urinary zinc excretion (1, 186). In human subjects a single dose of ethanol did not affect urinary zinc (187), but ethanol administered daily for 7 days in the rat significantly lowered liver zinc (188). These findings, coupled with the previously reported tendency for zinc administration to restore normal excretory patterns and liver function in postalcoholic cirrhosis, have been interpreted by Vallee to suggest conditioned zinc deficiency as a feature of this disease (1).

It was earlier pointed out (1) that the knowledge concerning relationships between zinc, insulin, and diabetes is in a state of confusion. The situation has changed little, and a more recent study involving sixty diabetic subjects failed to show a relationship between plasma zinc and insulin requirements (189).

As in the case of zinc and diabetes, relationships have been sought between zinc and cancer. Again, it has been pointed out (1) that the data are sketchy and contradictory. The earlier finding has been confirmed that leukocyte zinc is markedly lower in chronic leukemia patients and that an increase accompanies response to therapy (185). Analyses of zinc content of the hair of carcinoma patients (190) have shown no change from normal.

Recent interest in the use of hair zinc levels as a tool for studying zinc metabolism and status of humans stems from earlier observations that hair zinc content varies widely and, indeed, shares with bone the characteristic of being influenced markedly by dietary zinc supply (128). Two groups of investigators have reported that hair zinc levels in patients with atherosclerosis are about 50% of "normal" values (128, 191). Induction of abnormalities in zinc metabolism by alcohol (128) or by cadmium (180) does not, however, appear to affect hair zinc levels. Provocative evidence of a lowering of hair zinc in burned patients and of concentration of zinc in healing wounds, together with animal studies showing that a zinc-methionine complex speeds healing of wounds (192), points to a role of zinc in epidermal repair which should be studied in detail. It is interesting to speculate on the effect of zinc in the prevention of cadmium-induced injury to the vascular supply of selected tissues (177), and the observation that zinc accumulation in healing wounds coincides with the development of vascularity of the healing area (193).

From the immediately preceding paragraphs and the important observations of Prasad and co-workers relative to clinical evidence of zinc deficiency in humans (7–9) it appears that the role of zinc in human metabolism is a fascinating one and one likely to be of practical significance in a variety of clinical situations.

X. Toxicity

Effects of excess zinc ingestion have been reviewed and discussed (1, 2) from the viewpoints of industrial toxicology, public health, and animal experimentation. In spite of widespread warnings about the use of galvanized containers in food handling, instances of zinc poisoning in humans continue to occur (194). There appear to be species differences in zinc tolerance. Rats can tolerate at least 2500 ppm of zinc and swine up to 4000 ppm (195) without exhibiting symptoms of toxicity. Poultry seem to be more sensitive, with 1500 ppm of zinc in the feed adversely affecting growth rate (196, 197). Calves (198) and sheep (199) exhibit reduced gain when fed 1000–1500 ppm of zinc.

The laboratory rat has continued to be the animal of choice (convenience) in studies of mineral interrelationships involved in zinc toxicity The previously reported studies showing anemia to be a consequence of zinc toxicity which can be avoided or cured by copper supplements (200) have been extended by studies including tissue analyses for copper and iron. For example, zinc toxicosis was shown (201, 202) to result in an increase in liver zinc and a sharp decrease in liver iron. In the latter study it was shown that iron but not copper supplements would increase liver iron of zinc-toxic rats but that both elements were needed

to provide for complete cure of the anemia. It was subsequently reported (203) that the iron loss in the liver was in the form of both ferritin and hemosiderin, the former loss being the greater. Interestingly, in spite of a reduction of liver xanthine oxidase activity, liver molybdenum levels were not affected. The original finding that molybdenum toxicity is exaggerated by toxic levels of zinc (204) has been confirmed (205, 206), and a complex interrelationship between copper, zinc, and molybdenum has been revealed (206).

A further aspect of zinc toxicity, that is, the detrimental effect which normally innocuous concentrations of zinc may have on animals fed diets deficient in copper, has been revealed by Hill and co-workers (207, 208). These investigators have further developed the thesis that mineral biological relationships might be explainable on the basis of similarity of chemical parameters of the ions. They have demonstrated, in the chick, relationships between zinc, copper, cadmium, iron, mercury, and silver in agreement with this theory.

The source of protein has been reported to influence the production of zinc toxicity (209), soybean meal being distinctly protective in comparison to casein, and evidence has been presented (210, 211) that factors other than iron and copper are responsible for the protective effect of a variety of natural materials such as a methanol extract of liver, other liver fractions, distillers' dried solubles and soybean meal.

An effect of zinc toxicity on the metabolism of calcium, phosphorus, and magnesium has been detected (212). The absorption of all of these elements was reduced markedly by inclusion of 0.75% zinc in rat diets. Although the percent calcium and phosphorus in dried femurs was lowered, that of magnesium was not. The effects on bone composition and the toxic symptom of decreased weight gain were alleviated by supplements of calcium and phosphorus. At the same time, bone zinc accumulation was reduced, presumably by interference with zinc absorption by the excess levels of calcium and phosphorus.

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Toxicity of Some Trace Metals.

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(Received July 6, 1962)

In recent years, research on the significance of trace metals, their necessity in the nutrition of man, animals and plants, their role in metabolism and their part in many enzymatic reactions has greatly increased. For such research it is often important to administer the highest possible dosage of trace metals to the experimental animals, and for this the maximum tolerance level and the toxic level must be known. There are only isolated reports in the literature on the exact toxicities of metal salts, generally without specification of statistical safety levels. The best compilation of toxicities published up to 1955 is in Handbook of Toxicology (SPECTOR 1956). But even this work contains only few specifications on the fatal dosages of individual metal salts in the same experimental animal with the same means of administration.

We determined the lethal doses LD_1, LD_{50}, LD_{99} of 8 different trace metal salts -- all with the same anion as chloride (except sodiummolybdate) -- in white mice by intraperitoneal injection. In addition, we examined the toxicities of trace metal mixture and to what degree several injections over a few weeks can be tolerated.

Methodology

For each metal salt, 6-8 doses, increased in logarithmic intervals, were tested on 8-16 white mice (inbreeding strain "Agnus Bluhm") each. Evaluation of the experiments and the calculation of LD_1, LD_{50} and LD_{99} amounts in mg/kg and their 19/20 probability limits (alpha = 0.05) were based on LITCHFIELD and WILCOXON. The metal salts were dissolved in bidistilled water and the concentrations were chosen so that 1 ml/20g body weight was injected intraperitoneally. The concentrations ranged from 6×10^{-2} to 1×10^{-3} moles.

Results

The order of toxicity of the metal ions was found to be as follows: Mo(VI) < Cr(III) < Mn(II) < Fe(II) < Co(II) < Zn(II) < Ni(II) < Cu(II).

See Table 1. Toxicities of 8 trace metal salts for intraperitoneal injection in white mice in mg/kg with 19/20 probability limit.
(Wahrscheinlichkeitsgrenzen = probability limits).

(only one salt from each metal was examined. The amounts were calculated for the water-free salts and the metal ions.)

This order corresponds exactly to the Irving-Williams Rule, which states that the stability of complexes of bivalent ions of the first period of transition metals increases up to copper and decreases again at zinc, independent of the respective ligands. The order of stability for the complexes, eg. glycine and ethylenediamine, is accordingly: Mn(II) < Fe(II) < Co(II) < Zn(II) < Ni(II) < Cu(II). Therefore, the toxicity of these metal ions probably depends largely on the complex formation in the organism. A similar correspondence between toxicity and complex stability of metal ions has previously been found by other authors (SHAW and SHAW, and GRUSHKIN) in studies on water organisms.

See Table 2. Toxicities of sodium molybdate for subcutaneous injections in white mice in mg/kg with 19/20 probability limits.

Besides the toxicities for intraperitoneal injection listed in Table 1, we also determined the lethal dosages of sodiummolybdate by subcutaneous administration. Sodiummolybdate cannot be mixed with other salts, since the molybdate precipitates.

In order to administer all 8 metal salts to the experimental animals, we made a mixture of the 7 chlorides, which was then administered intraperitoneally, and injected sodiummolybdate separately, subcutaneously.

A single dose of all 8 salts in the respective LD_1 dosage proved fatal within a short period of time. Several injections at 2-3 day intervals were only tolerable when the mixture was diluted 1:4, i.e., when a dosage of 1/5 LD_1 from all 8 metals was administered.

Intraperitoneal administrations of single metal salts over several weeks in 2-3 day intervals were well tolerated in injections of the respective half-dosage of the lower LD_1 - probability limit and can be used in experiments in which the animals are supposed to remain under a high level of the respective metal.

Summary

The lethal dosages (LD_1, LD_{50}, LD_{99}) of 8 trace metal salts and their statistical safety limits for intraperitoneal injection in white mice are reported. In addition, tolerable amounts of individual metal salts and of mixtures of 8 salts in several administrations over a specific period of time are specified.

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Tabelle 1.

	$FeCl_3 \cdot 4H_2O$		$FeCl_2$		Fe^{II}	
Dosis	mg/kg	Wahrscheinlichkeitsgrenzen	mg/kg	Wahrscheinlichkeitsgrenzen	mg/kg	Wahrscheinlichkeitsgrenzen
LD_1	52,0	43,0 — 62,9	33,2	27,2 — 40,1	14,6	12,1 — 17,7
LD_{50}	92,5	82,0 — 104,2	59,0	52,3 — 66,4	26,0	23,0 — 29,3
LD_{99}	160,0	132,3 — 193,8	102,0	84,5 — 123,5	44,9	37,2 — 54,4
	$CuCl_2 \cdot 2H_2O$		$CuCl_2$		Cu^{II}	
LD_1	3,7	2,4 — 5,0	2,9	2,2 — 3,9	1,4	1,0 — 1,9
LD_{50}	9,4	8,0 — 11,1	7,4	6,3 — 8,8	3,5	3,0 — 4,1
LD_{99}	24,5	18,0 — 33,3	19,3	14,2 — 26,2	9,1	6,7 — 12,4
			$ZnCl_2$		Zn^{II}	
LD_1			20,0	15,9 — 25,2	9,6	7,6 — 12,1
LD_{50}			31,0	25,8 — 37,2	14,9	12,4 — 17,8
LD_{99}			48,0	38,2 — 60,5	23,0	18,3 — 29,0
	$CoCl_2 \cdot 6H_2O$		$CoCl_2$		Co^{II}	
LD_1	58,0	48,4 — 69,5	31,7	27,9 — 37,9	14,4	12,0 — 17,2
LD_{50}	90,0	82,5 — 98,1	49,1	45,0 — 53,5	22,3	20,4 — 24,3
LD_{99}	141,0	117,5 — 169,1	76,9	64,1 — 92,3	34,9	29,1 — 41,9
	$NiCl_2 \cdot 6H_2O$		$NiCl_2$		Ni^{II}	
LD_1	25,0	16,2 — 38,5	13,6	8,8 — 21,0	6,2	4,0 — 9,5
LD_{50}	48,0	36,0 — 62,0	26,2	19,6 — 33,8	11,9	8,9 — 15,3
LD_{99}	91,0	59,1 — 140,1	49,6	32,2 — 76,4	22,5	14,6 — 34,6
	$MnCl_2 \cdot 4H_2O$		$MnCl_2$		Mn^{II}	
LD_1	125,0	104,2 — 150,0	79,4	66,2 — 95,2	34,8	29,0 — 41,8
LD_{50}	190,0	166,8 — 216,2	120,6	105,9 — 137,3	52,9	46,5 — 60,2
LD_{99}	290,0	241,6 — 348,0	184,1	153,4 — 221,0	80,8	67,3 — 96,9
			Na_2MoO_4		Mo^{VI}	
LD_1			211,0	169,0 — 264,0	98,3	78,8 — 123,0
LD_{50}			344,0	308,0 — 385,0	160,3	143,5 — 179,4
LD_{99}			560,0	448,0 — 700,0	260,9	209,0 — 326,0
	$CrCl_3(H_2O)_6 \cdot Cl \cdot 2H_2O$		$CrCl_3$		Cr^{III}	
LD_1	340,0	276,0 — 419,0	202,1	164,0 — 249,0	66,4	53,9 — 81,8
LD_{50}	520,0	468,0 — 577,0	309,0	278,1 — 342,9	101,5	91,4 — 112,6
LD_{99}	782,0	636,0 — 961,0	464,7	378,0 — 571,1	152,7	124,2 — 187,6

Tabelle 2.

	Na_2MoO_4		Mo^{VI}	
Dosis	mg/kg	Wahrscheinlichkeitsgrenze	mg/kg	Wahrscheinlichkeitsgrenze
LD_1	278	192 — 403	130	89 — 188
LD_{50}	570	489 — 664	266	228 — 310
LD_{99}	1170	806 — 1695	546	376 — 790

Virchows Arch. path. Anat. 332, 481–493 (1959)

CONCERNING CHRONIC ZINC POISONING IN RATS

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received 30 July 1959

Zinc is the most important trace element in the organism after iron. B. L. Vallee performed extensive work concerning the entire zinc problem overview in his paper, The Metabolic Role of Zinc.

Zinc already in 1896 was recognized as being indispensible for the growth of fungi (Raulin). In 1940, it was determined being an ingredient of the carbo-anhydrase in cattle-erythrocytes (Keilin and Mann). Scott already 60 years before had stressed the significance of zinc in the crystallization of insulin. Glucagon, other island hormone, even loses its effectiveness if zinc is drawn from it (Weitzel). Zinc also playing a role in other metabolic processes, for instance as an ingredient of uricase, it is no cause for surprise, that several generations of animals fed on a zinc-free diet ultimately died from fermentation and hormonal dysfunctions. (Day and McCollun, Hove et al, Day and Seidmore).

The progress of zinc in the body also is well-known. Zinc administered perorally will be absorbed in intestine. Feaster et al indicate the proportion of re-absorbed metal as being 5%, as being determined from radio isotope tests. Hahn and Schunk on the basis of acute zinc poisoning examinations, obtained a value of 3.3%. Following absorption in the intestine, zinc reaches various organs by means of the blood. It will be present in loosely bound transport-form in the serum in the albumen and beta-globulin fraction (Wolff[1]). Examinations by Sheline et al show, that it will leave the blood very quickly: following intravenous administration of zinc^{65}, almost the entire zinc had vanished from the blood plasma after 10 hours. Sheline et al found that after intravenous administration of zinc Zn^{65}, the strongest isotope concentration was in the liver, pancreas, and kidneys. Other researchers also indicate

findings in the prostate (Gunn and Gould). According to Sheline et al, 50 % of the administered zinc will be eliminated in the stomach-intestinal canal in mice and dogs and only 2% in the urine. Feaster et al found values in the urine of only 4.2% in rats. Montgomery et al provided accurate information concerning the origin of the metal in the feces. They examined in isolation pancreatic juice, bile and duodenal juice. They determined, that after 14 days, approximately 11% of the administered zinc was eliminated in the pancreatic juice. Only very little (0.4% in 8 days) had been eliminated in the bile. Elimination ofthe radic isotopes by means of an isolated duodenal sling proved somewhat more ample. Birnstingel et al confirmed essentially such findings and extended them to this extent that already 15 minutes following injection, measurable radioactivity occurred in the pancreatic juice and in the bile. The highest values were measured about 2-3 hours following injection. On the basis of these examinations, the values listed by Sheline et al for the liver, pancreas and kidney are comprehensible, however they nearly cover the three organs which will eliminate the metal in the shortest time.

We raise the question, whether a chronic overloading of the organism with zinc will cause special pathological changes, such as are witnessed for chronic lead poisoning (Zollinger). We hoped furthermore that perhaps such overloading might provide insight into zinc metabolism, which for normal zinc loading and for acute zinc poisoning are less pronounced. Finally there was also the question whether the body is capable storing zinc under certain circumstances.

METHODS

Rats from 2 stocks, B D III a tumor-sensitive stock and another stock from the institute itself immune to tumors were used in the experiment. The animals from both stocks showed no differences in the course of the experiment as regards

their inner and external behavior, so that the findings of both stocks may be discussed in common. Twenty-five fully grown rats, 6 months old at the beginning of the test, received over a varying period of time (235-542 days) zinc acetate in peroral manner, the total amount for each animal lying between 47, 250 and 132, 825mg. In order to control precisely the amounts ingested, we placed the animals into individual cages and administered the easily soluble zinc acetate in the drinking water (from the faucet). We began with doses of 75mg per animal per day and increased those doses, taking precise weight control into account, to 600mg/animal/day. The doses were determined by the behavior of animal to the extent that we tried to determine the maximum tolerable doses over a long period of time by changing the amount of zinc acetate. Because the animals became increasingly cachetic for doses as high as 600mg (sometimes a loss of weight of 50-100gm occurred within 8 days) and because one animal evidently died of stomach perforation caused by direct zinc acetate effect, we reduced the doses to 400mg and later to 300 mg. Figure 1 shows the weight curve and the administered amount zinc for one animal, and this process is clearly visible. It was found that a dose of 300mg animal day is quite tolerable over a long period of time. Ten male rats 6 months old at the beginning of the test received the same nutrition as the control. These animals were weighed 7-10 days apart and recording were made at the same time of their general condition, of their pelt and of any specific suspicious particularities and symptoms.

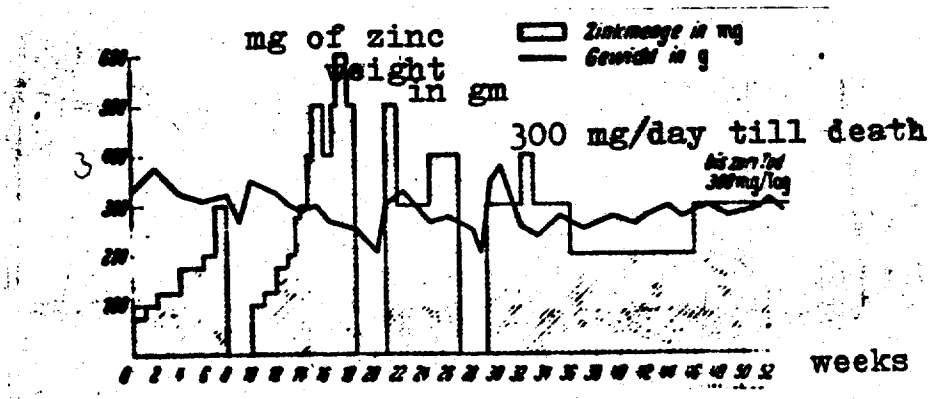

Figure 1. Influence of amount of zinc on the weight of a test animal.

Once the animals had gotten used to the bad taste of the water containing the zinc acetate, which took no more than 14 days, they showed at first no suspicious peculiarities despite increasing the doses from 75-350mg. Their pelts became rough after 8-10 weeks. In half the animals, there was a tremor ("zinc tremor", Hahn) after 18 weeks that extended to the entire body and was particularly noticeable, when the animals were lifted by their tails. This trembling vanished in the course of the 4 consecutive weeks. Even though the animal externally looked uncared-for and shaggy, they seemed to be particularly infection-resistant. A cage infection (broncho-pneumonic infection, that took place during the test period and which claimed the lives of many control animals from the same stable, and the test animals from other stables, did affect only one zinc subject animal. Also among the killed animals, the proportion of pneumonic or bronchectasis changes were relatively fewer than for the control animals, (6 of 25 as compared to 6 of 10). Despite the rough pelt of the subject animals, acariosis was more pronounced among the control animals than among the subject animals. Aside from one testicular tumor, no other suspicious microscopic changes in organisms were found.

Following the killing of the animals by means of a neck blow or by means of ether, the organs were examined histologically. Each time one or two parts of the pertinent organ were fixed in 4% neutral formalin and in 70% alcohol saturated with hydrogen sulphide, for zinc testing. At a time 2-3 sections were developed by the process indicated by Timm and further developed by Voigt, making use of silver sulphide, and as a control, a reaction also due to Timm and using diphenyl-thiocarbazon-(dithizone-). When fixing with hydrogen sulphide saturated alcohol without acid or alkali addition, and for the control according to the dithizone method, one may assume with certainty according to Stegner and Fischer, that zinc was selectively obtained from the heavy metals in question.

THE SILVER SULPHIDE METHOD.

Small pieces of tissue, as fresh as possible, will be fixed for 10-20 hours in 70% alcohol saturated with hydrogen sulphide, then they will be dehydrated for 10 hours in 96% and 100% alcohol and will be embedded in paraffin previously not used via xylol (twice for half an hour).

The sections 4-6 microns thick will be stretched on evaporated bidistilled water. Then they will be deparaffined in xylol. Whereafter one proceeds along a decreasing alcohol series down to bidistilled water and the sections that are on the cover glasses are placed into a flat dish and the solution indicated below will be poured over them and the sections will be developed under precise microscopic control and under artificial light for 1.5-2hours.

Following termination of development, the sections will be rinsed in running water and will be dyed with hematoxyline or with genuine red. Thereafter one rinses with distilled water. One proceeds then in ascending alcohol series. This is followed by xylol treatment "Eukitt".

SOLUTION.

60cc of 40% gum arabic[1] are shaken with 0.5cc of 10% silver nitrate solution for 10 minutes[2]. Then one adds 5cc of a reducing means[3] and shakes for exactly 30 seconds. Thereafter the solution

is immediately poured over the sections.

Important: in order to avoid precipitation, one must always make use of quartz glass and distilled water during the entire dying process, as well as when thinning the alcohol, when using the gum arabic solution, and when using silver nitrate and hydro-quinone-citric acid.

RESULTS.

The upper locations of the epithelium of the esophagus are penetrated with silver grains in the subject animals, and this indicates zinc impregnation of these layers. Also, the mucous covering the surface of the stomach mucous membrane is rich in zinc. The characteristic silver precipitates will still be seen beyond the dimple into the neck parts of the glands and occasionally are clearly arranged along the boundaries between the cylindrical cells (Figure2). Such changes are entirely lacking in the control animals.

When the silver sulfide method is applied, entero-chromaffin cells occur in the small and large intestines and are clearly seen because of the blackening of their grains, and in the same manner in subject as in control animals. There was a quantative comparison with sections following formaline fixing, and there was a conventional treatment according to the silver methods (Masson-Hamperl, Bodian); thus these were probably silver-affinity cells (Figure 3). The Paneth cells are shown very clearly both in subject and in control animals, and evidently they're very rich in zinc (Figure 4).

Silver grains are found in the mucous membrane stroma of the entire stomach-intestine track, but more are found among the subject animals, their occurrence being fairly scarce among the control animals (Figure 5).

This regards the excretory pancreas, 14 of the 25 test animals show either partly throughout the entire organ or partly in spots the secreting grains being strongly impregnated in the end pieces, whereas they were lacking in 11 test animals and in the control animals (Figure 6A and B). As concerns the islands, the subject and the control animals showed strongly silvered A-cells at the edges of the island, the strength of their reaction only varying slightly. The characteristic, grainy, black precipi-

tate could never be found in the B-cells, not even among the subject animals, however, there was a yellow-brownish tinting of the B-cells among several animals, which can be traced to the most minute silver precipitate (Figure 6A and B).

No silver deposit could be found that would have been traced back to zinc content among the liver of the control animals. But in 18 of 25 subject animals, and above at the center of th lobe, there were black granula in the cytoplasm of the liver cells and along thebile capillaries (Figure 7), Grains in the stellate cells could found only in very isolated cases.

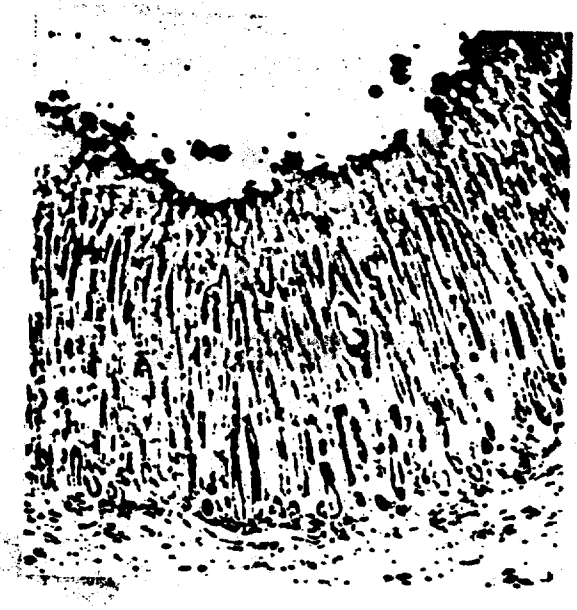

Fig 2

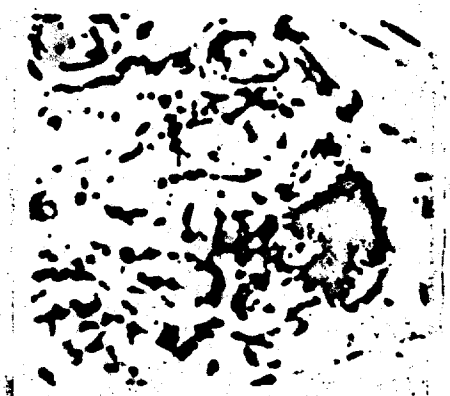

Fig 3

Figure 2. Rat (subject animal). Stomach. Silver sulphide process: core-genuine red. Impregnation of the upper layers with zinc. Amplification: 66.5x.

Figure 3. Rat (subject animal). Large intestine. Silver sulphide process: core-genuine red. Entero-chromaffin cell containing zinc. Amplification: 2100x.

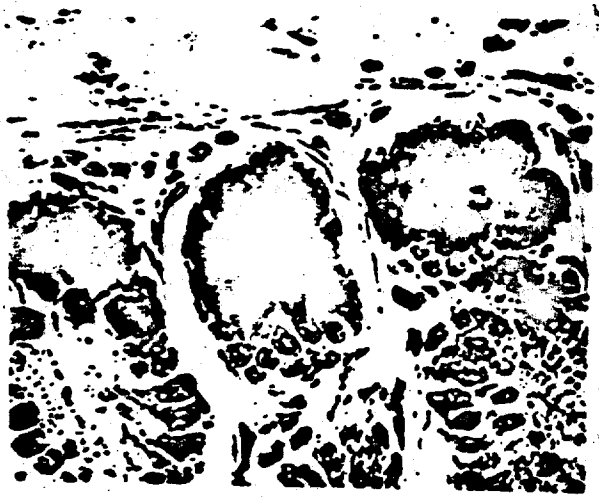

Fig 4

Fig 5

Figure 4. Rat (subject animal). Thin intestine. Silver Sulphide process: core-genuine red. Paneth cells containing zinc. Amplification: 665x.

Figure 5. Rat (subject animal). Thin intestine. Silver sulphide process: core-genuine red. a: zinc-laden fibrocytes in the villus-stroma. Amplification: 940x.

In the glandula submaxillaris, there was a fine blackish granulation in the cytoplasm of the exit of the epithelia only in 2 subject animals.

The cells of the dorso-lateral lobe of the prostate showed strong impregnation as well among control animals as subject animals, and also secretion in the clearing, whereas such was lacking in all other lobes of the organ.

Impregnated cylinders in the tubuli could be shown in the kidneys of almost all the animals (Figure 8). Furthermore, most finely impregnated grains were found among 12 subject animals and 8 control animals in the tubuli contorti of the first order (Figure 9) that always belonged to a nephrone. There was clear silver impregnation of the atrophied little canals that were evidently due to pyelo-nephritic inflammation.

Silver grains in the Sortoli support cells were found in individual testicular canals for 9 of 25 subject animals, and all support cells contained grains in all such little canals, whereas those cells of a neighboring little canal could very well be negative.

The adrenal marrow showed a yellow-brown tint as well in subject animals as in control animals, which is due to a most finely distributed silver grains.

There is a positive reaction whether for subject or for control animal in the lungs only for desquamated alveolar epithelia.

The epidermis will behave negatively for silver impregnation. A brownish toning in the corium could be determined for subject and control animals and is due to very fine silver grains.

In the brain, part of the Ammons-Horn is very beautiful, whereas all other parts proved negative.

As regards the spleen, the silver containing reticulum cells are

clearly more frequent among the subject animals than among the controls.

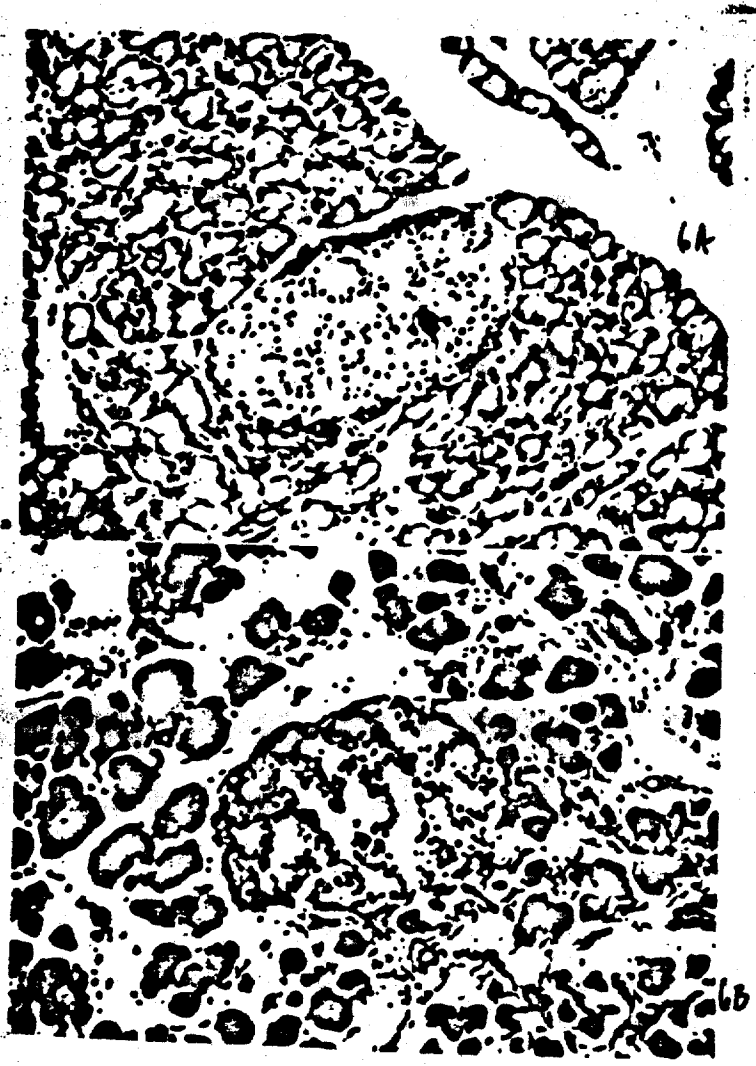

Fig 7

Figure 6 A, 6 B. Rat. Pancreas. Silver sulphite process: Core genuine red. a: subject animal. Zinc deposits in the A-cells of the islands. Excretory parenchyma is free of zinc deposits. B: subject animal. islands as in a. Appreciable zinc content of the excretory parenchyma. Magnification: 175x.

Figure 7. Rat. (Subject animal). Liver. Silver sulphite process: core genuine red. Clearly recognizable zinc deposits, preferably near the bile capillaries. Magnification: 1020x.

Figure 8. Rat. (Subject animal). Kidney. Silver sulphite process: core genuine red. a: zinc containing cylinder in the tubuli clearings. Magnification: 665x.

Ratte (Kontrolltier). Niere. Silbersulfidverfahren-Kernechtrot. a Zinkablagerungen in Tubuluspithelien, die zu einem Nephron gehören. Vergr. 700 ×.

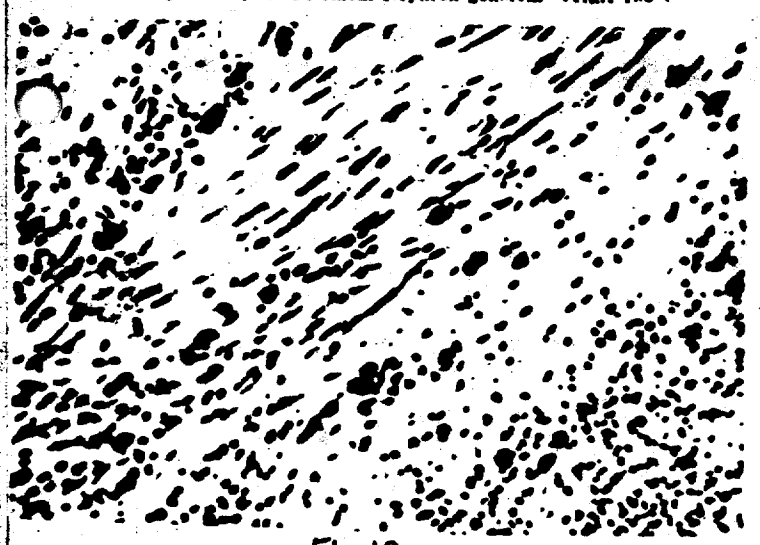

Fig 10

Fig 11

Figure 9. Rat. (control animal). Kidneys. Silver sulphite process: core genuine red. a: zinc deposits in the tubulus epithelia, belonging to a nephron. Magnification: 700x.

Figure 10. Rat. (Subject animal). Trachea. Silver sulphite process: core genuine red. Black mast cells. Amplification: 242x.

Figure 11 A and 11 B. Rat. Trachea. Silver sulphite process: core genuine red. a: subject animal. Calcified cartilage fundamental substance is amply loaded with zinc. Magnification: 35x. b: control animal. Calcified cartilage fundamental substance is free is zinc deposits. Magnification: 35x.

The histiocytes of the loose connective tissue and of the stroma of the individual organs are characterized among the subject animals by a greater amount of silver grains than is the case for control animals.

One will notice an appreciable blackening of the mast cells of the subject animals, whereas such are lacking among the control animals (Figure 10). The calcified cartilage fundamental substance and also the calcium-containing arterial walls are much blackened among the subject animals, whereas there is no silver reaction among the controls (Figure 11A and 11B).

DISCUSSION.

We failed just as did Drinker et al, to find noticeable organic changes that could be laid to chronic zinc poisoning, even though we fed zinc acetate in amounts 7-10 times higher. Obviously the zinc was eliminated so quickly that no toxic symptoms occurred.

Therefore we tried to determine the storage or the enrichment of the metal in given organs by means of histo-chemical methods.

According to the thorough testing of the specificity of the silver sulphite method at the hands of Stegner and Fischer, one may well assume, that for certain assumptions, it will indicate the presence of zinc with great reliability. Therefore we do believe we met all these re-

quirements (see above) and thus feel justified to attribute the previously described silver precipitations to the presence of zinc capable of reactions. Obviously one must take into account that even this histo-chemical reaction suffers from the drawback of not recording all of the actually present zinc, but only those parts that can be easily separated and are present in certain amounts.

The ingestion of the zinc by means of drinking the water probably accounts for the deposition in the uppermost locations of the esophagus epithelium and stomach mucous membrane, the penetration in the clearing of the stomach dimple and of the gland necks as well as further spreading along the cell boundaries being remarkable. There was no damage to the cells effected.

From the digestive tract, the absorbed zinc probably reached circulation and apparently was deposited at definite structures, which are particulary clearly shown in the overloading that was carried out in the test and as contrasted with the controls, and this is so first of all in the cells of the reticulo-histiocyte system. Already in the entire stomach-intestine mucous membrane, the reticulum cells of the mucous membrane stroma are particularly rich in zinc, but also, the histiocytes, which are present everywhere in the organic stroma, just as are the Kupffer stellate cells in the subject animals, are characterized by their higher zinc content. Surprisingly this is also the case for the mast cells.

Kidneys. Liver and pancreas are involved as elimination organs for zinc. For subject as well as control animals, there are equal amounts of zinc deposits in the kidneys in individual nephrons, so that

there is no indication in this case of increased elimination. The findings of McCaine and Widdowson, also those of Sheline et al, namely that the extent of the zinc deposits in the tubuli contorti will not increase in the subject animals, has been confirmed, since these authors could not ascertain an appreciable increase of the zinc in the urine following peroral or iv. zinc ingestion. However there was a difference regarding the controls as concerns the liver. Zinc containing grains could be shown in the subject animals in their gall path and on the side facing the liver cells, and this indicates an elimination function.

Zinc occurs in increased measure in the subject animals above all in the excretory pancreatic parenchyama, beside the liver. Even though the subject animals also may show completely zinc-free areas or even completely zinc-free pancreas, there are nevertheless 14 of the 25 subject animals that show unambiguous zinc deposits in the excretory pancreas paranchyma. Obviously what matters in this different issue as regards zinc reaction, is the phase of the elimination that one has encountered. The assumption presupposes that here too the explanation for the different amounts of zinc in the pancreas is similar to the investigations of Scott and Fisher and of Eisenbrandt and Sienz. Both these researchers found that the zinc content of the entire pancreas will fluctuate within slight limits for the diabetics and non-diabetic sufferers. They encountered the fact that often there was more zinc in all of the pancreas than could have been expected from the zinc content of insulin, and this is probably due to the deposition of zinc in the excretory pancreas. The finding of much zinc in the excretory pancreas parenchyama when overloading the animals with zinc probably agrees with the findings of Montgomery et al, also those

of Birnstingel et al, according to whom the amount of the ingested zinc will be eliminated in the pancreas whereas kidneys and liver only effect elimination in smaller proportions. In fact one does already find normal zinc deposits in the liver, but obviously the elimination capacity of the organ is restricted, whereas the pancreas may be enlarged in the manner of an emergency valve in case of need.

For subject as well as for control animals, a series of organs contained equal amounts of ample zinc, and this obviously in an indication that the zinc in this case was only absorbed according to the function of the organ and was so deposited, where the function evidently does not depend on the zinc supply. It is not in the B-cells of the Langerhans islands of the rats, as for other animals, that one finds ample zinc, but in the A-cells. Furthermore, Stegner and Fischer could already determine appreciable zinc amounts in the boundary cells of the rats' islands. These findings are in agreement with the fact that it was not feasible previously in rats to induce a dithzon-diabetics in rats (DeMoor and Hoet). We are in no position to state on the basis of these experimental results whether the rat regulates its sugar metabolism differently or whether it does not require insulin build-up in the case of zinc. We could not find an increase of zinc in theB-cells as Voigt determined following chronic zinc administration in 2 rats.

We find most remarkable the fact that the entero-chromaffin cells of the rats were characterized both for the subject animals and for the control animals by equivalent amounts of zinc. In order to determine whether we were dealing in this case with silver-affin or argyrophile cells, we could only count the cells in two neighboring sections of intestine that had been fixed in different manners, the silver reaction initially described being used for the intestinal section that had been fixed with hydrogen sulphide alcohol, the formalin-fixed segment being subjected to the Masson-Hamperl reaction for representation of silver-affine cells, and the Bodian reaction being used for representing argyrophile cells. The numbers obtained indicate that the zinc content applies to the silver-affine cells. Furthermore, the

silver reaction of Masson-Hamperl is also positive for the material which is fixed with hydrogen sulphide alcohol, because the silver nitrate reacts with the silver sulphide. To us it seems a very fine confirmation of the view of Feyrter who, just as Weitzel et al, shows the high zinc content of the carcinoid.

When examining the small intestine, one notices the appreciable zinc content in the Paneth cells, a finding that was already described by Okamoto and also by Stegner and Fischer. A further indication will be found in a remark in the discussion by Mawson, who reports high zinc content of the basal cells in the intestine, without however becoming more precise about their relationship. Wollf (2) only discusses zinc-richness in the mucus of the thin intestine. Since it is precisely the zinc elimination in the intestine which is minor, and since there was no difference between subject and control animals, we are inclined not to consider the zinc in those cells as an elimination product, but rather wish to assume that they are related to the function of the Paneth cells.

Lastly it must also be stressed that zinc overloading had no effect in the well-known deposit sites of this substance, namely at the "Ammonshorn" (Maske) and in the dorso-lateral lobe of the prostate (Mawson and Fisher). An increase in the lateral portion of the dorso-lateral lobe, as had been described by Gunn and Gould following zinc^{65} ingestion, could not be determined by means of our method, since the presence of zinc was already so high in the control animals, that a further filling of the cells with zinc could no longer be clearly detected.

Finally we wish to point out to certain zinc deposits that have this in common, that they affect tissues of cells of reduced vitality--in this instance the zinc seems to assume a

certain similarity with the behavior of calcium. This is particularly clear in calcification as for instance in tracheal cartilage, which besides calcium will also contain ample zinc in all subject animals (see above), a finding that was already reported by Okamoto in the rabbit. The same applies to the occasionally calcified arteries in the subject animals. In similar manner, the zinc content of desqua ated alveolar epithelia and the degenerated kidney tubuli could be interpreted within the scope of a pyelo-nephritic shrinkage.

A number of organs seem to take little or no part in the zinc metabolism. These organs include the spleen, the seminal vesicle and the supra-renal cortex. We could not achieve rendering visible the metal in the heart even though Sheline et al indicate that the heart participates in the rapid conversion of the zinc. A "negative" result in the skin and in the supra-renal marrow probably should not be evaluated, since these organs show a yellow-brownish toning, such as we could not determine in other organs. This is a finding which in our view would rather indicate a fine, diffused distribution of zinc.

Summary

With long term administration of excessive amounts of zinc to rats the following findings were made:

There were no specific visceral changes produced which could be attributed to the long term zinc overloading.

It was shown, however, that especially the cells of the reticulo-endothelial system (very Kupffer cells, reticulum cells of the intestinal lamina propria) and the mast cells have the tendency to store the excess zinc.

On the organs which normally excrete zinc (the liver, kidney, and pancreas), we were unable to demonstrate an increase in the zinc content only in the excretory parenchyma of the pancreas.

In addition it was observed in both the experimental animals as well as in the controls that besides the already known localization of zinc in Langerhans' islets, in Paneth cells, in prostate, and in pancreatic islet cells: there was an increased content of zinc in the argentaffine cells of the intestine.

Literature

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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 72, 302-315 (1957)

The Action of 2,4-Dinitrophenol on Myosin

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Received January 30, 1957

Introduction

2,4-Dinitrophenol has been shown to stimulate, or release, the ATPase[1] activity of mitochondria (1-3), and also to activate myosin ATPase (4-6). An examination of the experimental procedure used by Greville and Needham (4) and by Chappell and Perry (5) in establishing the stimulating effect of DNP on myosin ATPase revealed that rather high substrate concentrations were used ($1-5 \times 10^{-3}$ *M*). These concentrations were in the range usually regarded as above the optimal for studies on the myosin enzyme system, and it occurred to us that the apparent DNP stimulation might have been, in fact, a shift in the optimal substrate concentration of the same sort as that reported first by Hasselbach and Weber (7) and later by Perry and Grey (8) for the Marsh-Bendall factor. Although the validity of the results of these workers was in question, in view of the limitations of the Fiske and SubbaRow (9) technique for P estimation reported by Blum and Chambers (10), nevertheless it was clear that the relation of DNP activation to substrate concentration required investigation.

In their preliminary note on the stimulation of myosin ATPase by DNP, Chappell and Perry reported that DNP did not activate the enzyme at 0°C. This phenomenon, also, has been investigated more fully in the experiments reported below. Data from these two lines of

[1] The following abbreviations are used: ATP, adenosine triphosphate; ITP, inosine triphosphate; GTP, guanosine triphosphate; CTP, cytosine triphosphate; UTP, uridine triphosphate; ATPase, adenosinetriphosphatase; DNP, 2,4-dinitrophenol; GSH, reduced glutathione; EDTA, ethylenediaminetetraacetic acid; PCMB, *p*-chloromercuribenzoic acid; PMA, phenylmercuric acetate; Tris, tris(hydroxymethyl)aminomethane.

attack have enabled us to propose a general theory for the mode of action of DNP.

Methods

Myosin

Actin free myosin was prepared by the method of Szent-Györgyi (11), water redistilled in glass being used throughout. The myosin was dissolved in 0.6 *M* KCl and stored at a temperature of less than 5°C. The activity of different preparations, expressed as micromole of ATP split/mg. protein/min. at 25°C., varied from 0.35 to 0.85.

ATP

This was purchased as the dibarium salt (Schwarz) and converted to the sodium salt by treatment with Amberlite IR-100.

DNP

DNP was precipitated with HCl from a solution of the sodium salt. The precipitate was washed twice with ice-cold water and dried in air.

Albumin

Crystalline bovine plasma albumin was from Armour and Co. A molecular weight of 69,000 was used in calculating concentrations. It was dissolved in water at a concentration of about 10 mg./ml.

Insulin

Crystalline bovine insulin, from the Commonwealth Serum Laboratories, was freed of metal by shaking an aqueous solution with 8-hydroxyquinoline in chloroform. After separation of the aqueous phase, metal-free insulin was precipitated isoelectrically, washed, and dried. A molecular weight of 6000 (12) was used in calculating concentrations. Insulin was dissolved at a concentration of 5 mg./ml. by addition of NaOH, and the pH was brought to 7.0 before addition to the reaction mixture.

Growth Hormone

Crystalline bovine hypophyseal growth hormone made by the Wilhelmi, Fishman, and Russell (13) method, was donated by Prof. A. H. Ennor of the Australian National University. A molecular weight of 44,000 was assumed (14). It was dissolved at pH 9.0 at a concentration of about 10 mg./ml., and the pH was reduced to 7.5 before addition to the reaction mixture.

Hemoglobin

Crystalline hemoglobin was prepared from freshly collected rat blood. A molecular weight of 65,000 was used in calculation. The hemoglobin was dissolved

at pH 9.0 at a concentration of about 10 mg./ml., and the pH was reduced to 8.0 before addition.

Tests of Enzyme Activity

ATPase activity was measured in a mixture containing 0.05 *M* Tris buffer of pH 7.2, 0.10–0.12 *M* KCl, and 0.01 *M* $CaCl_2$, with other additions as noted in the text. Limiting enzyme concentrations (0.08–0.12 mg. protein/ml.) were used. The reaction was started by the addition of substrate to the enzyme mixture, cooled in ice water, and a blank sample taken at zero time. Incubation times were 3-7 min. at 25°C. and 20–70 min. at 0°C.

Measurement of P Evolved

Enzyme activity was stopped by the addition of trichloroacetic acid to a final concentration of 6%, and inorganic P was measured by the method of Fiske and SubbaRow (8), the colour being developed for 15 min. (accurately timed) and read on a Coleman Junior spectrophotometer at a wavelength of 700 mμ. Under the conditions of our experiments this method gave true readings of P provided the ATP concentrations did not exceed 2×10^{-3} *M*.

Results

Influence of Substrate Concentration on DNP

Activation of Myosin ATPase

The findings of Greville and Needham on the activation of myosin ATPase by DNP at 25°C. were confirmed. In our experiments the increase in activity at a substrate concentration of 1×10^{-3} *M* in the presence of Ca^{++} produced by 4×10^{-3} *M* DNP varied between 80% and 95%.

The relationship between enzyme activity and substrate concentration at 25°C. is shown in Fig. 1. It was clear from the experiments that the extent of the activation by DNP was very closely dependent on the substrate concentration. Very marked stimulation could be demonstrated at substrate concentrations in excess of 1×10^{-3} *M*, while at substrate levels below 1×10^{-4} *M*, DNP inhibited the enzyme. Whereas the optimal ATP concentration for untreated myosin was in the neighborhood of 5×10^{-4} *M*, the activity of the DNP-treated enzyme was still increasing at a substrate concentration of 1.7×10^{-3} *M*. Higher substrate concentrations than this were not investigated because of the limitations of the Fiske and SubbaRow technique mentioned above.

We have also confirmed the activation by DNP of myosin ATPase

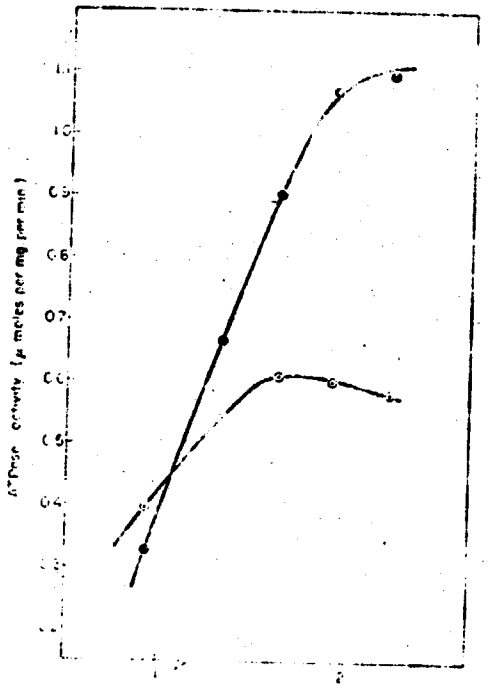

FIG. 1. The relationship between enzyme activity and substrate concentration at 25°C. ○ Myosin alone; ● myosin plus DNP. The reaction mixture contained myosin 0.08 mg./ml., KCl 0.12 *M*, $CaCl_2$ 0.01 *M*, Tris 0.05 *M*, DNP 4×10^{-3} *M*.

in the presence of higher KCl concentrations (5). In one experiment run at 0.5 *M* KCl and 9×10^{-4} *M* ATP, DNP at 4×10^{-3} *M* produced a 65% increase in enzyme activity.

Effect of DNP on Enzyme Activity at 0°C.

Whereas Chappell and Perry (5) reported no activation by DNP in experiments done at 0°C., our results demonstrated a marked inhibition of myosin by DNP at this temperature. This inhibition was exhibited over a wide range of substrate concentrations. The relationship is illustrated in Fig. 2. The influence of varying DNP concentrations at constant substrate concentration is shown in Fig. 3.

A comparison of the four curves in Figs. 1 and 2 suggested to us that

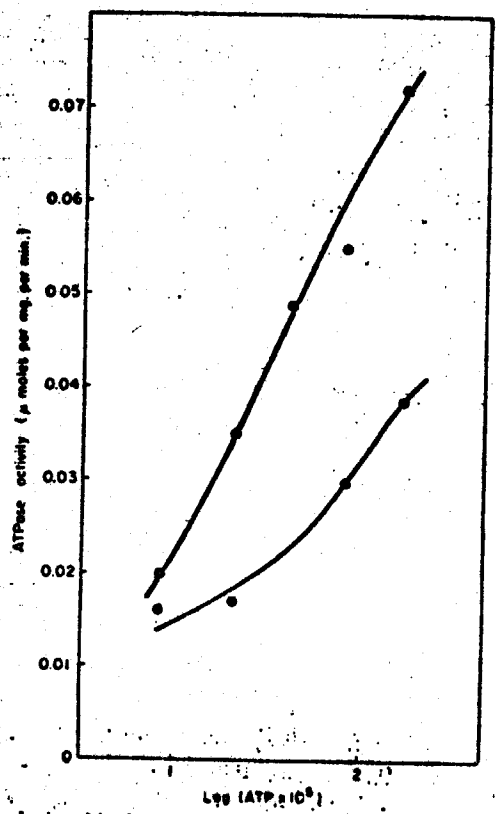

FIG. 2. The relationship between enzyme activity and substrate concentration at 0°C. ○ Myosin alone; ● myosin plus DNP. The reaction mixture was the same as for Figure 1.

there must have been a fairly profound difference in the nature or state of the enzyme molecule at 25°C. as compared with 0°C. It was as if the enzyme were in some way self-inhibited at 25°C. and that the DNP was acting to release the inhibition. In order to examine this hypothesis, a series of tests on the effect of various inhibitors and activators on the enzyme at the two different temperatures was initiated. These were all done at substrate concentrations in the neighborhood of 1×10^{-3} *M*.

Effect of Zinc

Heavy metals are known to inhibit myosin. The effect of differing concentrations of Zn^{++} on the enzyme, in the presence or absence of

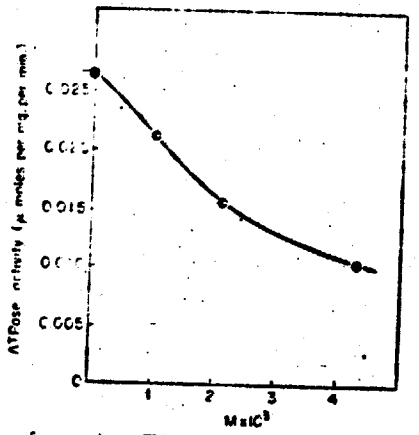

FIG. 3. Inhibition of myosin ATPase by DNP at 0°C. The reaction mixture contained myosin 0.12 mg./ml., ATP 1.13×10^{-3} *M*, KCl 0.1 *M*, $CaCl_2$ 0.01 *M*, Tris 0.05 *M*.

DNP at 25°C., and the untreated enzyme at 0°C., are illustrated in Fig. 4. At 25°C. Zn^{++} was found to activate myosin ATPase in concentrations up to 1.5×10^{-5} *M*. Beyond this point its effect was inhibitory. In the presence of DNP, Zn^{++} inhibited over the whole range of concentrations. At 0°C., also, its effect was inhibitory.

Effect of p-Chloromercuribenzoate

Greville and Needham (4) showed in experiments run at 25°C. that phenylmercuric acetate at low concentrations activated myosin and at higher concentrations inhibited myosin, whereas the effect on the DNP-treated enzyme was inhibitory over the whole concentration range. Kielley and Bradley (15) have investigated this activating effect of —SH reagents more fully by relating the binding of —SH groups by PCMB to the ATPase activity of the enzyme. They showed that up to the point at which approximately half the —SH groups had been titrated the enzyme was progressively activated. Further titration beyond this point led to inhibition, complete inhibition coinciding with elimination of all —SH groups.[2]

We have confirmed this action of PCMB on myosin ATPase in the experiments illustrated in Fig. 5. The series of curves obtained are very similar to those resulting from treatment of the myosin with Zn^{++}.

[2] The article by Kielley and Bradley does not state the temperature at which ATPase activity was assayed.

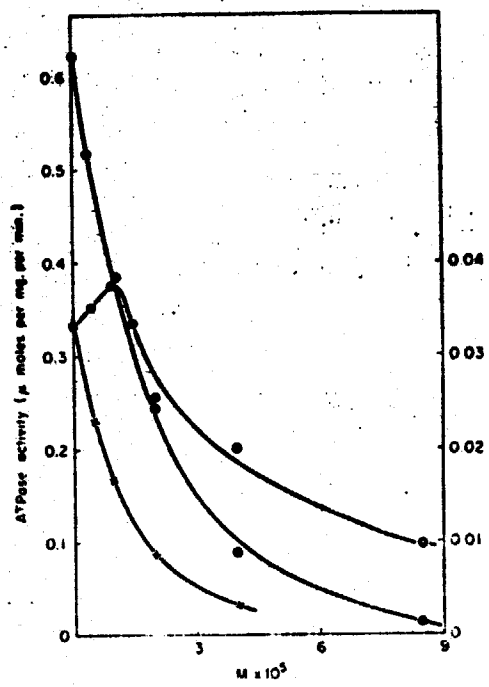

FIG. 4. Effect of Zn^{++} on myosin ATPase under various conditions. ○ Myosin alone at 25°C.; ● myosin plus DNP at 25°C.; × myosin alone at 0°C. The reaction mixture contained myosin 0.12 mg./ml., ATP 1.13×10^{-3} *M*, KCl 0.1 *M*, $CaCl_2$ 0.01 *M*, DNP 4×10^{-5} *M*, Tris 0.05 *M*. The scale on the ordinate at the left refers to activities at 25°C., that on the right to those at 0°C.

Whereas myosin alone at 25°C. was activated by low concentrations and then inhibited by higher concentrations of PCMB, the effect on the enzyme in the presence of DNP at 25°C. and on myosin alone at 0°C. was progressively increasing inhibition. The same concentration of PCMB that produced a 20% stimulation of myosin ATPase at 25°C., caused a 35% inhibition of the enzyme in the presence of DNP and a 50% inhibition of the untreated enzyme at 0°C.

Effect of Proteins

Proteins enhanced the DNP activation at 25°C. This is illustrated in Fig. 6 for bovine plasma albumin. Similar results were obtained with bovine hypophyseal growth hormone, rat hemoglobin, and metal-free insulin (Table I). Protein also activated myosin alone at 0°C., but had

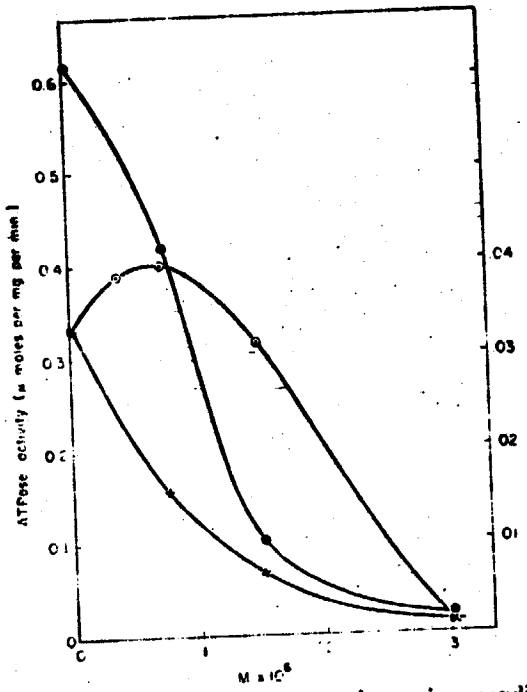

FIG. 5. Effect of PCMB on myosin ATPase under various conditions. Symbols and reaction mixture as for Fig. 4.

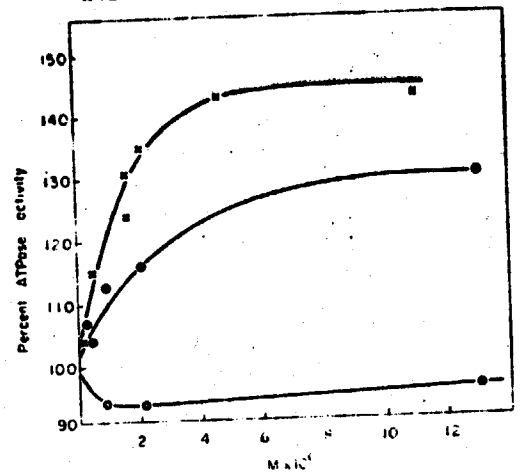

FIG. 6. Effect of serum albumin on myosin ATPase under various conditions. Symbols and reaction mixture as for Fig. 4.

TABLE I

Influence of Proteins on Myosin ATPase under Various Conditions

The reaction mixture contained myosin 0.1 mg./ml., ATP 8×10^{-4} *M*, KCl 0.12 *M*, $CaCl_2$ 0.01 *M*, DNP 4×10^{-3} *M*, Tris 0.05 *M*.

Protein added	Myosin: activity at 25°C.		Myosin + DNP: activity at 25°C.		Myosin: activity at 0°C.	
	μmoles P/mg./min.	*per cent*	*μmoles P/mg./min.*	*per cent*	*μmoles P/mg./min.*	*per cent*
None	0.35	100	0.65	100	0.029	100
Growth hormone						
1.1×10^{-5} *M*	0.37	106	0.73	113	0.040	138
2.3×10^{-5} *M*	0.37	106	0.95	146		—
Hemoglobin						
4.8×10^{-6} *M*	0.37	106	0.77	119		—
1.5×10^{-5} *M*	0.37	106	0.81	124		—
Insulin						
2.7×10^{-5} *M*	0.37	106	0.75	116		—

very little effect on the untreated enzyme at 25°C., albumin being slightly inhibitory, whereas the other proteins produced a small stimulation. Since the "protective" action of proteins on enzymes is generally believed to be due to combination with stray heavy metals, the protein activation was re-examined in the presence of 0.1 *M* histidine buffer in place of the Tris buffer used in all other tests. Replacement of Tris by histidine resulted in an increase in enzyme activity, but an additional effect of protein over and above this could be demonstrated (Table II). Moreover, glutathione did not activate the enzyme, as would have been expected if there had been some inhibition by stray metals. GSH had no effect, either in the presence or absence of DNP, in concentrations up to 4×10^{-5} *M*, while at 8×10^{-4} *M* it brought about a 20% inhibition of untreated myosin and a 10% inhibition of the enzyme in the presence of DNP.

TABLE II

Activation of Myosin by Albumin in the Presence of Different Buffers at 0°C.

The reaction mixture contained myosin 0.12 mg./ml., ATP 1.13×10^{-3} *M*; KCl 0.1 *M*, $CaCl_2$ 0.01 *M*.

Albumin concn.	Activity: *μmoles P/mg./min.* Tris buffer 0.05 *M*	Histidine buffer 0.1 *M*
None	0.031	0.039
1.6×10^{-6} *M*	0.041	0.043

Effect of Ethylenediamine Tetraacetate

Several workers have shown that EDTA activates myosin, and it had been suggested that this action may have a common basis with that of DNP. Thus Kielley and Bradley (15) have shown that —SH— binding reagents inhibited the EDTA-activated enzyme, just as in our experiments PCMB inhibited the DNP-activated enzyme at 25°C. and the untreated enzyme at 0°C. It was, therefore, of interest to compare the effects of EDTA on the enzyme at 0°C. and 25°C. This comparison is illustrated in Table III. Experiments with EDTA were run at high KCl concentration (0.5 *M*), since EDTA does not activate at low KCl concentrations, and in the absence of Ca^{++}, so that the direct effect

TABLE III

Activation of Myosin by EDTA at 0°C. and 25°C.

The reaction mixture contained myosin 0.12 mg./ml., ATP 1.03×10^{-3} *M*, KCl 0.5 *M*, Tris 0.05 *M*.

Temp. °C.	Ca^{++} *moles/l.*	EDTA *moles/l.*	Activity *μmoles P/mg./min.*
0	—	—	0.003
	0.01	—	0.017
		4.5×10^{-3}	0.010
25		—	0.019
	0.01	—	0.130
	—	4.5×10^{-3}	0.246

of EDTA on the enzyme would not be confused with its action in chelating Ca^{++}.

Our results agreed with other workers in showing that at 25°C. activation by EDTA in the absence of Ca^{++} was stronger than that achieved by Ca^{++}. At 0°C., however, whereas EDTA did activate the enzyme, its effect was much less marked than that of Ca^{++}.

Discussion

Several workers have reported that myosin in solution at temperatures above 0°C. tends to aggregate. Thus Parrish and Mommaerts (16) have shown that the sedimentation constant of myosin increases with temperature, while Laki and Carroll (17) have reported an apparent polymerization of myosin at higher temperatures. This polymerization is reversible if exposure to a temperature of 25°C. lasts for only 2 hr., but becomes irreversible on longer exposure.

The results of these investigations suggest a possible explanation for the anomalous behavior of the enzyme at 25°C. in our experiments. It is clear there is a close parallelism in the behavior of the enzyme at 0°C. and its behavior in the presence of DNP at 25°C., whereas the untreated enzyme at 25°C. departs consistently from this pattern. These results become understandable if it can be supposed that the enzyme aggregates at 25°C., that this process of aggregation masks or modifies the active center for the hydrolysis of ATP, and that DNP splits the polymer and restores the enzyme to the state in which it exists at 0°C. A similar sort of explanation might be true for the effect on the mitochondrial ATPase, since in this case the action of DNP seems to be an uncoupling or unmasking of the bound enzyme.

If the modifications of ATPase activity we have studied are a reflection of changes in aggregation, as suggested, then it seems that —SH groups are concerned in the aggregation process, since the addition of the specific —SH reagent PCMB activates the enzyme at 25°C. Other —SH groups are necessary for the hydrolysis of ATP, however, since higher concentrations of PCMB cause inhibition. In the unpolymerized enzyme at 0°C., which in our view corresponds to the state of the DNP enzyme at 25°C., the —SH groups of the active center for ATP hydrolysis are exposed and available for combination with PCMB which under these circumstances produces a decrease in enzyme activity.

The action of all three reagents, DNP, PCMB, and Zn^{++}, appears to be similar. They may be regarded as causing a simultaneous depolymerization with a resultant increase in the concentration of active enzyme, and an inhibition by combination with the active centers or modification of the attachment of ATP. The interaction of these opposing effects could result in the typical curve of at first rising and then falling ATPase activity with increasing concentration of the reagent at 25°C. Such curves have been demonstrated by us for Zn^{++} and PCMB, and by Greville and Needham for DNP. The combination of either Zn^{++} or PCMB with the concentration of DNP needed to produce full activation at 25°C. then results in inhibition. Similarly, all three reagents inhibit at 0°C. Although —SH groups are apparently concerned in both the processes of activation and inhibition, it cannot be concluded that all three reagents either act on —SH groups or produce their effect in the same way.

Whether EDTA has the same action as DNP, Zn^{++}, and PCMB remains uncertain. Certainly the activation it achieves is relatively much less (in relation to Ca^{++} activation) at 0°C. than at 25°C. Nevertheless, it does have a pronounced effect at 0°C., so that even if it can be supposed that EDTA achieves a depolymerization at 25°C., a direct effect on the unpolymerized enzyme must be postulated. However, experiments with EDTA are complicated by the fact that they had to be run at high KCl concentrations, since EDTA does not activate at low KCl, and in the absence of Ca^{++}, because of the high affinity of EDTA for Ca^{++}. Since DNP activates myosin at 25°C. at a KCl concentration of 0.5 *M*, it seems probable that K^+ itself does not cause depolymerization of the enzyme.

The mechanism of the enhancement of the DNP activation by proteins is also obscure. It seems not to be entirely due to the fixation of heavy metals, since the effect is not duplicated by glutathione, and only partly by histidine, and histidine itself may be duplicating some unspecific effect of protein other than the binding of metals. The protein effect apparently is manifested only in the fully activated, "depolymerized" enzyme, since the addition of protein to the untreated enzyme at 25°C. has very little effect.

The participation of —SH groups in what seems to be a polymerization of myosin recalls earlier work on the role of such groups in the binding of actin to myosin, which established that —SH groups were involved in this interaction (18), but not necessarily the same ones as were concerned in the hydrolysis of ATP (19). The suggestion that the same —SH groups are involved is further reinforced by the profound effect on the ATPase activity that results from the combination of actin with myosin. Moreover, Chappell and Perry have shown that the addition of actin to myosin progressively lowers the DNP effect, a result which could be interpreted to mean that both reactants were competing for the same —SH groups.

Greville and Reich (6) have studied the effect of DNP and PMA on the nucleoside triphosphatase activity of L-myosin with a number of different substrates (all added at 2×10^{-3} *M*). They found that with ATP and CTP as substrates DNP caused activation, and PMA activated at low concentrations and then inhibited at higher concentrations, while with GTP and ITP both DNP and PMA inhibited. With UTP the effect was intermediate: slight activation at low concentrations followed by inhibition at higher. Examination of the absolute rate of nucleotide hydrolysis in these experiments shows that ITP, GTP, and

UTP were hydrolyzed more rapidly than ATP and CTP. In the light of the hypothesis developed above, these results suggest that polymerization of myosin is facilitated in the presence of the 6-amino nucleotides and inhibited or reversed by the 6-hydroxy nucleotides. Similar results with different nucleotide substrates have been obtained by Kielley, Kalckar, and Bradley (20), in experiments in which the effect of EDTA was examined. This investigation further reinforces the view that EDTA and DNP have similar effects on the myosin molecule.

The shape of the velocity *vs.* substrate concentration curve at 25°C. (Fig. 1), and the fact that at low substrate concentrations DNP actually inhibits the enzyme at 25°C., also point to the involvement of ATP in the inhibitory process. This and other aspects of the problem, particularly the physical state and particle size of myosin ATPase under various conditions will be the subject of further investigations.

Summary

1. The activation of myosin ATPase by 2,4-dinitrophenol at 25°C. is closely dependent on substrate concentration. Marked stimulation by DNP occurs at ATP concentrations in excess of 1×10^{-3} *M*, whereas at concentrations below 1×10^{-4} *M* DNP inhibits the enzyme.

2. At 0°C., DNP inhibits the enzyme over a range of substrate concentrations of $0.8\text{–}17 \times 10^{-4}$ *M*.

3. At 25°C., Zn^{++} activates myosin ATPase in concentrations up to 1.5×10^{-5} *M*. At 0°C. and in the presence of DNP at 25°C., Zn^{++} is inhibitory at this concentration.

4. At 25°C., *p*-chloromercuribenzoate activates the ATPase at concentrations up to 7×10^{-7} *M*. At 0°C. and in the presence of DNP at 25°C., PCMB inhibits at this concentration.

5. Proteins enhance DNP activation of myosin ATPase at 25°C. In the absence of DNP the effect of proteins is negligible at 25°C. At 0°C. proteins enhance the ATPase activity.

6. At 25°C. and at high KCl concentration, activation of myosin ATPase by ethylenediamine tetraacetate in the absence of Ca^{++} is much greater than that due to Ca^{++} alone. At 0°C., Ca^{++} is the better activator of the two.

7. These results are discussed in relation to published data on the polymerization of myosin at higher temperatures.

Note

Since the work reported above was completed, two papers have appeared which have a bearing on the hypothesis presented. A Holtzer [*Arch. Biochem. Biophys.* **64**, 507 (1956)] has confirmed the conclusions of Laki and Carroll with regard to the spontaneous aggregation of myosin, and found that after 45 hr. at 25°C. the aggregation was reversed neither on cooling nor by the addition of ATP or EDTA. N. B. Madsen and C. F. Cori [*J. Biol. Chem.* **223**, 1055 (1956)] have shown that muscle phosphorylase is split into four parts of equal size by PCMB.

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ZINC TOXICITY IN THE RAT AND ITS INTERRELATION WITH COPPER

by D. R. GRANT-FROST AND E. J. UNDERWOOD

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(*Accepted for publication 25th March, 1958.*)

Most of the early work on zinc toxicity in animals is difficult to evaluate since neither the zinc intake nor the detailed nature of the rest of the diet is given. The first quantitative studies are those of Heller and Burke (1927) and Sutton and Nelson (1938), who added zinc to otherwise satisfactory diets in concentrations ranging from 0·1 p.c. to 1·0 p.c. and found that the effects on rats ranged from no effect at the 0·1 p.c. level to severe anaemia, markedly subnormal growth, reproductive failure and some mortality at the 1 p.c. level. Stimulated by these findings, Smith and Larson (1946) carried out more critical studies which revealed that the addition of zinc to an otherwise adequate diet for rats induces at least two distinct effects—a microcytic, hypochromic anaemia that can at least partially be overcome by an additional supplement of copper salts, and subnormal growth that can be partially prevented by an additional supplement of liver extract. The copper did not affect the growth-rate, nor did the liver extract affect the anaemia. Van Reen (1953) confirmed and extended these findings. He found that 0·5 to 0·7 p.c. zinc as the carbonate, added to a diet containing 8 p.p.m. copper, reduced growth, food consumption and haemoglobin levels in young rats and produced a marked reduction in liver catalase and cytochrome oxidase activities. Feeding additional copper at the rate of 0·2 mg. or 0·4 mg. Cu daily as the sulphate maintained the liver catalase and cytochrome oxidase activities of the zinc-fed rats at normal levels but had no effect on the growth of the animals.

It is apparent that these workers have left certain important questions unanswered. For instance, it is not known whether the subnormal growth which occurs in zinc toxicity is due wholly or in part to reduced food consumption or whether the anaemia is caused by a depression in copper assimilation in the animals, by increased excretion or by an antagonistic effect of the zinc on the copper within the tissues. The present experiment is designed to throw some light on these problems.

MATERIALS AND METHODS.

An experiment of simple factorial design was carried out, in which 8 groups of 6 rats in each group were fed as follows:

Group A: Basal diet; Group B: Basal diet + copper; Group C: Basal diet + zinc; Group D: Basal diet + copper + zinc; Group E: Basal diet (food consumption restricted to that of Group C).

Wistar hooded male rats, 5-6 weeks of age at the commencement of the experiment, were selected from six litters and distributed so that as far as possible litter mates occurred in different groups and the mean weights of the groups were similar. Each animal was allotted an individual metabolism cage at random and each group was given a position in the housing room at random. The cages of the first four groups were constructed of galvanised iron, coated with hard plastic enamel to exclude all contact with zinc and fitted with aluminium funnels which led to glass collection vessels allowing the separate collection of urine and faeces. The grid floor of each cage was made of chrome-plated steel. Group E was housed in chrome-plated wire mesh cages raised to avoid access to faeces.

The basal diet consisted of a mixture of 80 p.c. whole crushed wheat and 20 p.c. whole dried milk and contained an average of 2·1 p.p.m. Cu. This diet was fed *ad libitum*, but a daily record of feed consumption was kept. Copper-free water was available at all times. Zinc was added at a level of 0·5 p.c. Zn as the oxide and mixed into the feed of Groups C and D. This level was chosen because it was expected that it would produce anaemia and subnormal growth without heavy mortality. Supplementary copper was given as copper sulphate to each animal of Groups B and D each day at the rate of 0·4 mg. Cu daily. This was administered in 5 ml. of fresh milk in small glass jars and was offered to the animals *before* being given their dry feed. It is possible that this routine could have an effect on the results of the experiment as will be explained later. Groups A, C and E were given 5 ml. of milk only each morning in the same manner.

The rats were weighed three times each week and haemoglobin determinations made at the beginning, every two weeks and at the end of the six weeks' experiment. Feed residues were weighed daily and the food consumption of the animals of Group E maintained each day at that of the zinc group (Group C) on the previous day. Some spillage of milk and of faeces and contamination of faeces by urine and urine by faeces occurred so that complete copper balances, as originally intended, were not attempted.

At the end of six weeks' experimental regime all animals were anaesthetised with ether, their body cavities opened and blood removed for analysis by heart puncture. Liver and kidneys were removed, weighed, dried at 105° C. and the dry weight determined. The alimentary canal was removed and discarded and the remaining part of the body dried and ground. With blood, liver and kidney the whole sample was analysed for copper, but the copper and the fat contents of the body were determined on suitable aliquots. All copper values were obtained by a modification of the method of Eden and Green (1940).

RESULTS.

Growth and food consumption. The average live-weights of the rats given the different treatments at the beginning and end of the six weeks' experimental period are presented in Table 1, together with the food consumption data.

The results for all five groups were submitted to an analysis of variance and, in addition, Groups C and E were compared by the "t" test. A highly significant variation between groups was obtained both with the weight gains and the food consumption data. In neither case was the copper-zinc interaction significant, but the effect of zinc on growth and food consumption was highly significant at the 0·1 p.c. level. Variation due to copper was significant at the 5 p.c. level

TABLE 1.

The effect of copper and zinc on the growth and food consumption of rats.

(Wts. in gm.)

Group	Treatment	Av. wt. at 0 weeks	Av. wt. at 6 weeks	Av. wt. increase	Av. food consumption per week	Food eaten per gm. wt. gain
A	Basal	85	202	117	87	4·5
B	Basal+Cu	84	208	124	68	4·3
C	Basal+Zn	80	116	36	54	8·9
D	Basal+Cu+Zn	78	135	57	59	6·3
E	Basal (R)	79	111	32	54	10·0

in the case of the weight gains, but not in the case of the food consumption. There is no significant difference between Groups C and E.

Haemoglobin values. The mean haemoglobin values of the rats in relation to treatment at 0, 2 and 6 weeks are given in Table 2.

Differences between groups are highly significant at 2 weeks and at 6 weeks, but at neither time is the copper-zinc interaction significant. The effects of copper and of zinc are significant at both times and the difference between Groups C and E at 6 weeks, when compared by the "t" test, is also highly significant ($p < 0·001$).

Copper concentrations. The average concentrations of copper in the blood, liver, kidney and body of the rats following six weeks of each treatment are given in Table 3.

With each tissue, the copper, zinc and copper-zinc effects are all highly significant ($p < 0·001$), while these effects are significant ($p < 0·01$) for the whole body. The treatment differences between Groups C and E are also all highly significant.

TABLE 2.

The effect of copper and zinc on the haemoglobin levels of rats.

(gm. Hb per 100 ml. blood)

Group	Av. value at 0 weeks	Av. value at 2 weeks	Av. value at 6 weeks	Av. change in 2 weeks	Av. change in 6 weeks
A	11·7	12·6	13·7	+ 0·9	+ 2·0
B	11·8	14·2	15·4	+ 1·5	+ 3·6
C	12·3	10.3	7·3	− 2·0	− 5·0
D	12·6	12·0	11·3	− 0·6	− 1·3
E	—	—	14·5	—	—

TABLE 3.

The effect of copper and zinc on tissue copper concentrations.

(p.p.m. Cu on dry basis)

Group	Blood	Liver	Kidney	Body	Body (fat-free)
A	1·03	12·8	21·6	3·7	6·0
B	1·22	15·0	23·4	3·7	5·8
C	0·25	6·7	10·0	2·3	2·9
D	1·34	16·0	22·2	3·8	5·5
E	1·05	15·0	19·8	4·4	6·3

Total copper values. The average total copper contents of the liver, kidneys and whole bodies (less blood, liver, kidneys and intestinal contents) of the rats in each group at the end of the treatment period are set out in Table 4.

TABLE 4.

The effect of copper and zinc on total tissue copper contents.

(Amounts in μg. Cu)

Group	Liver	Kidney	Body
A	164·7	48·6	1417·1
B	191·2	52·9	1443·7
C	43·3	15·5	396·8
D	121·3	36·2	857·5
E	100·7	29·8	886·7

As with the copper concentrations, the differences between groups are highly significant ($p < 0·001$); the copper and zinc effects are similarly highly significant; and the copper-zinc interaction significant ($p < 0·01$). Application of the "t" test to Groups C and E showed that the treatment differences are highly significant for liver and significant for body and kidney values.

Body fat values. The mean percentage body fat for the various treatments were as follows: Group A, 38·6; B, 35·7; C, 21·6; D, 30·3; E, 30·3.

The copper effect is non-significant, the zinc effect highly significant and the copper-zinc interaction significant ($p < 0·05$). An analysis of variance on Groups C and E showed the difference to be significant ($p < 0·05$) but when the "t" test was employed the difference appears non-significant.

DISCUSSION.

The earliest and most obvious sign of zinc toxicity was found to be a marked reduction in food consumption, accompanied by a depression in growth rate. Food intake was reduced in the zinc-fed animals from the first day of treatment, pointing strongly to unpalatability as the main causal factor. The total reduction in food consumption in Group C was 38 p.c., compared with a 25 p.c. reduction in the experiments of Van Reen (1953) where similar levels of zinc, as zinc carbonate, were used. This suggests that zinc oxide is more unpalatable, or more toxic, or both, than zinc carbonate. From a comparison of the weight gains and food consumption figures for the zinc-toxic animals of Group C and those of Group E, in which the food intake was maintained at the same level as that of Group C, it would appear that the effect of zinc on growth can be wholly accounted for by the lowering of food consumption and of food utilisation (Table 1).

It should be noted, however, that the addition of copper to the zinc-fed animals (Group D) had a small but significant effect in improving growth without any corresponding effect on food consumption, and the rats of this Group had significantly lower haemoglobin values than those of Group E. Lowered food consumption could not, therefore, of itself, be responsible for the anaemia and the possibility exists that the zinc-induced anaemia contributed to the poor growth. It seems that the major cause of the poor growth in zinc toxicity is a reduction in food intake, due in this case to unpalatability of the ration, but that metabolic effects of zinc contribute also to this effect.

According to Gallagher (1957) anaemia is a relatively late sign of simple copper deficiency in the rat and this anaemia is consequent upon a severe depletion of cytochrome oxidase activity following depletion of the animal's copper reserves. In our zinc-toxic animals (Group C), also copper deficient, a highly significant reduction in haemoglobin concentration occurred within 2 weeks of feeding the zinc and could have occurred earlier. This difference between the two types of copper deficiencies again suggests that zinc, besides reducing copper absorption, clearly indicated by the low blood and tissue copper concentrations and amounts (Tables 3 and 4), also had an effect at the cellular level. In other words, the zinc-toxic animal is unable to utilise its copper reserves because of an antagonistic effect of the zinc absorbed from the diet. There is no direct experimental support for this hypothesis, but additional evidence in this direction can be adduced from a comparison of Groups A and D. The haemoglobin levels for the animals of Group D (copper + zinc) are significantly lower than those of the controls at the end of the experiment, although there is certainly no lack of copper in their blood and tissues. Either, therefore, the absorbed zinc is reducing the availability of the tissue copper, as just suggested, or a deficiency of some other dietary essential affecting haematopoiesis is occurring as a consequence of the high zinc intakes.

The outstanding features of the copper values are the very low levels in the animals receiving the zinc supplement alone and the complete maintenance of these values at normal levels by the further addition of 0·4 mg. Cu per day. The former can mean either that the zinc has reduced copper absorption, promoted excretion or both. In the absence of copper balance studies, which were planned but rejected owing to spillage and faecal and urine contamination,

conclusive evidence for either one of these cannot be presented. The normal copper contents of the animals receiving the copper supplement in addition to the zinc might be taken as support for the contention that the main effect of zinc is to increase excretion of absorbed copper, but the timing and form of the copper supplementation may be of considerable significance. In our experiment, and indeed in all previous experiments involving zinc and copper feeding, the copper was presented in a small quantity of milk and was consumed prior to the main ration containing the zinc. Copper, therefore, entered the alimentary canal at a time when it was relatively free from zinc and could have been absorbed before any effect of zinc on copper availability in this site was fully exerted. In the future, it would appear essential to administer the copper and zinc together. In spite of this, there is no doubt that zinc, at the level used in this experiment, profoundly reduces copper *retention* and induces a marked copper deficiency within the animal. The blood copper values are, in fact, so low that normal haematopoiesis could not take place.

The observation of Sadasivan (1951) that excess zinc in the diet reduces the liver fat content of rats finds support from our findings. The fat content of the bodies of the zinc-fed animals of Group D was significantly lower than that of any other group, including Group E which received the same amount of food but no zinc. Lowered food consumption is clearly therefore not the reason for this effect. It can be argued, however, that the reduction in body fat is not due, as Sadasivan states, to a lipotropic action of zinc by depressing lipase activity, but to the copper deficiency. Gallagher (1957) has shown that phospholipid synthesis is depressed in copper deficiency and concludes that it is the attachment of fatty acids of glycerophosphate which is affected. This process needs studying under conditions of zinc toxicity.

SUMMARY.

An experiment was carried out to study the effect on growing rats of the addition of 0·3 p.c. of dietary zinc, as zinc oxide, and of the relation of supplemental copper to zinc toxicity.

The zinc was found markedly to reduce the growth, food consumption, haemoglobin levels, copper retention and body fat content of the animals.

The addition of 0·4 mg. Cu daily to the diet of the zinc-fed rats was found to have no effect on food consumption, to improve growth slightly but significantly, to maintain the copper contents of blood and tissues at normal levels and to afford considerable protection against the anaemia.

It was concluded that:

(a) The depressing effect of zinc on growth is caused largely but not entirely by reduced food consumption, probably due to the unpalatability of the zinc-containing diet; (b) that anaemia is caused by a zinc-induced copper deficiency in the animals; and (c) the zinc not only profoundly reduces the copper concentrations in the blood and tissues but probably also antagonises absorbed copper at the cellular level.

The importance of the nature and timing of the zinc and copper supplements to a proper understanding of zinc-copper interrelations is stressed.

Acknowledgments. Grateful acknowledgment is made to the University of Western Australia for a research grant to one of us (D.R.G.F.); to Mr. A. B. Beck and Mr. N. S. Stenhouse, both of C.S.I.R.O., for assistance with the copper determinations and the statistical analyses, respectively; to Messrs. R. J. Moir, M. Somers and P. B. Scott, of the Institute staff, for helpful discussions on many occasions; and to the Physiology Department for animal house facilities and assistance in the care of the animals.

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VITAMIN DEFICIENCY SYNDROME IN THE ALBINO RAT PRECIPITATED BY CHRONIC ZINC CHLORIDE POISONING*

PAUL GROSS, M.D., ZABOJ HARVALIK, Dr.Sc., AND EDITH BUNNE, A.B., M.A.

The beneficial effect of parenteral liver therapy in arsphenamine reaction and gold dermatitis has been recognized by clinical investigators. Although there may be other detoxifying substances in liver extract, we have been interested in the rôle which the Vitamin B complex plays in the therapeutic action of liver extract. While planning experiments along these lines, we discovered a toxic effect of zinc chloride in rats under certain experimental conditions which promises to shed some light on the mechanism of chronic metallic poisoning.

EXPERIMENTS

Group 1—Controls

Female albino rats, at ages from 21 to 24 days and weighing around 40 grams, were kept in individual cages and fed the following ration:†

Casein (Labco)	20
Cerelose	73

* From the Department of Dermatology, College of Physicians and Surgeons, Columbia University.

This work was supported in part by a grant from the John and Mary R. Markle Foundation.

Read before the Fourth Annual Meeting of The Society for Investigative Dermatology, Cleveland, Ohio, June 3, 1941.

† The Thiamin chloride, Riboflavin, Pyridoxin, Nicotinic acid and Calcium Pantothenate Dextrorotary, were furnished by Dr. J. M. Carlisle of Merck & Company.

The Ethyl linoleate was made and supplied by Dr. G. E. Martin of the William R. Warner Laboratories for Therapeutic Research, and the Wheat Germ Oil was made available by Dr. Ezra Levin of the Viobin Corporation.

We wish to express our appreciation for these gifts.

Salts (Osborn Mendel)	4
Wheat germ oil	2
Cod liver oil	1

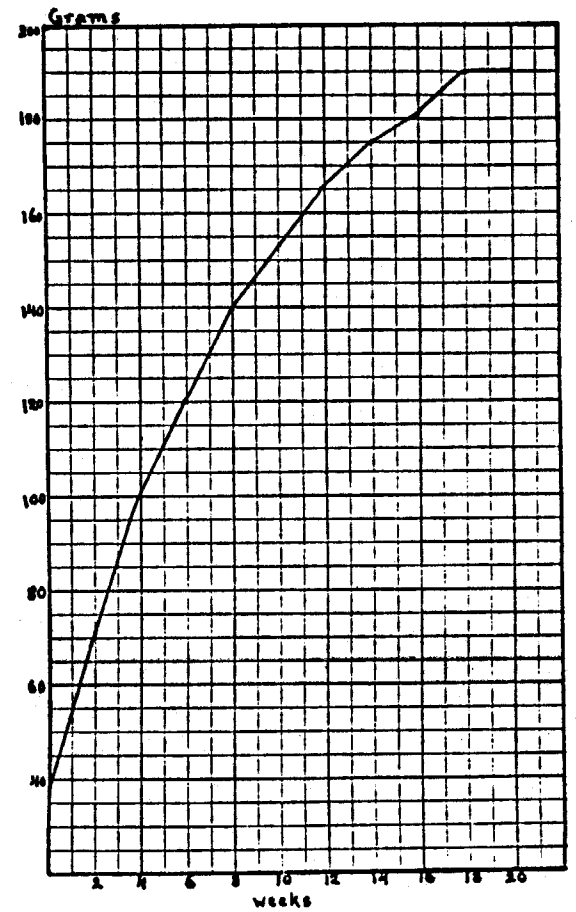

FIG. 1. WEIGHT CURVE OF CONTROL RATS (GROUP 1) RECEIVING A SYNTHETIC DIET SUBOPTIMAL ONLY IN PANTOTHENIC ACID, SUPPLIED BY 100 MGM. OF LABCO RICE POLISH FACTOR II (DIET S.O.)

The supplements consisted of:

Choline chloride	15 mgm. for the first four weeks and 1 mgm. thereafter

Thiamin chloride	20 micrograms
Riboflavin	50 micrograms
Pyridoxine	40 micrograms
Rice polish factor II (Labco)	100 mgm. (filtrate fraction)
Nicotinic acid	1 mgm.
Ethyl Linoleate	2 drops

All supplements were fed daily by syringe except on Sunday.

Animals on this diet have a normal appearance but their weight curve does not come up to the standards established for a synthetic diet of the rat.

Supplee (1) and co-workers have shown the effectiveness of the filtrate fraction in Labco rice polish factor II, when added in daily doses of 100 mgm. to a diet similar to ours, and supplemented with thiamin 12.5 micrograms, riboflavin 10 micrograms, pyridoxine 10 micrograms, and Crisco 3 per cent. They found it was adequate for a normal rate of development of about 12 to 15 grams per week. The average weight curve of our control animals compares favorably with those reported by Supplee and co-workers, but the weekly gains are lower than those obtained with a synthetic diet supplemented with optimal amounts of pantothenic acid, according to Unna (2).

Outside of the inferior weight curve and rusting of the otherwise well kept fur, which cleared up spontaneously, control rats observed over a period of 20 weeks showed no skin lesions associated with pantothenic acid deficiency of the albino rat.

We have designated this diet with the initials "S.O." to indicate its suboptimal nature.

Group 2

Eight albino rats under the same experimental conditions as Group 1 were fed diet S.O., and four hours after the syringe feeding of supplements, 4 mgm. of zinc chloride dissolved in cod liver oil[1] were instilled into the mouth of the animal by syringe using a simple device to spread the mouth. This was done daily except on Sundays.

Three to five weeks after the beginning of the experiment, five

[1] A saturated solution of zinc chloride in alcohol was mixed with the oil shortly before each feeding.

of the eight animals showed retardation of growth, expressed by a plateau of the weight curve, and various skin manifestations

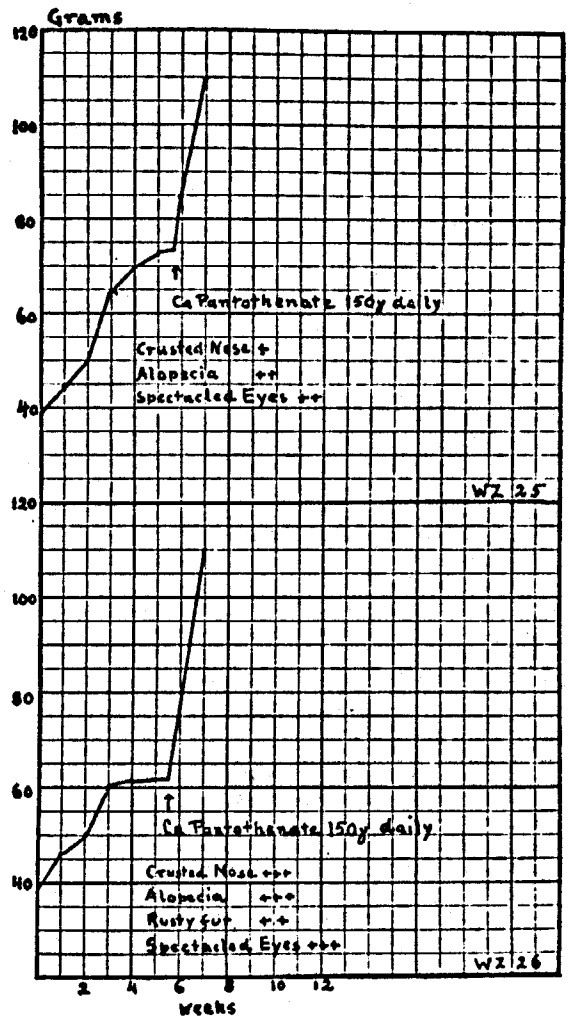

FIG. 2. WEIGHT CURVE OF WHITE RATS (GROUP 2) FED DIET S.O., DEVELOPING A DEFICIENCY AFTER DAILY SYRINGE FEEDING OF 4 MGM. ZINC CHLORIDE Recovery after addition of 150 microgram calcium pantothenate per day

described by György and coworkers (3), and commonly identified with pantothenic acid deficiency. Four animals developed a

severe alopecia, especially on the face, head, neck, back and belly; three a mild alopecia; five animals showed severe rusting of the fur and a ruffled appearance; four had crusting of the nose and chin; and three animals developed crusting of the eyelids and alopecia, giving the well-known spectacled appearance. One of these animals died five and one-half weeks after the beginning of the experiment.

At the end of the sixth week, the seven surviving animals were fed 150 micrograms of synthetic calcium pantothenate daily in addition to 100 mgm. of the filtrate fraction. Although the zinc chloride feeding was continued at the same level, the animals immediately began to gain weight and showed increases from 12 to 27 grams for the first week. The deficiency symptoms responded promptly and the spectacled eye condition disappeared within ten days and the re-growth of hair was complete in three weeks. No rusting of the fur was noted at the end of this period.

Group 3

Six black and eight white rats kept under the same experimental condition and fed diet S.O. received 5 to 6 mgm. of zinc chloride in olive oil, four hours after the feeding of supplements. The black rats developed severe greying of the fur in the fourth to fifth week, and the white rats showed severe rusting. In both groups manifestations of deficiency developed, consisting of crusting of the snout, blood caked whiskers, ruffled and matted fur, with alopecia of varying degree especially on the back of the neck, belly and hind back, redness of the skin, especially on the belly and hind back, in some animals accompanied by scaling; hemorrhagic ears and crusted tails. So far only one animal has developed a spectacled eye condition. This group developed the deficiency later than Group 2, and therefore the weight curves reached higher levels. The experiment is still being continued.

Group 4

Twenty-four white rats were fed the diet S.O. under the same experimental conditions, receiving in addition to 100 mgm. of filtrate fraction, a daily dose of 100 micrograms of synthetic cal-

cium pantothenate per day. They were divided into six groups, receiving zinc chloride in oil in amounts varying from 4 to 15 mgm.

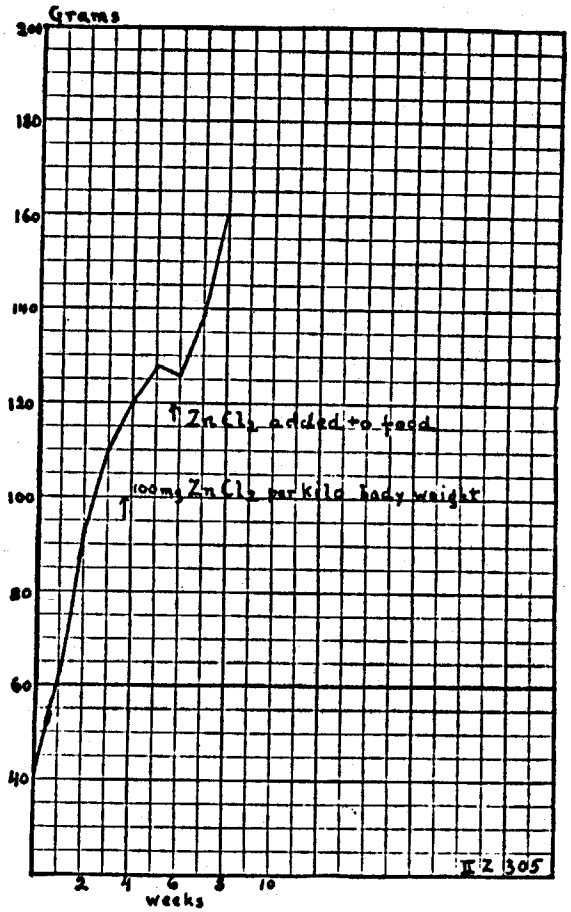

Fig. 3. Weight Curve of a White Rat (Group 4) Fed 100 Microgram Calcium Pantothenate Daily in Addition to 100 Mgm. Filtrate Fraction of Diet S.O.

Zinc chloride fed at 6 mgm. level per day, shows no toxic effect; increase to 100 mgm. zinc chloride per kilo body weight causes weight decrease. Recovery after mixing the same amount of zinc chloride with food.

of zinc chloride per day, fed about seven hours after the vitamins were given. The weight curves of these animals, averaging a gain of 20 grams per week, registered only a slight toxic effect of

zinc chloride. Therefore, the amount of zinc chloride was increased in the fourth week of the experiment so that the animals received 75, 100, 125, 150, 200 and 250 mgm. per kilogram body weight. The weight curves of most of the animals under this regime reached a plateau or declined in the sixth week. No pronounced skin lesions had developed, but the fur of most of the animals was rusted. The feeding of zinc chloride in these larger quantities gave considerable difficulties due to the caustic and nauseating effect of the salt even when dissolved in oil. Therefore, the desired amount of zinc chloride was incorporated in 5 grams of the food mixture which was offered the animals after 16 hours fasting and before the regular food cups were returned to the cages. Some animals refused the food containing the zinc chloride but most animals completely ate up the mixture. This change of experimental conditions produced a prompt increase in weight even in the animals which had eaten the full amount of zinc chloride mixed in food. After it was found that the weight increase improved in the second week of this feeding technic, the experiment was terminated.

ANALYSIS OF EXPERIMENTS

By feeding a group of rats a diet suboptimal in its content of pantothenic acid but protective against clinical manifestations of pantothenic acid deficiency, it was possible to demonstrate a toxic effect of zinc chloride. This toxic action was visualized by a vitamin deficiency syndrome consisting of retardation of growth, expressed by weight, and skin manifastations which have been known to result from pantothenic acid deficiency. The curative effect of synthetic calcium pantothenate added in amounts of 150 micrograms per day to the ration S.O. further suggests that a pantothenic acid or filtrate fraction deficiency had been precipitated by the feeding of zinc chloride. One is justified to argue that the action of zinc consisted in the destruction of the unsaturation of the ingested essential fatty acids in the intestinal tract.

We raise this question for the following reasons: Animals kept on diet S.O. from which all fatty acids had been removed by eliminating wheat germ oil, cod liver oil and ethyl linoleate,

developed a severe deficiency which is characterized by very poor weight curves, spectacled eye condition, panophthalmia and death within six weeks. Identical symptoms developed if biologically inactive fatty acids were added to this fat free diet. In several of our experiments of this kind we used a condensation product resulting from heating the linoleate in the presence of zinc chloride. Against the inactivation of the unsaturated fatty acids by zinc chloride *in vivo* speaks the fact that 150 micrograms of calcium pantothenate per day had no curative effect on the deficiency

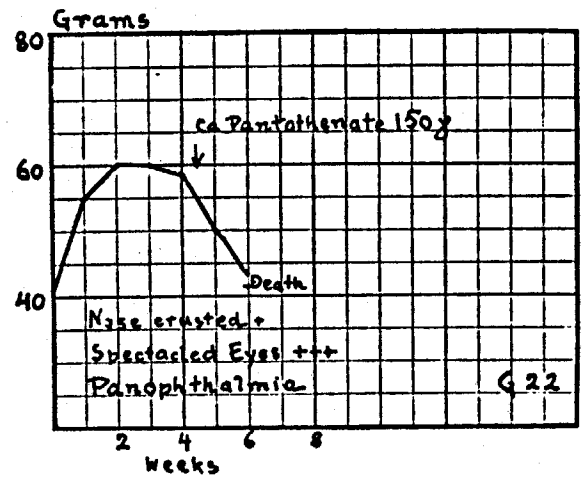

FIG. 4. WEIGHT CURVE OF A WHITE RAT FED A FAT FREE DIET S.O., 150 MICROGRAM CALCIUM PANTOTHENATE PER DAY HAS NO EFFECT ON ADVANCED DEFICIENCY

produced by the fat free diet low in pantothenic acid. The animals thus tested died within a week to ten days with a rapidly declining weight curve. The prompt weight response and the time of cure of the deficiency syndrome as observed in Group 2 of our animals following the feeding of 150 micrograms of pantothenic acid corresponds perfectly to the effect of high pantothenic acid supplements on rats which had developed filtrate fraction deficiency.

The observation that the mixing with the food neutralized the toxic effect of zinc chloride suggests that a chemical combination had been formed with a substance in casein. This would agree with the statement of Eichholtz (4) that milk inhibits the toxic

effect of zinc chloride. The same author also refers to the lipo solubility of zinc chloride which accounts for its high toxicity compared to other zinc salts. We believe that the lipo solubility and higher reactivity of zinc chloride is responsible for the successful demonstration of zinc toxicity in the rat. A caustic effect on the intestinal wall can be ruled out since the animals receiving curative doses of pantothenic acid recovered despite the combined feeding of zinc chloride. It seems more probable that zinc in the form of zinc chloride exerts an action on metabolic processes in the intestinal wall or that the absorption of zinc ions into the portal circulation is higher when zinc chloride is used.

DISCUSSION

An indication of the toxic effect of prolonged feeding of zinc in rats has been reported by W. R. Sutton (5) who, using zinc carbonate in amounts of 0.5 per cent of the food ration for 39 weeks, observed the following symptoms: Retarded growth to 80 per cent normal weight, diminished food consumption and anemia due to lowered hemoglobin of the erythrocytes which were not diminished in number, loss of fertility in female rats, and greatly increased urine volume in male rats.

Zinc has been proven an essential metal for the nutrition of the rat by Hove, Elvehjem and Hart (6, 7) as well as by Day and McCollum (8). Zinc deficiency, according to these authors, results in the reduction of the alkaline phosphatase of the serum. The action of zinc on various enzymes has been studied in vitro. Hove, Elvehjem and Hart (7) have shown that zinc activates the hydrolysis of sodium hexosediphosphate and sodium pyrophosphate by crude intestinal phosphatase while crude bone phosphatase was inhibited progressively by increasing concentration of the zinc ions. In another publication, the same authors (9) reported on a marked inhibition in vitro of carbonic anhydrase by relatively large amounts of inorganic zinc. The diphasic action of zinc was also shown by Lohman and Kossel (10) in the case of yeast decarboxylase which was inhibited or stimulated according to concentration.

If an enzymatic function is activated by small (physiologic)

amounts of the metal and inhibited by large (toxic) quantities, one would expect similar biological effects from the deficiency of an essential metal as one obtains from its toxic inhibitory action. It is, therefore, not surprising that Day and McCollum (8) described retarded growth, marked eczema, and some alopecia in several of their zinc deficiency animals, a symptomatology closely resembling that of our zinc poisoned rats.

We are now engaged in enzyme studies of rats showing the effect of chronic zinc poisoning and hope to obtain some information on the relationship of pantothenic acid and unsaturated fatty acids to the metabolism of the rat.

Our experimental conditions using suboptimal doses of pantothenic acid have been helpful in demonstrating the biologic effects of chronic zinc chloride poisoning, but have also brought out the importance of an optimal diet for the protection against chronic metal poisoning. They have further suggested that pantothenic acid has a specific curative effect on zinc chloride intoxication.

The prevention of metal poisoning by dietary measures has become a fruitful field since Eisner (11) first proved that green fodder, beets, etc. prevented death in rabbits poisoned by uranil nitrate.

Recent studies on chronic selenium poisoning of the rat by Smith (12, 13) and also by Lewis (14) and co-workers have called attention to the protective action of a high protein diet against the toxicity of seleniferous wheat flour.

CLINICAL IMPLICATION

Since it has been shown in our experiments that a metallic poisoning can produce a clinically recognizable deficiency disease, one is justified in asking how many of the symptoms due to chronic heavy metal intoxication are subclinical manifestations of vitamin deficiency, produced by the injurious effect of the therapeutic metal used. The varying sites of organic injuries by heavy metals may have a close relation to the resulting impairment of enzymatic functions.

A better knowledge of the nutritional factors which exert a protective action against metallic poisons may eventually be of

help in the successful prevention of arsphenamine and other heavy metal reactions, not based on an allergic mechanism.

Only then will it be possible to place our dietary regulations for the patient receiving anti-syphilitic treatment on a rational basis with regard to the protein-fat-carbohydrate balance and to make the proper selection of the vitamins which offer the best protection against a given heavy metal.

SUMMARY AND CONCLUSIONS

Young female rats kept on a synthetic diet and a filtrate fraction low in pantothenic acid were fed zinc chloride dissolved in oil in daily doses of 4–6 mgm. by syringe. Most animals developed deficiency symptoms which were completely absent in control animals receiving the identical diet with the omission of zinc chloride.

The deficiency syndrome thus produced in a group of animals could be cured by additional feeding of 150 micrograms of calcium pantothenate per day.

Larger amounts of zinc chloride were necessary to produce a weight plateau in rats fed the same diet with the addition of 100 micrograms of calcium pantothenate.

If zinc chloride was added to 5 grams of the food mixture and fed, its toxic effect was apparently lost.

It is believed that the toxic action of zinc chloride in oil directly introduced into the intestinal tract is due to the higher activity of zinc ions thus fed.

The interaction of zinc with known enzyme systems and the rôle of pantothenic acid are under investigation.

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DISCUSSION

Dr. Paul Gyorgy, *Cleveland*: My simple excuse for discussing this paper is that the speaker has asked me to comment on his paper with the emphasis on "*kindness*." It is very easy for me to be "kind" because the paper is a very important contribution to experimental dermatology, and that makes my task even easier, because this paper is "right down my alley" for two reasons: (1) it popularizes experimental dermatology, in which I have been interested for the last 12 years, and (2) it is a very good example of the problem which has lately become very acute—and that is the question whether we are dealing with intoxication in a special pathologic condition or deficiency. I will not go into details. Here, you see, one could, from one point of view describe this condition as zinc intoxication and the production of the disease by the effect of zinc being added to the diet. On the other hand, it is a deficiency, because the effect of zinc chloride can be neutralized. The common denominator is pantothenic acid deficiency. The need for panththenic acid is greater.

I am reminded in this connection of only one other example where the intoxication versus the deficiency consists of metal and vitamin, that is manganese and vitamin B-1. Perla has shown a few years ago that the addition of manganese or a manganese compound to the diet had a toxic effect on rats, leading to cannibalism, diminished fertility, and by raising the B-1 level the effect of manganese can be neutralized. There is a definite relationship between manganese and thiamin,

vitamin B-1. The explanation may be easily furnished. Vitamin B-1 is very important and necessary for decarbolylation of pyruvic acid, which is in turn, absolutely essential for the metabolism of carbohydrates, and manganese is one of the most important catalytic activators of the system.

I think Dr. Gross is on the right track when he also looks for such an interrelationship in the enzyme system and pantothenic acid.

DR. ARTHUR SCHOCH, *Dallas*: I would like to ask a question and make one observation. It seems to me that the most obvious explanation for the problem just before us is that zinc combines with pantothenic acid to give an insoluble substance. That is so simple and obvious that I hesitate to mention it, but it has not been mentioned at all to date.

I would also like to recount an observation made in our clinical practice for the past six months, and that is that giving large doses, of 40–50 mgm. intravenously has sharply reduced the incidence of gastro-intestinal reactions to pentavalent arsenicals. This has been mainly vomiting, but the same individuals, when given 40-50 mgm. of vitamin B-1, lose all of their gastro-intestinal intolerance to the quinqui and pentavalent drug; this is not true in every case, but in 50 to 75 per cent of our cases this has been found to be true.

DR. LOUIS SCHWARTZ, *Bethesda, Md.*: I realize the great importance which this paper plays in our national defense. If the observations which were made for animals hold for human beings, then it is of great importance in the industrial fields. A great deal of the materials, air planes, etc., being manufactured for national defense require the manufacture of dyes made of zinc and lead alloys, and the industrial engineers are busy devising ventillating systems to prevent zinc and lead poisoning among the workers who make these dyes. If pantothenic acid is given to these people, and has the same effect as it had on the animals, then I think Dr. Gross really deserves a medal of honor.

DR. J. GARDNER HOPKINS, *New York, N. Y.*: This paper has interested me particularly because it throws light on a thing which has been puzzling us for so long—that conditions which we call vitamin deficiencies because they are cured by high vitamin administration, occur in patients whose intake of vitamins seems to have been normal.

Many hypotheses have been presented to explain this phenomenon, such as failure of absorption, and inadequate utilization in the liver. Probably the explanation is not the same in all cases. This work indicates that inactivation of an enzyme by a poison may create a demand for an excessive amount of the vitamins which form that enzyme.

DR. PAUL GROSS, *New York*: I am grateful to Dr. György for his discussion. His reference to manganese and vitamin B_1 is very interesting and would run parallel to our findings. On the other hand, we know that manganese deficiency leads to graying of hair in rats.

In answer to Dr. Schoch's question, zinc has no destructive effect on pantothenic acid. Actually, zinc pantothenate has been used in the early experiments and found to be an effective supplement. As far as the beneficial effect of liver

therapy in arsphenamine reactions is concerned, we cannot definitely state the factor in the B-complex responsible for the effect. That detoxication is not a function of vitamins only has been demonstrated recently by Martin and Thompson, especially for amino acids.

It was very gratifying to hear the remarks of Dr. Schwartz and we hope that our work will eventually lead to a rational prophylaxis against chronic metallic poisoning in industry.

I am obliged to Dr. Hopkins for the support given by his discussion and particularly for the encouragement offered to us throughout the whole experiment.

J. AMER. PHARM. A. (SCI. ED.) 42: 700-702. (1953).

Blood and Bone Marrow Effects of Feeding Zinc Sulfate to Rats and Dogs*

By ERNEST C. HAGAN, JACK L. RADOMSKI, and ARTHUR A. NELSON

Because of its emetic properties zinc sulfate has been proposed for inclusion in barbiturate preparations to prevent deaths due to overdosage of these hypnotics. Since this would lead to chronic ingestion of zinc sulfate in persons receiving prolonged barbiturate medication, we have studied the chronic toxicity of this emetic. Rats were fed zinc sulfate ($ZnSO_4.7H_2O$) incorporated in ground commercial Purina Laboratory Chow at 1,000, 500, 100, and 0 parts per million for twenty-one months. Four of six dogs all maintained on Purina Chow received zinc sulfate by capsule for seventy weeks. The initial daily dose for dogs was 200 mg./Kg. Emesis forced a change of dosage to 100 mg./Kg. at seven weeks, and again in three of four dogs at thirty-two weeks to 50 mg./Kg. The other dog was changed because of emesis to 50 mg./Kg. after three weeks on the 100 mg/Kg. level. Effects observed in rats were microcytosis, coupled with polychromasia in some cases, and hyperchromia in others, and a hypochromic anemia in dogs. The blood effect noted in rats was reversed despite continued zinc feeding. The average myeloid-erythroid ratio of rat bone marrow smears taken at the termination of the experiment ranged from 1.16 to 1.35 for the various groups of zinc animals and read 2.14 for the untreated controls. Dog bone marrow also studied at the termination of that experiment was slightly hyperplastic. Although no differential marrow count was made, the myeloid-erythroid ratio appeared unchanged.

ZINC SULFATE is used medicinally as an emetic, and this has led to the suggestion by Miskimon and Miskimon (1) that it be added in small amounts to barbiturate preparations so as to cause vomiting when these drugs are taken in overdosage either accidentally or with suicidal intent. A corollary of the use of this drug combination would be the ingestion and retention of zinc by the person using reasonable daily amounts of barbiturates. When confronted with this latter possibility we felt it desirable to study the chronic effects of feeding zinc sulfate to laboratory animals.

Smith and Larson (2) fed zinc carbonate to rats for five weeks at a level providing 1.0 per cent zinc and observed a microcytic hypochromic anemia together with retardation of growth. The hemoglobin deficiency failed to develop when a mixture of iron, copper, and cobalt were fed in the anemia-producing diet, while dietary liver produced significant growth response in the retarded animals. Sadasivan (3) fed zinc oxide to rats at 0.5 and 1.0 per cent for fifteen days, and found it to exert a lipotropic effect. Further findings were failure to grow normally and retardation in development and mineralization of bone. At the 1.0 per cent level of zinc oxide feeding Sadasivan (4) observed rats to excrete increased amounts of nitrogen through urine and feces. At 0.5 per cent dietary zinc oxide urinary excretion of phosphorus and sulfur decreased, while urinary excretion of uric acid and creatinine increased. Fecal excretion of phosphorus and sulfur increased. Sutton and Nelson (5) fed rats zinc carbonate at levels corresponding to 0.10, 0.50, and 1.0 per cent zinc. The 1.0 per cent level was found overwhelmingly toxic while impairment of reproduction and decrease in hemoglobin concentration occurred at 0.5 per cent. Hegsted and co-workers (6) have reviewed the literature pertaining to zinc. Drinker, *et al.* (7), reported negative results for production of anemia after administering zinc oxide to dogs and cats. Doses fed to dogs were 1,000 and 500 milligrams per animal for feeding periods of fifteen, and three to nineteen weeks, respectively. Doses ranging from 600–1,000 milligrams were poorly tolerated by cats, and the maximum time such levels were fed was twenty-one weeks. The longest period of feeding at any level was that fed to 1 cat and measured 175 milligrams for eight weeks, then 354 milligrams for forty-five weeks. Drinker, *et al.* (8), fed zinc to rats in daily doses of 0.5 to 34.4 milligrams for from thirty-five to fifty-three weeks. Zinc was fed as the oxide, acetate, citrate, and malate. Blood studies made prior to autopsy failed to reveal abnormalities.

EXPERIMENTAL RESULTS

A.—Four groups of eight Osborne Mendel weanling rats (4 males and 4 females) were fed *ad libitum* a basal diet of ground commercial Purina Chow containing 1,000, 500, 100, and 0 p. p. m. of zinc sulfate ($ZnSO_4.7H_2O$). The rats were individually housed in wire mesh cages. Food consumption and animal weight were recorded weekly. Eight complete blood counts were made which were confined to the

* Received March 25, 1953, from the Division of Pharmacology, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.

first three and last five months of the experiment. A study of bone marrow smears was made at the termination of the experiment.

During the approximately twenty-one-month feeding period, food intake and weights of the rats fed zinc varied within normal limits. Hemoglobin values, red cell, white cell, and differential counts remained normal throughout the experiment. However, inspection of smears showed the occurrence of microcytosis, coupled with polychromasia in some cases and hyperchromia in others. These blood changes occurred at all levels of feeding, and were first observed at the 1,000 p. p. m. level on the resumption of blood studies at the sixteenth month. One month later this change was noted at the 500 and 100 p. p. m. levels of feeding. Despite the continuation of these rats on the zinc-containing diets the red cells subsequently returned to normal size. Counts (500 cells each) of the bone marrow smears taken at autopsy revealed the average myeloid-erythroid ratio (M/E) of rats fed the basic diet to be 2.14 and of the rats fed all levels of zinc sulfate to vary from 1.16 to 1.35. The bone marrow data are given in Table I.

At autopsy, there were no gross pathological changes attributable to zinc, with one possible exception. The kidneys of the male rats on 500 and 1,000 p. p. m. of zinc sulfate were larger and more granular than those of 100 p. p. m. or the controls. Unfortunately, the kidneys were not weighed. The kidneys of the females were only rarely enlarged or granular, and there was no difference between groups. Liver weights, when expressed as percentage of body weight, showed no difference between groups. All 32 rats were to at least some extent examined microscopically. Six animals given 1,000 p. p. m. zinc sulfate and 6 controls, evenly divided by sex, were examined in detail, while for the remaining 20 animals sections were limited to liver, kidney, testis (if male), and in 8 instances bone and bone marrow. Only the kidney and bone marrow showed microscopic changes which could be attributed to zinc. In the bone marrow sections the cellularity of the marrow was alike for all groups, and within normal limits, but the M/E ratios appeared altered, as was better shown by the actual counts of smears. The kidneys of the males, both control and treated, commonly showed at least a slight degree of the spontaneous nephritis (sometimes called nephrosis) which is frequently seen in older rats. The condition is described and illustrated by, among others, Saxton and Kimball (9). Among all the females there was but one in which this condition was present in as much as a moderate degree. Among the males, however, there were 6 with the condition in moderate degree, and 5 with it in severe degree. All the latter 5 animals were in the 500 and 1,000 p. p. m. groups, suggesting, if not proving, that zinc increased the severity of the normally occurring nephritis or nephrosis in males.

B.—The toxicity of zinc sulfate to dogs was also determined. A litter of 6 Dalmatian puppies, ten weeks old, was selected for the experiment. Four of the 6 dogs were given zinc sulfate by capsule at a daily level of 200 mg./Kg. to start. Complete blood counts were taken every two weeks at the start of the experiment and at longer intervals thereafter. No counts were taken from the eighth to the thirtieth week of the experiment because of the absence of a technician. After seven weeks of zinc sulfate administration at this level most of the dogs began to vomit consistently after their doses, and the daily dosage level was reduced to 100 mg./Kg. After three weeks on this dose, dog No. 5 began to vomit again, and its dose was reduced to 50 mg./Kg. daily. After thirty-two weeks on 100 mg./Kg. daily, the other 3 experimental dogs began to vomit fairly consistently after dosage and their daily dose was likewise reduced to 50 mg./Kg. At this time dog No. 6 was losing weight and appeared to be in bad physical condition, though all other dogs showed normal appetite and growth throughout experimentation. This dog was sacrificed *in extremis* one week later. After seventy

TABLE I.—BONE MARROW COUNTS—ZINC SULFATE

1,000 P. P. M.				500 P. P. M.			
Animal Number	Total Myeloid, %	Total Erythroid, %	M/E Ratio	Animal Number	Total Myeloid, %	Total Erythroid, %	M/E Ratio
5605	49	44	1.11	5613	50	38	1.32
5606	48	41	1.17	5614	51	41	1.24
5607	51	40	1.27	5615	48	45	1.07
5608	49	43	1.14	5616	49	36	1.33
5609	55	45	1.22	5617	50	44	1.14
5610	44	46	0.96	5618	47	40	1.17
5611	50	48	1.04	5619	49	47	1.04
5612	58	40	1.42	5620	46	46	1.00
Av.	50.5	43.0	1.17	Av.	48.7	42.1	1.16

100 P. P. M.				Control			
Animal Number	Total Myeloid, %	Total Erythroid, %	M/E Ratio	Animal Number	Total Myeloid, %	Total Erythroid, %	M/E Ratio
5621	52	40	1.30	5629	60	28	2.14
5622	60	33	1.52	5631	58	29	2.00
5623	57	40	1.43	5632	63	29	2.17
5624	53	40	1.33	5633	67	21	3.19
5625	54	37	1.73	5634	64	28	2.28
5626	45	46	1.00	5635	64	34	1.88
5627	49	45	1.09	5636	54	38	1.42
5628	53	37	1.43				
Av.	52.8	39.8	1.35*	Av.	61.4	29.6	2.14

* Probability <0.01.

weeks the experiment was terminated and all surviving dogs were sacrificed.

Blood studies of the dogs receiving prolonged daily doses of zinc sulfate showed that although all 6 dogs had, because of their young age, low hemoglobin values at the start of the experiment, they soon attained normal adult hemoglobin values. However, the 4 experimental dogs all eventually developed hypochromic anemia, in that the hemoglobin values declined while the red cell counts remained at normal levels (Table II). No significant effects on the white blood count, total or differential, were noted. Histopathological examination of from 20 to 25 tissues of each of these dogs revealed the only change attributable to zinc to be in the bone marrows, which were uniformly slightly hyperplastic as compared with the controls.

TABLE II.—THE EFFECT ON THE BLOOD HEMOGLOBIN OF ZINC SULFATE ADMINISTRATION TO DOGS

	Hemoglobin—Gm. per 100 Cc. of Blood					
	Untreated			Treated		
Weeks	Dog No. 1	Dog No. 2	Dog No. 3	Dog No. 4	Dog No. 5	Dog No. 6
0	10.0	12.0	10.0	10.0	8.5	10.5
3	10.5	11.0	11.5	11.5	10.5	11.5
5	12.2	12.0	14.0	13.0	13.0	11.9
7	13.0	12.0	11.0	13.0	11.0	11.5
31	14.0	14.0	12.0	13.5	12.8	12.5
39	13.5	15.0	13.5	13.5	11.2	9.0[a]
53	14.0	15.0	12.5	14.0	13.0	...
61	15.0	14.0	10.8	11.0	12.0	...
70	14.0	14.0	10.5	10.0	9.0	...

[a] This value was taken before the dog was sacrificed after 33 weeks on experiment.

Marrow counts were not done on the dogs, but by inspection of sections and smears the M/E ratio did not appear altered.

C.—It was believed desirable to attempt to confirm the results reported by the Miskimons that zinc sulfate is effective in preventing poisoning by fatal doses of pentobarbital. We determined that 8 Gm. of zinc sulfate consistently produced vomiting in twenty to forty-five minutes in dogs concomitantly receiving 450 Gm. of horse meat. Then, to a group of 4 dogs, 8 Gm. of zinc sulfate and 4 Gm. of pentobarbital were administered along with 450 Gm. of horsemeat. This experiment was repeated, omitting the horsemeat. Whether or not the administration of the zinc sulfate-pentobarbital combination was accompanied by the feeding of horsemeat, all 4 of the dogs exhibited emesis in twenty to forty-five minutes. Only 1 of the 4 exhibited sedation, this lasting in both experiments from fourteen to eighteen hours. The other 3 dogs showed no effects from the pentobarbital administration. However, when the dogs were given the same dose of pentobarbital alone, all 4 succumbed within thirty hours. These results indicate that zinc sulfate is effective in preventing poisoning by fatal doses of pentobarbital.

DISCUSSION

Because of the relative leveling off of the growth of rats at about the sixteenth week, the amounts of zinc sulfate subsequently ingested measured about 4–6 mg./Kg. daily, at the 100 p. p. m. level. To correlate this and human consumption it is felt that the ingestion by a human being of a single overdose of 10 or even fewer therapeutic doses (usually 100 milligrams each) of a barbiturate, could easily have serious consequences. The human emetic dose of zinc sulfate is commonly accepted as being from 1 to 2 Gm. The Miskimons found emesis to occur in dogs with 0.3- to 0.5-Gm. doses of zinc sulfate given in single capsules. An increase in the amount of zinc sulfate was found necessary when the dose was divided into several capsules. These investigators also found that the usual dose of zinc sulfate required for emesis in dogs was considerably increased by the simultaneous administration of pentobarbital. It seems probable therefore that each hypnotic capsule intended for human use might have to contain several hundred milligrams of the emetic. The daily intake of zinc sulfate per kilograin of body weight in humans would then be comparable to that which was found to produce adverse effects in rats. It is felt, therefore, that this emetic should not be taken over prolonged periods in amounts that might be included in reasonable barbiturate dosage.

SUMMARY

Zinc sulfate was fed to weanling rats at 1,000, 500, and 100 p.p.m., and to dogs at 200 mg./Kg. day initially, then 100 mg./Kg., and finally 50 mg./Kg. The dose in dogs had to be reduced because of emetic effects. The effects resulting in the rat were microcytosis coupled in some cases with polychromasia and in others with hyperchromia. No reduction of red cell numbers or increase in hemoglobin values occurred. Hypochromic anemia was observed in the dog. The blood changes observed in the rat returned to normal despite continued zinc feeding. The rat bone marrow showed a myeloid-erythroid ratio average of 1.16 to 1.35 in the various groups of zinc animals in contrast to the value of 2.14 found in the untreated controls, all taken on 500 cell counts. The intake of zinc sulfate at the lowest level of rat feeding was approximately 4–6 mg./Kg. daily for the last seventy-seven weeks of feeding. The dog bone marrows showed slight hyperplasia as compared to the controls, with no change in the myeloid-erythroid ratio apparent on inspection.

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From the Pharmacologic Institute of the Medical Academy Düsseldorf
(director: Prof. Dr. F. Hahn)

Investigations about acute Zinc-intoxication

by

F. Hahn and R. Schunk

(received on June 20, 1955)

Zinc is one of the metals, the toxic effect through peroral intoxication of which is very much dominated by the local irritant effects (Eichholtz). It is not possible to clearly picture the share of resorptive effects to oneself on the basis of the literature. In the casuistic literature corresponding indications are only partially existant (comp. Esser). In renowned text-books (Sterkenstein; Flury and Zangger; Patty; Godman and Gilman) the opinion can be found that the zinc is not sufficiently resorbed in order to cause general symptoms. Most probably the practical experiences with zinc-chloride are a factor, which, disregarding some few cases of zinc-sulphate intoxication, dominates the chemistry of lethal intoxications (comp. to synopsis by Esser). Very often high percent (50%) $ZnCl_2$-solutions have been drunk, whereby the strong corrosive actions were specially noticed and led to the conclusion that with zinc intoxications the concentration rather than the dosis is important (Flury and Zangger).

The clinical material should hardly be enough to clarify the relations of the effects of zinc to the dosis and to the concentrations independantly from each other. When drinking a high percent zinc-chloride solution usually the assimilated amount of zinc is larger than in the case of low percent solutio resp. in the case of other zinc-salts, where as high percent a solution as with the zinc-chloride do not appear. Clearer pictures of a resorptive effect not overlapped by the corrosive action are therefore less likely to be seen.

The question about the toxic effect of low concentrations is significant for those substances as well, which become soluble in the acidity of the stomach;; as is the case with e.g. zinc-oxide and zinc-sulphide. Here for the occuring chloride-concentration there is a limit set by the intensity of the density (o.3-0.4%).

This question is also interesting, because a mixture of zinc-sulfide and barium-sulphate, the so-called lithopone, is a constituent of the whites paint in children's watercolour paint-boxes. The hazards of a possible swallowing of

such white paint can be judged with the more accurate knowledge of the toxic properties of lithopone. Lithopone of 1 mol of zinc-sulphide and 1 mol of barium-sulphide and a zinc-sulphide content of about 30% is called "Rotsiegel" (comp. Ulmann). A lithopone of 60% zinc-sulphide content is called "Silbersiege. A "sacchtolith" is a zinc-sulphide preparation without barium-sulphate with special pigment properties. The zinc-sulphide has been exposed to an annealing and chilling process in order to increase the opaqueness of these products.

Zinc-chloride ($ZnCl_2$)

During experiments on rats we examined the dependence of a peroral zinc-intoxication of dosis and concentration. Over a concentration range of 2-50% the average lethal dosis of almost 0.75g/kg does not change and this shows that the zinc-chloride intoxication is lastly independant of concentration . Only the course of the intoxication showed differences dependant upon concentration. The animals died faster on the average, whenever the average lethal dosis was administered in low concentrations. The death rate decreased within the first 24 hours from over 50% with 2-5% solution to 0% with 50% solutions. An equilibrium appeared with the 20-50% solutions, caused by late death cases after 2 1/2 to 11 days. In these cases a difficult local diagnosis revealed the cause of death: open rupture of stomach ulcers into the free abdominal cavity plus peritonitis, concealed perforations with epilones of penetration into the liver tissue as well as pylorusstenoses.

Smaller local damages (scarred shrinkage of the stomach, plain ulcers of the mucous membrane, recovering penetrated ulcers) were found in a number of animals still alive, killed after 4-12 weeks. Out of table1 it can be seen that these local diagnoses were dependant upon dosis and concentration with increasing percentage.

The early cases of death only showed a superficial corrosion of mucous membranes and bledding. As the cause of death we see the resorptive effect of the poison in the first place, as we mostly observed the typical, central-nervous symptoms of zinc-intoxication in these animals: tremor, disturbed movement coordination, the fur standing on end(temperature depression), respiration difficulties and cyanosis. With increasing $ZnCl_2$-concentration these symptoms became weaker and rarer. The specially characteristic zinc-tremor was finally missing in the experiments with 50% solutions.

Local and resorptive effects thus show a mutual dependence on the dosis however, a reciprocal dependency on the concentration within the middle and lower range.

Obviously local and resorptive zinc-chloride effects exclude each other to a certain extent. The independence of $ZnCl_2$-mortality of the concentration,

which at the first sight appeared to be amazing, thus finds an explanation.

table 1. Peroral zinc-chloride intoxication of rats (probang-feeding). The numbers in brackets show the number of animals with a pathologic diagnosis. (see text)

dosis g/kg	concentration %	death after hours	death after days	surviving no.	killed after (days)

1 Using "% solution the dosis of 0.75 g/kg for 7 animals was devided in to 2 evenly large sizes, administered with an intermission of 3 hours. Of these 4 animals died. Of the remaining 8 animals, which received all of the dosis at one time, 6 died. The dosis of 1 g/kg in a 2% solution was only devided into 2 administrations.

table 2. Effect of perorally administered zinc-chloride on rabbits.

dosis g/kg	concentration %	no.	death after 1 day	2 days	3 days	13 days
					perforated ulcer	perforated ulcer

With the absolutely lethal dosis of 1 g/kg the late cases of death with severe local diagnosis were also missing at the higher concentration. Obviously enough is resorbed, to cause an acute death.

Informing experiments with 5 and 20% solution showed a similar picture (table 2): late cases of death plus perforation after 3-13 days only with the higher concentrations, only cases of death only dependant on the dosis. A reciprocal dependency on the concentration might not be discernible, because of small number of experiments.

Out of the size of the intrvenous lethal dosis for rats (table 3) it follows that at least 1/30 of the administered dosis is resorbed through the stomach-intestinal canal. About the same amount is assimilated through subcutanous injection. In contrast to this the resorption through intraperitoneal injection of a diluted solution (0.5%) is considerably more favourable, as the lethal dosis is only about twice as big, in comparison to the intravenous.

table 3. $ZnCl_2$-effect with intravenous, intraperitoneal and subcutanous administration on rats.
mortality = number of dead animals/total number

dosis g/kg	concentration %	intraven. mortality	intraven. time	intraperiton. mortal.	intraperiton. time	subcutan. mortal.	subcutan. time
			7 min. to 3 days		3 hours to 5 days		7 hours, 12-34 days

When discussing the resorption-value a certain decontamination factor for slow resorption has to be accounted for. The low tendency of accumulation which e.g. allows to inject intravenously 2/3 of the absolutely lethal dosis (i.e. 0.02 g/kg) on at least 7 subsequent days, supports a fast decontamination of the zinc. A habituation to the zinc is thereby unimportant, at least inasmuch as we found 0.03 g/kg fully effective on the 5. day after 4 administrations of each 0.02g/kg.

The same typically nervous symptoms appear also intravenously. At one time or the other the supposition has been expressed, that the effect of the intravenous $ZNCl_2$ injection has to do with the precipitation processes on the blood-protein (comp. Schwartze and Alsberg), According to in vitro- experiments of our own protein precipitations occur in the serum with concentrations, which could be reached with the intravenous injection of lethal doses. However, the serum-precipitation can be neutralized by sodium thiosulphate (7.22 parts per 1 part $ZnCl_2$, comp. Lebduska), wihtout preventing the lethal effect. Thiesulphat alone was intravenously toleratdd in a dosis 5 times as large.

In contrast to this the peroral poisonousness of $ZnCl_2$ was increased by sodium thiosulphate. The average lethal dosis can be lowered to 0.25 g/kg (5%), i.e. 3 times by addition of 1.8 g/kg thiosulphate.

Table 4. Effect of Sulfactin (S) on the intravenous and peroral zinc-chlori intoxication of rats.

dosis and form of administration	No. of animals	dead after						survivir and killed after 3E
		15min.	2-5hours	6-12hours	1day	2-3days	etc.	

U_3= small ulcer of the muccus membrane, U^1= penetrated ulcer, U^2=perforated ulcer U^3= ulcer plus pylorustenosis

Technique of the Sulfactin administration.

1. Intravenous zinc-chloride-injection: 3 hours before, 1/2 hour after the zinc-dose and on the following day each 50 mg/kg S. subcutaneously. Or: 1 hour before, 5 hours after the zinc-dose and on the following day each 20 mg/kg S.i.p as well as on the day after the next 10 mg/kg.

2. Peroral Zinc-intoxication: 1 1/2 hours before, 15 min. and 4 hours after the zinc-feeding each 50 mg/kg S subcutaneously. Or: 1 hour before, 1,3,and 5 hours after the zinc-dose as well as on the following day 20 mg/kg and on the day after the following 10 mg/kg S.i.p.

6 times the amount of this sulphate alone was survived. The thiosulphate did not have a poisonousness increasing effect on the effect of intravenous subletha doses (0.02 g/kg). Undoubtedly by suppression of the local corrosive action the resorption under the influence of thiosulphate is favoured, whereby the solidity of the complex does suffice to suppress the toxic effect after the resorption.

The mortality after intravenous $ZnCl_2$ doses can be clearly reduced by

parenteral pre- and after-treatment of the rats with BAL (Sulfactin "Homburg") diminishing the nervous symptoms, and the course of intoxication of the animals not rescued can be temporary delayed.

With parenteral Sulfactin treatment the resorptive effect of peroral zinc doses had to be affected and hereby its share of the over-all intoxication could be shown. Table 4 shows that the acute mortality is actually very much reduced. On the other hand the number of late cases of death with the appearance of severe ulcers, which are not usual with these concentrations, is increased. Thus the animals are brought into a state by the sulfactin treatment, where the not influenced local damages can take effect. Administeirng a usually not accutely lethal dosis of 1 g/kg we once saw with Sulfactin a severe local damage (Pylorusstenosis) with late death. The decreasing mortality by Sulfactin thus is only small.

table 5. Acute poisonousness of zinc-sulphate and zinc-acetate on rats (death after 1-4 days) mortality=number of dead animals/total number of guinea-pigs.
The highest zincsulphate concentration and zinc-acetate concentration had to be prepared hot and kept warm (30°C)

Salt and form of administ.	dosis g/kg expressed by the isomolar $ZnCl_2$-amount	concen. %	mortality	concen. % see first concen.	mortality	concen. % see first concen.	mor.	cc % se 1s cc

1. Two more animals died after 12 resp. 16 days with stomach diagnosis, out of those one because of severe degenerative heart deformation.
2. Another animal died after 15 days because of stomach perforation and pertonit

II. (ZnS) Zinc-sulphate and zinc-acetate

There is no difference in the lethal doses of zinc-sulphate and zinc-acetate in comparison to isomolar amounts of $ZnCl_2$ supplied perorally, subcutaneously, intravenously, and intraperitoneally (table 5). The cases of late death with a perforation of the stomach wall after peroral application were missing. (with the exception of one rat, which died 15 days after administering zinc-acetate, corresponding to 1g/kg $ZnCl_2$ in a 20% solution.) The zinc-acetate could not be examined in its top concentration, because of its lower solubility. Corresponding to the lower corrosive action of the sulphate and acetate with high concentrations as well the fast resorptive effects are emphasized. When dissecting the surviving animals killed after 4 weeks the mucous memebrane after the sulphate intoxication was free of macroscopic obvious deformations. Only after zinc-acetate with an average lethal dosis in 20% solution in one of 7 animals a penetrating ulcer, furthermore in one of the remaining animals a

recoverod ulcer with scar formation and a scarred and coalesced with the ulcer were to be observed.

After subcutaneous injection within about a week the same skin-necroses were developed as with $ZnCl_2$. After interperitoneal application the dissection diagnosis of the animals killed after 4-5 weeks showed the same signs of a passed peritonitis as after $ZnCl_2$.

III. Zincsulphide, lithopone, sacchtolith, and Zinc-oxi

1. Experiment with rats: for these experiments a suspension of 20% with an addition of tragacanth gum was fed with a probang. For 4 weeks the general behaviour and the weight were observed.

Technical ZnS: Experiments on 18 days showed a compatibility up to 20 g/kg, 10 animals 40 g/kg.

Sacchtolith: 5 animals survived 20 g/kg, 10 animals 40 g/kg.

Lithopone "Rotsiegel": 3 animals survived 10 g/kg, 3 20 g/kg.

Lithopone "Silbersiegel": 5 animals got 20 g/kg, 10 animals 40 g /kg.

Of each of the two groups one animal died after 13 resp. 10 days because of pneumonia. The remaining animals survived.

Zinc-oxide: The compatibility went up to 12 g/kg (2 rats).

2. Experiments with rabbits: 2-3 kg heavy animals got the zinc-compounds with tragacanth gum in a suspension with 25-50 cm^3 water. Within the following 4-9 weeks the weight, blood and urine was constantly controlled.

Technical ZnS: 3 g/kg was tolerated by 2 animals. After feeding 6 g/kg 2 out of 3 animals died after 7 and 21 days. One animal had dissentry, the other pneumonia and histologically a severe diffuse damage of the liver-parenchyma. One animal survived.

Sacchtolith: 3 animals survived 6 g/kg.

Lithopone "Silbersiegel": 5 animals toelrated 5 g/kg.

Experiments with 10 g/kg proved to be difficult to be carried out, because an overfilling of the stomach was caused by this amount.

ZnS thus as sacchtolith and lithopone practically not poisonous for rats and rabbits. For the rat the technical ZnS is not poisonous as weel, however, not for the rabbit with an optimum dosis (6 g/kg). Zinc-oxide seems to behave similar to ZnS.

IV. vomitive effect of $ZnCl_2$, ZnS, sacchtolith and lithopone for dogs.

The voitive effect for dogs, which allows the registration of the presence of small amounts of zinc-chloride, provide a contribution to the question, which significance the rate of solubility has for the toxic effect of zinc-sulfide.

Altogether 104 vomit experiment were performed. Out of this number 91 experiments were performed on dogs in an interval of 2-3 days, the remaining on 4 other dogs. The zinc-compounds were administered with a probang in a 2% solution resp. suspension (without tragacanth gum)into the empty stomach. The high dosis of the insoluble compounds were administered in such a way, that the supplied amount of liquid never exceeded 100 cm^3. The weight of the dogs was between 5.5 and 9.1 kg. Each dog get all zinc-compounds in different doses at least once.

table 6. vomitive effect of $ZnCl_2$, ZnS, sacchtolith, lithopone "Silbersiegel" for a dog.

Dosis g/kg	$ZnCl_2$	technical ZnS	Sacchtolith	Lithopone "Silbersiegel" (dosis of ZnS counted)

Ø no animal, + less than half of them, ++ half of them, +++more than half, ++++ all animals vomited.

Table 6 shows the differences of the vomitive effect between $ZnCl_2$, technical ZnS, sacchtolith and lithopone, related to doses of the same zinc-content. The vomitive effect decreases in this order. The difference between ZnS and sacchtolith permit only the interpetation, that a different grain-size of the sacchtolith reduces the rate of solubility in the hydrochloric acid of the stomach. It is more difficult to interpret the difference between sacchtolith and lithopone. We could not answer this question by simple mixing experiments of $ZnCl_2$ and barium-sulphate, as there was no retarding influence of the barium-sulphate on the vomitive effect of the ZnS.

Discussion of the results

Because of our results the significance of the resorptive component of the zinc intoxication is again more emphasized. Only for high concentrations of $ZnCl_2$, which indeed played practically the main role, the general experience is valid, according to which the resorptive effects are insignificant in comparison with the local effects. The experience gained by diluted solutions and thiosulphate that the zinc resorption is inversely proportionate to the local effect, is according to known facts from the pharmacology of metals. As the thiosulphate is recommended for the therapy of zinc intoxications, this observation in combination with the parenteral ineffectiveness might not be unimportant for a valuation of its antidotic value. The solidity of thiosulphate zinc complex is obviously inferior to the BAL-Komplex.

The lower corrosive action of acetate and sulphate in comparison to chlorid commonly accepted in the literature, is confirmed by our experiments in its lower resp. missing local diagnosis as well as the increased out-standing of

acute-toxic effects. It is surprising that the acetate takes a mid-position between chloride and sulphate.

By the way the commonly accepted order of corrosive action (chloride> sulphate> acetate) in vitro by precipitation experiment with protein cannot be proven. The necessary concentrations for precipitation are, counted by mol, the same for chloride, acetate, and sulphate. There was also no difference in the subcutis and in the peritoneal area with the used concentrations of 0.5 and 5%.

We believe that differing penetration possibility has to be held responsible for the differences of the corrosive action on the mucous membrane of the stomach. $ZnCl_2$ is lipoid-soluble.[1] The zinc-acetate could in its undissociated form, in which it partially appears in the solution, penetrate into the cell. Both possibilities on the other hand are unlikely for the ZnS.

The zinc-tremor served as a well measureable symptom for the resorption that had taken place. But most probably it caught the important central factor for the acute deadly effect of zinc. The histologic deformations of the neurons indicate this, which Esser could prove on acutely died animals. We did not find any histologic damages on those other organs (heart, lungs, liver, kidneys. milt) of 14 examined animals, which had died within 24 hours after peroral intoxicatio 4 animals, which died in 4 days, we observed a turbid swelling of the kidney- and liver-cells and on another animal, which had died after 7 days, necrosis of liver-cells. We deal with animals, which had gotten a 2-5% solution. For higher concentrations histologic diagnoses were missing, even when death did not occur acutely (4 animals), furthermore with the surviving animals after 46- 85 days, (17 animals) as well.

Thus the histologic examinations also let the relation between concentratio and resorption become clear. Besides this they show that deformation of parenchyma organs only very gradually become apparent. Therefore they only appeared in severe forms with intravenous injections (necroses of the tubulus), (fatty degeneration of the liver), whenever death exceptionally occurred only after 3-5 days, as it happened once spontaneously and twice by Sulfactin-treatment.

Out of zinc-oxide and zinc-sulfide by reaction with the hydrochloric acid in the stomach only $ZnCl_2$ concentrations of less than 2% can be formed. We believe according to our diagnoses that there are no more severe damages of the mucous membrane of the stomach. It only was sufficient to cause a vomit effect. On the other hand just with these low concentrations we have count on a zinc resorption. Obviously the changing process into $ZnCl_2$, however, is insufficient, to cause toxic amounts. The limiting factor lies, besides the amount of HCl in the stomach, in the insufficient rate of solubility. This is very much influenced by the physical condition of the zinc-sulphide, as can be seen in the difference between technical (unglowed) ZnS and the sacchtolith

(glowed ZnS). Out of our experiments about the vomitive effect on the dog one may figure, that about 20% of the supplied technical ZnS, 2% of the supplied sacchtolith and even only about 0.5 to 1 % of the ZnS contained in the lithopon is transformed into $ZnCl_2$.

This is of course also important for the consideration of hazards, which could arise for a child by swallowing the white colour-plate in his paint-box, The ZnS-content of a colour-plate is 0.27 g. The amount of $ZnCl_2$ which would be formed in the stomach according to the given calculations is practically indignificant, even if one considers a higher lethal effect for human beings than for animals, because of the literature, the lower limit of lethal doses 3-5 g were indicated. However, it is also reported that considerably higher doses have been tolerated.

Summary

1. It is reported about the deadly doses and about the corrosive action of $ZnCl_2$, ZnS, and -acetate through peroral, intravenous, intraperitoneal, and subcutaneous intoxication of rats.

2. There is a certain antagonism between the local and resorptive effect through peroral supply, which is expressed in the large independence of the lethal effect of concentration, furthermore also in the effect of thiosulphate and BAL on $ZnCl_2$ intoxication.

3. The toxic effect of zinc-oxide and -sulphide (incl. sacchtolith and lithopone was examined on rats, rabbits and dogs and the significance of the rate of solubility was pointed out.

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Table 1
(see p. 3)

Dosis g/kg	Konzentration %	Tierzahl	Tod nach 2—24 Stunden	24—48 Stunden	48—84 Stunden	6—11 Tagen	Überlebende Zahl	getötet nach (Tagen)
1. 0,5	2	2	—	—	—	—	2	85
	5	2	—	—	—	—	2	85
	10	2	1	—	—	—	1	85
	20	5	—	—	—	—	5 (2)	60—85
	50	4	—	—	—	1 (1)	3 (2)	28
2. 0,75	2[1]	15	7	1	1	1	5	50—85
	5	12	8	—	1	—	3 (1)	51—82
	10	5	2	—	—	—	3 (1)	51—83
	20	10	3	—	—	3 (3)	4 (2)	50—83
	50	6	—	—	3 (3)	1 (1)	2 (2)	28
3. 1,0	2[1]	2	2	—	—	—	0	—
	5	2	2	—	—	—	0	—
	10	2	1	—	1	—	0	—
	20	5	3	2	—	—	0	—
	50	2	2	—	—	—	0	—

Table 2

Dosis g/kg	Konzentration %	Zahl	Tod nach 1 Tag	2 Tagen	3 Tagen	13 Tagen
0,25	5	2		—	—	—
	20	2	—	—	—	1 perfor. Ulcus
0,5	5	3	—	1	—	—
	20	3	1	1	1 perfor. Ulcus	—
1,0	5	2	1	1	—	—
	20	—	—	—	—	—

Table 3

Dosis g/kg	Konzentration %	intravenös Letalität	intravenös Zeit	intraperitoneal Letalität	intraperitoneal Zeit	subcutan Letalität	subcutan Zeit
0,02	0,5	0/11		—	—	—	—
0,03	0,5	10/10	2 min bis 3 Tg.			—	—
0,05	0,5	—	—	3/6	6 Std bis 5 Tg.	—	—
0,1	0,5		—	2/2	5—12 Std	—	—
0,5	5		—			3/6	7 Std, 12 u. 34 Tg.
1,0	5		—		—	5/6	12 Std bis 3 Tg.

Table 4
(see p. 4)

Dosis und Verabreichungsform		Tierzahl	Tod nach 15 min	2—5 Std	6—12 Std	1 Tag	2—3 Tagen	4—5 Tagen	9—14 Tagen	38 Tagen	Überlebende und getötet nach 38 Tagen
0,03 g/kg i.v. 0,5%	ohne S	10	5	1	—	2	2	—	—	—	—
	mit S	10	1	—	—	—	2	2	1	—	4
0,75 g/kg per os 5%	ohne S	12	—	2	5	1	1	—		—	3 (1)
	mit S	12	—	—	2	—		1	3 (1 U)[1]	1 U[2]	5
1 g/kg per os 5%	ohne S	2	—	1	1					—	—
	mit S	7	—	4	2	—	—	1 U[3]	—	—	—

Table 5 (see p. 5)

Salz und Verabreichungsform	Dosis g/kg	Konzentration %	Letalität	Konzentration ausgedrückt durch die isomolare $ZnCl_2$-Menge %	Letalität	Konzentration ausgedrückt durch die isomolare $ZnCl_2$-Menge %	Letalität
	ausgedrückt durch die isomolare $ZnCl_2$-Menge						
$ZnSO_4 \cdot 7 H_2O$							
per os	0,5	5	0/2	20	0/4	50	0/4
	0,75	5	3/4	20	7/12[1]	50	3/6
	1,0	5	4/4				
subc.	0,5	5	0/4				
	1,0	5	3/4				
i. p.	0,05	0,5	1/4				
	0,1	0,5	4/4				
i. v.	0,02	0,5	1/3				
	0,03	0,5	2/2				
$Zn(CH_3COO)_2 \cdot 2H_2O$ per os . .	0,5	5	0/2	20	0/4		
	0,75	5	2/4	20	5/12		
	1,0	5	3/4[2]				
subc.	0,5	5	0/4				
	1,0	5	4/4				
i. p.	0,05	0,5	1/4				
	0,1	0,5	4/4				
i. v.	0,02	0,5	0/2				
	0,03	0,5	2/2				

Table 6 (see p. 7)

Dosis g/kg	$ZnCl_2$	technisches ZnS	Sachtleben	Lithopone „Silbersiegel" (Dosis auf ZnS berechnet)
0,01	+	∅	—	—
0,025	++	∅	—	—
0,05	++++	+	—	—
0,1	—	++	∅	—
0,2	—	++	∅	∅
0,50	—	+++	∅	∅
1,0	—	+++	++	∅
1,8	—	—	—	+
2,4	—	++++	++	—

VITALSTOFFE- ZIVILISATIONKRANKH. 6: 59-66. (1961).

On the cancer-causing effect of Zn-containing drinking water*

(Temporary News) by E. Halme

Although the teratome inducing and cancer-causing effect of ZnCl is already known by the works of Michalowsky and Bagg, since 1936 most of the research on Zn up to now has led to the conclusion that this metal, administered even in excess and internally, and over a longer period of time, is relatively harmless to the health of man and animals. Zn has also been determined as a first group integrating trace-element.

Within the past ten years I have experimentally examined the effect of Zn-containing drinking water on tumor-resistant as well as on tumor-sensitive mice families. At first for some years I made small, orienting experiments only, with a mixed stock of white mice from the anatomic institute of the University of Helsinki. Using pieces of Zn in drinking water, the pH value of which was reduced to 5.0 by HCl, there were two ulcers in a group of 15 mice (pic. 1 and 2). As all other tumors of my experiments during the later years, they were examined by the Finnish cancer scientist, Professor of pathologic anatomy at the University of Helsinki, Harald Teir.

The PAD of the first tumor, which appeared seven months after the start of the experiment, was as follows: "Macroscopically: In the lower part of the abdominal cavity a considerable, about plum-size, ulcer with brittle contents. Experimental sample: might remind one of a teratome tissue. In the dorsal part one can see a bump of bean-size, which is connected to a capsule. Microscopically: Medular tumor tissue with distinct gland-structures. Reminds one of pancreas, partially of a seminone. Very few mitoses. At one place osseuous tumor tissue is seen." The second tumor was formed about eight months after the start of experiments. PAD: "Macroscopically: in the lower part of the abdominal cavity a large encysted ulcer of about 2.5 x 2.5 x 1.5 cm, which contains hard material. Sample: Microscopically: similar to theone before. Obviously a seminome."

As the sminomes are about the most malignant of all there is in cancer pathology, the results required to continue the experiments on a larger scale. However, as the work at the pathologica-anatomic institute of the University of Helsinki had to be done in the evening after normal working time, some hundreds of mice could be fed simultaneously at best.

Pic. 1. The first ulcer induced by Zn-containing drinking water, in 1952.

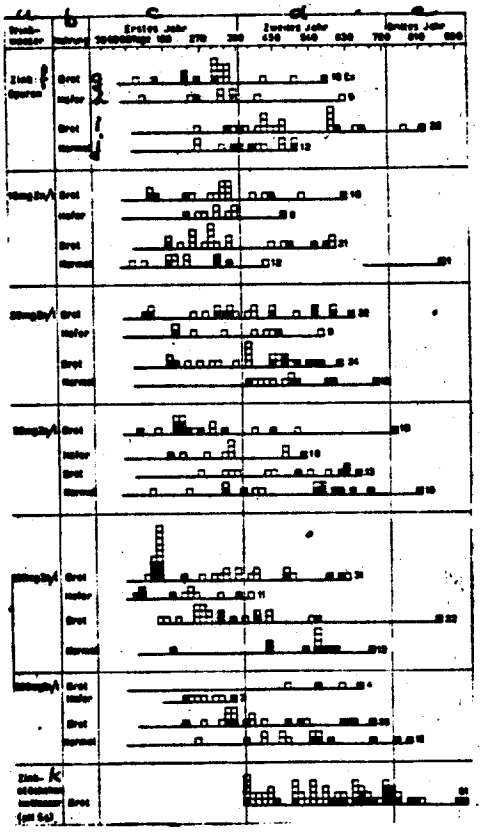
*The subject was discussed at the 6th International vital-substances and nutrition convention.

The base material of the test series were 540 mice of a mixed stock of the institute ("mice family I"). The spontaneous tumor frequency of these mice is very low, at most 0.004% (Setälä 1956, (3), Merenmoes 1959, (4)). The animals were divided into groups of ten and were fed in glass containers of 10 l. In the drinking water they got a ZnCl-admixture of 0-200 mg Zn/l to the tap water, which itself had traces of Zn, only. during the feeding either a deficiency diet (only bread and oats) or a full diet (bread, butter, cheese, vegetables and a special nutrition for mice, called "normal nutrition" there) were administered. Furthermore, smaller special arrangements, for example regarding the collection of material for the examination of the Zn-excretion, were made.

Later, a second, tumor-resistant mixed stock of the pathologic-anatomic institute ("mice family II") was used for the experiments. The spontaneous tumor frequency is also very low with this stock, but was not examined statistically, however. For this, two tumor-sensitive stocks of the institute were used, the C3H stock and the A/Sn stock. Only females of this family show spontaneous ulcers; we never observed any on the male. The total spontaneous tumor frequency is supposed to be about 15%, that of the females 30%, that of the males, accordingly, 0%.

Pic. 2. The second ulcer induced by Zn-containing drinking water, in 1952.

The results of the experiments with the F_0 generation of the tumor-resistant mice family I are shown graphically in picture 3. All these mice were at least two months old at the beginning of the experiments. In the diagram the horizontal lines indicate the periof of the experiment, starting with birth. The white square on the left indicate death cases, where no anatomic or physiologic disturbances during or after the experiment could be found; where disturbances of the stated kind could be observed, those squares are marked with a cross, the squares with a black dot showing the time of death of the tumor-mice. All entries are based on bi-weekly periods.

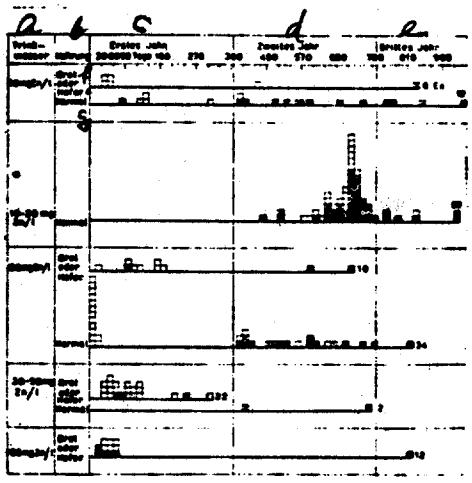


Picture 3. Experimental results with the F_0 generation of the tumor-resistant mice family I; square = death case. Explanation in the text.
a = drinking water, b = nutrition, c = 1st year, d = 2nd year,
e = traces of Zn, g = break, h = oats, i = bread, j = normal,
k = pieces of Zn in the water (pH 5.4)

As one may see, in these series with more than two month old mice of the tumor-resistant family I only three ulcers were observed. The first of these tumors proved to be an adenocarcinome, the second an epitheliome, the third a malignant, marrow-like tumor, probably of mesnchymal origin. In contrast to this different inflammations and swellings of the glands were frequent, which in some cases externally reminded one of an ulcer; when microscopically examined, however, no tumor cells were found.

Among the tumor-resistant mice family I, no ulcers were formed in the F_0 mice.

Picture 4 shows those mice of the tumor-resistant family I, which were born during the period of the experiments. These mice received Zn-containing drinking water from birth on, and their parents algo got it for a longer or shorter period. One can see that ulcers were formed significantly more often than in the F_0 generation. Among these mice of the F_1, F_2, F_3 and F_4 generation, one spindle-cell sarcome, two fibro-sarcomes, four adenocarcinomes, one haemangiom, in the liber and one in the ovaries, one granula-cell tumor in the ovaries, one leiomyoma in the lungs and altogether eleven lung adenomes of differeing grades of malignancy were to be observed. The tumor frequency of both genders was about the same. The most effective Zn concentration in the drinking water was as before with the F_0 generation 10-20 mg Zn/l.



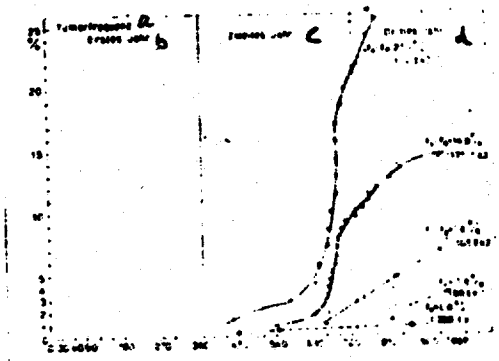
Picture 4. Experimental results with the F_1 -F_4 generations of the tumor-resistant mice family I. Square = death cases. Explanation in the text.

a = drinking water, b = nutrition, c = 1st year, d = 2nd year, e = 3rd year.

Picture 5 shows the tumor frequency curves of the named series. As stated, the spontaneous tumor frequency of this family is lower than 0.004%. The F_0 generation of the guinea pigs had a tumor frequency of about -.8%, F_1 25.7%, F_1-F_2 7.6%, F_3-F_4 25.7%. The highest number of ulcers were induced in those mice which had received An in the drinking water since their birth. One also sees that the tumor frequency of the following generations increases considerably more and more. The time of induction of the ulcers decreases also. Altogether, the time of induction of tumors in this tumor-resistant family corresponds to the so-called age of cancer of a human being, if one year of a mouse equals thirty years of a human being.

Provided that these results are generally valid, the differences of the tumor frequency curves of the different generations mean that the reason for the later appearance of the Zn ulcers is not only to be found in childhood but also is already present in the sex cells and in the parents. In other words, the effect of Zn is accumulated in the following generations.

I also have experimented on quite a scale with An powder in the nutrition and with normal tap water as drinking water. No tumors were generally to be identified in these experiments. Among the tumor-resistant mice families I and II, two benign ulcers were formed. One of them was a necrotic swelling of the glands in the testicles, which very much reminded one of a pancreas tissue with Langenhaus' Islands, the other was also located in the testicles, where a lot of epithel-covered cavities with partial proliferations were to be found. Among those already stated experiments at a concentration of more than 50 mg Zn/l in the drinking water, only one tumor was induced.

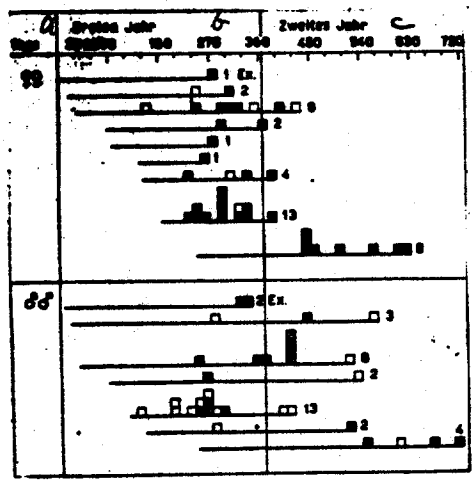


Picture 5. Tumor frequency of the F_1-F_4 generation of the tumor-resistant mice family I.

a = tumor frequency, b = 1st year, c = 2nd year, d = 3rd year.

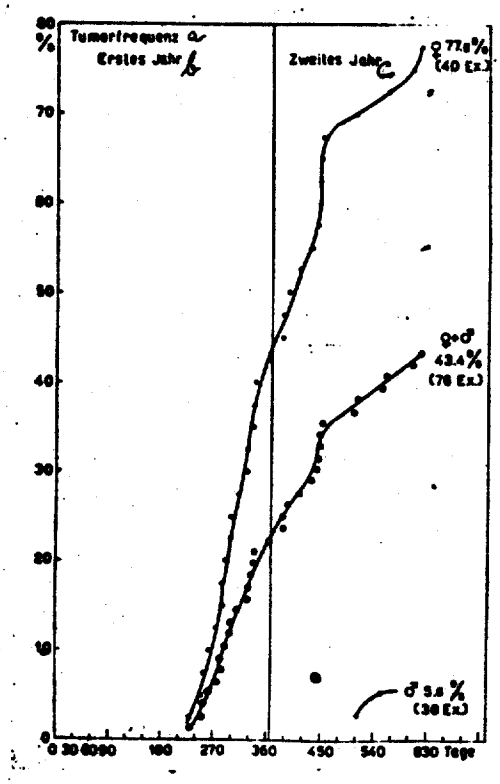
Therefore, large amounts of An do not appear to be standard. The ulcers were formed on a deficienty diet as well as on a full diet.

For the experiments of the last years with the tumor-sensitive mice families C3H and A/Sn, therefore, only 10-29 mg Zn/l were used in drinking water and in the nutrition (normal diet). The tumors spontaneously formed in the mice are mostly subcutaneous adenocarcinomes, generally malignant, marrow-like ulcers with small cells, which form alveoli and sinus-like structures. Necroses are frequent.



Picture 6. Results with the tumor-sensitive mice family C3H.

a = tumor frequency, b = 1st year, c = 2nd year.

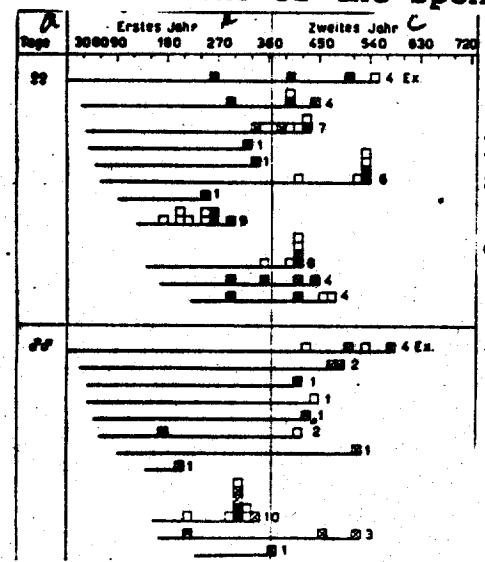


Picture 7. Turn or frequency curves of the tumor-sensitive mice family C3H.

a = tumor frequency, b = 1st year, c = 2nd year.

Picture 6 shows the results with the mice family C3H. Altogether 33 tumors were diagnosed, of those two on male. One of the tumors of one of these male mice was an adenoide carcinome, which typically appears in the female, the other was found in the liver, where cell infiltrates are abundantly to be found there. The condition of the liver of this mouse very much resembled the one with leucemia. In the females most of the tumors consisted of typical adeno-carcinomes; however, epithelial neoplasma and lympho-sarcome were observed as well.

Picture 7 contains the tumor frequency curves for these mice. The total tumor frequency in these Zn tests increased from 15% (spontaneous frequency) to 43.4%. For the females 77.5% (spontaneous 30%), for the males 5.6% (spontaneous 0%) were registered. The time of induction altogether corresponds to the one of the spontaneous tumors.



Pic. 8. Results with the tumor-sensitive mice family A/Sn.

a = days, b = 1st year, c = 2nd year.

The tumor-sensitive mice family A/Sn showed quite a similar picture in the test (pic. 8). Altogether 24 tumors were found, 40 of which in males. Most of these ulcers were also typical adenocarcinomes. However, two adenomes of the lungs and another deviating ulcer, probably a lymphome malignum, was noticed in the females.

Picture 9 shows the tumor frequency curves in the test with these mice. The total tumor frequency increased to 32.4% (spontaneous about 15%), the one of the females to 42.6% (spontaneous 30%) and the one of the males to 14.8% (spontaneous 0%). The relatively high frequency of tumors for the males, which normally do not show any spontaneous tumors, is striking. Here again, the time of induction of the ulcers corresponds to the one of the spontaneous tumors.

The fact that the time of induction of Zn tumors did not differ from that of the spontaneous tumors for the C2H as well as A/Sn mice shows that Zn did not have a distinct carcinogenous of tumor-causing effect, but functioned as a true carcinogenous factor. As most of the Zn-caused ulcers, among these families were typical adenocarcinomes, naturally it may be concluded that the spontaneous adeno-carcinomes which appeared in these mice could have at least partially been Zn ulcers, caused by the Zn content in the nutrition and the drinking water; in most of the laboratories the guinea pigs are kept in Zn galvanized cages with a container made of Zn for the drinking water.

During the last ten years I also have made many Zn analyses of faeces, in order to get an impression of the Zn excretion of the guinea pigs as well as human beings. The dithizone method used, developed mainly because of a paper about heavy metals by Koroloff (1950) (5) and was further developed by the named analysts, is the following:

Reagents: 1. Dithizone or di-phenylthiocarbazone is solved in carbon tetrachloride in an approximate concentration of 65 γ DzV 10 ml, which corresponds to 8.3 γ Zn. Then the exact Dz concentration of the solution is determined spectrophotometrically. In order to avoid an oxydation of the solution it is covered by a layer of hydroxylamine-hydrochloric solution. Storage is in a dark place. Because of the slow oxydation taking place, the solution has to be changed at least every two months.

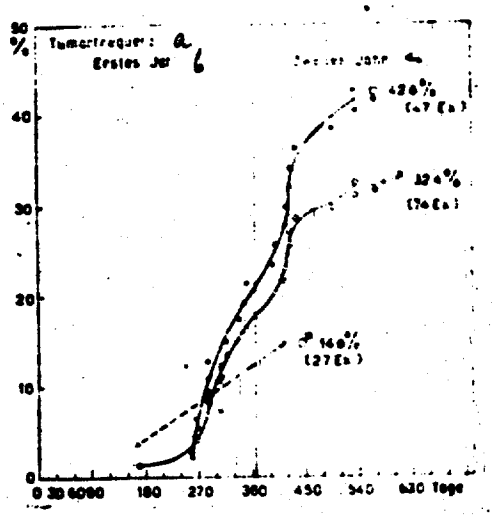
2. One prepares a sufficient amount (e.g. 4l) of 0.1-n-sodium-acetate solution which is washed free of metal in a diaphragm funnel with concentrated Dz solution.

3. For the diligent rinsing of the pipette, etc., redistilled, metal-free water has to be used. For this, the above mentioned sodium-acetate solution may also be used.

Method: 1. 500 mg air-dried discharge is grinded in such a way in a mortar, into which 40 ml of sodium-acetate solution have been added, so that one gets an evenly thick paste.

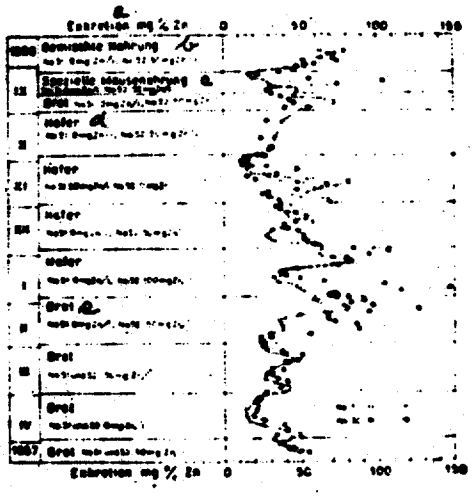
2. 10 ml sodium-acetate and exactly 5 ml of the above mentioned Dz solution with the determined Dz content are poured into a small funnel shut by a glass-valve.

3. After this, small amounts of the discharge-paste are added to the funnel and then it is shaken for about 5 minutes, so that the green color of dithizone changes into the red of the Zn dithizone. The color is determined in the light of a constant electric lamp. Out of the tirtration result the amount of Zn is figured in mg%.



Picture 9. Tumor frequency curves of the tumor-sensitive mice family A/Sn.

a = tumor frequency, b = 1st year, c = 2nd year.



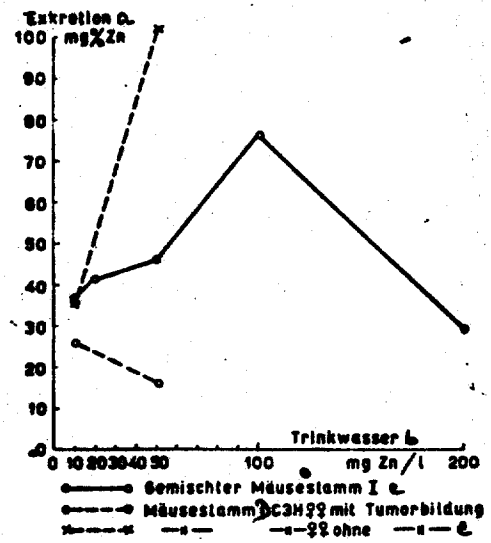
Picture 10. Variations of the Zn excretion for different diets and Zn concentrations of the drinking water of the tumor-resistant mice family I.

Remarks: It is important to determine the color always in the same light, in order to get exact results. Because this is a time reaction, in which the equilibrium at usual room temperature is reached in 5 minutes, one has to shake exactly this long during the last phase of the analysis, before determining the color. It is remarkable that those discharge samples dried in a heater provide considerably higher values than the air-dried. This is obviously based on the fact that in the haeter, parts of the unsolved Zn of the nutrition in the intestines is set free. The same happens if the samples are stored too long.

In order to prevent a reaction of other metals with the dithizone, it is necessary to use complex organizers, e.g. sodium-thiosulphate. However, as the amount of Zn appears to be relatively small and as there is a bothersome S-preparation for a color determination with sodium-thiosulphate, it appears to be more practical to do the analyses without any complex organizers.

Picture 10 shows the results of a series which lasted several months. Here the diets and the Zn contents of the water were changed a number of times. For experiments No. 51 and 52 as well, the faeces of seven mice were collected. Because of the very differing Zn content of each sample the curves were balanced by the formula a + 2b + c. Disregarding this, it seems to be quite difficult to conclude anything regarding the effect of different diets and Zn concentrations of the water. The best probably would be to use radio Zn for these experiments.

Picture 11, however, shows that one can get clear results with the median of larger amounts of An analyses of the faeces. One can see that for the tumor-resistant mice family I the excretion of Zn is increased, if the Zn concentration of the drinking water is increased from 10 mg Zn/l to 20, 50, 100 mg An/l, but after this decreases with increasing concentration. The healthy animals of the tumor-sensitive mice family C3H even react by excreting a relatively larger amount of An, if the Zn concentration of the drinking water is increased from 10 mg to 50 mg/l. In contrast to this, those animals with a tumor formation show a distinctly worse excretion.

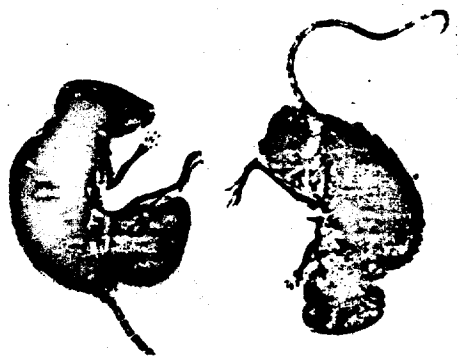


Picture 11. Medium Zn excretion of the tumor-resistant family I and the tumor-sensitive family C3H and with different Zn concentrations of the drinking water.

a = excretion, b = drinking water, c = mixed mice family I, d = mice family C3H with tumor formation, e = without tumor formation.

With the development and growth of the ulcer the Zn excretion becomes weaker and weaker and the animals do not seem to react on the increase of the Zn concentration of the drinking water like healthy animals with an accordingly increased Zn excretion.

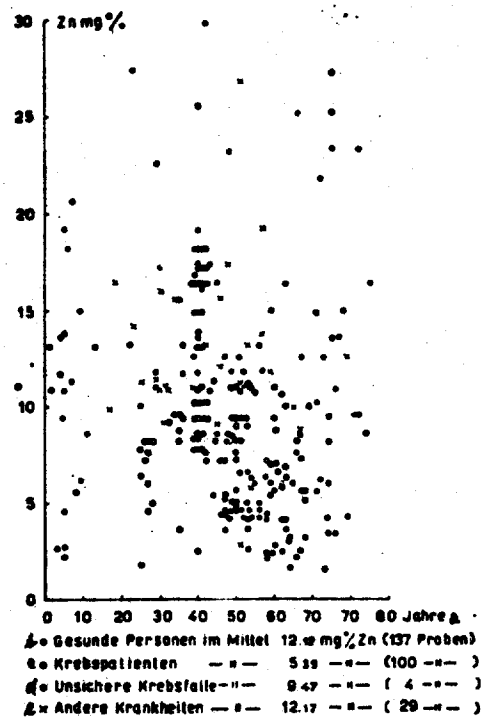
Thus, an accumulation of the Zn must take place in the body of the mice having the tumor. In fact, an X-ray of two tumor mice of the family C3H shows that especially the skeleton, but also the tumor tissue can store considerable amounts of Zn (Picture 12). In this case the ulcers of the two mice were formed spontaneously, simultaneously and at the same spot between the back legs. Before taking the picture the mice with the darker tumor got Zn of 50 mg Zn/l in the drinking water for 50 days, the other just ordinary tap water. Both of them were kept on an oats diet.



Picture 12. Zn accumulation of two tumor mice.

It could have been suggested that the same phenomenon is valid for human beings. Therefore, I collected for several years samples of discharges of cancer patients (mainly from the Radium Hospital in Helsinki), of patients of the second university hospital in Helsinki (internal diseases), and of healthy people, among others from the institution for the aged in Helsinki.

Picture 13 shows the results. The Zn excretion of most of the patients is quite low, on the average only 5.39 mg%. For the healthy people of different ages it is much higher, on the average 12.12 mg%. It should be mentioned that the lowest cross indicates the sample of a 51-year old man who was taken care of in the clinic for internal medicine. Much later it was discovered that he had leucemia. It is also remarkable that a bad Zn excretion was found amoung the youngest cancer patients. The good excretion of some very old, healthy people is also interesting. In general, a bad Zn excretion is found in just those people who later on may possibly contact cancer. Altogether, I would not exclude the possibility that the applied, quite simple and fast dithizone method - further developed - would be useful for mass control and early diagnosis of cancer. Because of the carrying Zn content of single samples and in order to get a dependable result, it would be necessary to examine several samples in each case.



Picture 13. Zn excretion of the examined cancer patients of differing age, of healthy people and of people with internal diseases.

a = healthy people on the average, b = cancer patients on the average, c = unclear cases of cancer on the average, d = other diseases.

Evaluation:

The results of the experiment thus show that Zn is a carcinogene, the nature of which and its contribution to the cancer problem of today is not known enough. Obviously the long time of induction of the Zn ulcers and under the experimentation condition the relatively small amounts of Zn necessary to form them give an explanation for the fact that amoung the present experiment there were no ulcers.

The most effective concentration of Zn in the drinking water was 10-20 mg Zn/l. It is to be noted that just these concentrations are not rare in highly civilized communities. For good drinking water a Zn content of 5 mg Zn/l is internationally acceptable; to be sure, this is a recommendation subject to change if more becomes known about the effect of this metal (WHO 1956 (6)). In practice there seems to be considerably higher Zn concentrations in the drinking water of different countries. 35% of all drinking water samples examined in Finland contain more than 8 mg Zn/l (Johansson and Weijola 1955 (7)). The Zn galvanized water pipes and the intensity of corrosion especially seem to be standard here. Because the daily need of human beings for water is quite high, 3.5 l roughly, many people daily and for decades get just these concentrations and with them the according amount of Zn, inducing considerable amounts of ulcers of different nature in the above mentioned animal experiments.

In reality the dialy need of this trace element for human beings is estimated to be 4500-9000 γ (Schweigart 1956 (8)). If we consider the fact that many of our nutritives, especially some products of the nutrition industry, contain considerable amounts of Zn, one can say that

almost every human being daily receives a multiple of this necessary amount of Zn starting from birth. The calculation and the results of the experiments given here led to the conclusion that too high a content of Zn in the nutrition and drinking water of certain countries would be a very important cause of the present frequency of cancer and that today, in reality, many of the causes of cancer are real metal cancers, which have mainly been induced by a decade-long daily excess of Zn. The results also show interesting perspectives for the discussion about the frequently mentioned assumption that cancer and especially cancer of the lungs has become more common rather than early appearing in cultivated countries during the last decades. One should be reminded of the fact that just among the younger generations of the tumor-resistant mice there were quite a lot of lung tumors and that these ulcers were mainly induced with a concentration of only 10-20 mg Zn/l in the drinking water. From this aspect it is interesting, as Schweigart has stressed in a letter to me, that the ferment in the blood for the transportation of the CO_2, the carbo-anhydrase, is a Zn proteid. According to the results found it appears desirable to order a total elimination of the Zn from the water when filtering the drinking water in order to prevent Zn-induced cases of cancer in the future, as we already receive a sufficient supply of this metal from other daily nutritives. The results indicate that a considerable reduction of the Zn contents of the nutrition and of the drinking water for children and those elderly people who have a bad Zn excretion is especially important. Besides this, it would also be necessary to quickly start thorough and varied wide-spread research concerning the metabolism and nature of the cancerogenous effect of Zn.

Summary

The author [illegible] the [illegible] of zinc [illegible] drinking water (10-20 mg/l) [illegible] (10-20 mg of Zn/l) [illegible] 5 mg per litre [illegible] 35% [illegible] 8 mg of Zn/l [illegible] 50-60 mg of Zn/l [illegible]

Literature

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2. [illegible] (1958): Amer. J. Canc., 28, 69
3. [illegible] Scand., Suppl. 115
4. [illegible] Scand., Suppl. 130
5. [illegible] Gesundheitsfürsorge, Gemeinde [illegible]
6. [illegible] Z. [illegible] 4

Anschrift des Verfassers:
Prof. [illegible] D. Erkki Hämäläinen
[illegible] Helsinki, Finnland

BRITISH JOURNAL OF INDUSTRIAL MEDICINE 15(2):130-132, 1958.

MISCELLANEA

Some Enquiries into the Toxicology of Zinc Stearate

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(RECEIVED FOR PUBLICATION OCTOBER 23, 1957)

At the International Congress of Industrial Medicine in Naples (Noro and Uotila, 1954), it was stated that "the inhalation of zinc stearate dust is known as a cause of pneumonia in children, but its effects in the working environment are not so well known. The authors describe the fatal case of a worker employed for 29 years in a rubber plant. Clinical examination and necropsy showed diffuse fibrosis of the lungs. Histological tests for zinc were positive". These authors (Uotila and Noro, 1957) have since given some further details of their patient, who was first seen by them in July, 1952, after having spent 29 years as a rubber cutter using zinc stearate powder in his work. From the autumn of 1951 he had increasing dyspnoea on exertion, cough, and much sputum. When examined, his general condition was considered fairly good, he had a desquamative dermatitis, clubbed fingers, and "watch-glass" nails. A radiograph of the chest showed slight thickening of the left pleura diminishing towards the apex; small nodules radiating from the hilum; emphysema on the right side posteriorly; and enlarged hilar shadows that were considered to be vascular and not lymphoid. A diagnosis of "pneumoconiosis with probable heart failure" was made. He was in hospital for two further periods and died in June, 1954, approximately two years after he was first examined and after his last exposure to dust. At necropsy the lungs were more fleshy than normal, especially the upper lobes, but without calcification or fibrous bands; the lower lobes showed slight oedema: each ventricle of the heart was 8 mm. thick: the liver was granular and there was ascites. Histological examination of the lungs showed old bleeding and a notable increase of connective tissue with abundant chronic inflammation in which were foreign-body giant cells. A test for zinc showed numerous granules and needles in the connective tissue that contained the metal.

In 1925 a committee on accidents from zinc stearate dusting powders appointed by the Board of Trustees of the American Medical Association reported that as the result of a questionnaire they had received reports of 131 cases of poisoning, 28 of which had terminated fatally. They had also heard of many others unofficially. They recommended that all manufacturers of zinc stearate powders for infants should use a self-closing container of a type which does not lend itself to manipulation by an infant, and should place a uniform caution label on the container. They also recommended that the use of zinc stearate as a dusting powder for infants should be discouraged by the medical profession because of lack of therapeutic evidence of its value. Murray (1926), in recording 60 cases of drug poisoning in infants, stated that one fatality, out of a total of three cases, resulted from the aspiration and ingestion of zinc stearate powder. A baby of 14 months was taken to hospital immediately, she developed a diffuse bronchopneumonia next day, and died in less than a further 24 hours. No record of poisoning by zinc stearate powder has been found in the British literature, and none of the many British paediatricians who have been asked has heard of babies suffering from pneumonia due to this powder.

Zinc stearate is used commercially as a substitute for talc in the rubber tyre industry to prevent adhesion. H.M. Factory Department has no evidence that any disability has been caused by its use, but I know of no examinations for possible harmful effects. Stearate kindly supplied by the management of a rubber tyre company was used in the experiments described here.

Experimental

Zinc stearate powder was suspended in tap water and autoclaved. Approximately 100 mg. of the powder suspended in 1 ml. water was injected into the lungs through the larynx of six rats anaesthetized with ether. All the animals died within 10 minutes. Approximately 100 mg. was injected into the peritoneal cavity of six cavies. All the animals became rapidly unwell and two died within a few hours: the others appeared quite normal next day and remained so until they were killed at intervals up to 35 days. All but one, killed at the latest date, showed small granulomata in the peritoneum. Examination of the suspension showed that the stearate was difficult to keep suspended in water, and the fluid was notably acid. All further experiments were therefore done with zinc stearate suspended and autoclaved in a mixture of skimmed milk and saline, and the dose was halved.

Fifty rats were given approximately 50 mg. of zinc stearate suspended in 1 ml. and injected into the lungs through the larynx under ether anaesthesia. Twenty animals died within less than 24 hours, most of them within an hour. Those that were examined showed severe oedema and congestion of the lungs and sometimes small haemorrhages. Animals that survived more than 24 hours appeared quite normal until the time they were killed. Animals killed seven days after injection showed small cellular islands of histiocytes scattered through the lungs. None of the injected dust was visible

FIG. 1.—Peritoneal granuloma in cavy after 28 days. Stained with haematoxylin and eosin. × 25 approximately.

FIG. 2.—Peritoneal granuloma after 42 days. Stained with haematoxylin and eosin. × 30 approximately.

in ordinary paraffin sections, but it is possible that some was still present, since it was later found that the stearate is moderately soluble in fat solvents. Animals killed 14 days or longer after the injection showed no abnormality in the lungs. Rats were killed up to 259 days after the injection and in none was any fibrosis detected in the lungs.

Six cavies were given 50 mg. suspended in 1 ml. water and injected into the peritoneal cavity. None of them showed any apparent ill effects. One animal killed at seven days showed a few small white nodules in the peritoneum that on section had a granulomatous character with a centre of polymorphonuclear leucocytes. After 14 days the stearate lay in a mass of dead cells surrounded by a layer of histiocytes and an outer layer of early fibroblastic proliferation. At 28 days the small granulomata were slightly more defined (Fig. 1). At 42 days a formless or crystalline centre was ringed round by histiocytes outside which was a narrow band of young fibrous tissue (Fig. 2). At 100 and 105 days nothing abnormal was visible in the peritoneum, and no granulomatous or fibrous reaction was found in sections.

Discussion

From these experiments it appears that zinc stearate in suspension in tap water or in a mixture of skimmed milk and physiological saline is acutely irritant, and in doses of 50 mg. kills nearly half the animals if injected through the larynx into the lungs of rats. Death results from acute oedema of the lungs. Given intraperitoneally to cavies, 100 mg. killed two of six animals within a short time, but 50 mg. was tolerated well.

It appears further that zinc stearate disappears within a period of about a fortnight from the lungs of rats, presumably by processes of phagocytosis and solution. It is therefore not surprising that no fibrosis results in the lungs of these animals after a single injection through the larynx.

In the peritoneum of cavies the powder provokes a granulomatous reaction, at first with a polymorphonuclear leucocytic response. These cells die and are surrounded, and eventually digested, by histiocytes. The stearate remains visible in the peritoneum of cavies much longer than in the lungs of rats, possibly because it is rapidly aggregated into a very few masses, and it can still be found after 42 days enclosed in a ring of histiocytes outside which is a narrow zone of fibroblastic activity. However, somewhere between the 42nd and the 100th days the stearate disappears and the granulomatous process regresses until no sign of it can be found.

These experiments, then, while suggesting that inhalation of zinc stearate might produce acute inflammation of the upper respiratory tract or of the lungs, provide no evidence of any fibrogenic permanent effect. Indeed, its solubility suggests that such an effect is unlikely to occur.

Summary

Industrial zinc stearate dust in suspension is acutely irritant when injected into the lungs of rats or the peritoneum of cavies.

No fibrosis has resulted from single injections into the lungs of rats or the peritoneum of cavies. Foreign-body granulomata were present for several weeks in the peritoneum but ultimately resolved.

I am grateful to Dr. A. I. G. McLaughlin, H.M. Medical Inspector of Factories, for drawing my attention to the report of Noro and Uotila, for trying to obtain further information from Dr. Noro, and for helping to obtain the sample of zinc stearate. The photomicrographs were taken by Mr. A. W. Collins, F.I.M.L.T.

The expenses of this investigation were met by a grant from the Research Fund of the South-West Metropolitan Regional Hospital Board.

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J. Biol. Chem. 74: 85-93 (1927)

TOXICITY OF ZINC.

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(Received for publication, May 2, 1927.)

During the course of an investigation undertaken by this station ...at 3 years ago in regard to the possibility of poisoning from ...ermilk which had been stored in zinc-lined containers, we were ...essed, first, by what seemed to be the contradictory findings ...erning the toxicity of zinc; second, by the more general dis-...ution of zinc in animal and plant tissue than commonly is ...ned by those who have not investigated the subject; and third, ... the inadequacy of methods for separating and estimating ...titatively this element when it appears in traces in organic ...ter.

Since starting this study a series of articles by the coworkers of ... Department of Physiology of Harvard School of Public Health ... gone far to clear up much of the uncertainty pertaining to ...ain branches of the problem. The excellent bibliographies of ...z (1) and of Drinker and Collier (2) call one's attention to the ...e distribution of zinc throughout all living matter, and even ...gest that it may have a favorable function in life processes. The completeness of their references makes it unnecessary to ...er further to the historical works, and those interested in this ...ject are referred directly to the work of these authors.

The investigations of Batchelor, Fehnel, Thompson, and Drinker ... who have made an extended study of the physical condition ... workers long in contact with zinc and its compounds, likewise ...d one to conclude that the earlier investigators might have been ...what misled in drawing their conclusions. Facts repeatedly ...d by these recent investigators indicate that formerly lead, ...nic, or cadmium were probably the contaminating elements ...ich were really responsible for many of the pathological condi-...ns reported.

In our first attack on this problem we were confronted with several difficulties which have been eliminated somewhat by these latter reports. First, we were not certain whether the alleged poisonous conditions reported came from the zinc of the containers or from other elements present. In the course of the investigation we were hampered by the lack of methods of analysis for the element in such small amounts as are to be found in the organs of the rat, which was the animal chosen for our work. We were familiar with and were using the methods of Scott (4), Bodansky (5), and Birckner (6). These methods gave satisfactory results where materials were plentiful, but the completion of the work has been greatly simplified by new methods and modifications as suggested by Lutz (7), Fairhall (8), and Thompson (9). Even with the addition of this later series of investigations, certain phases of the problem concerning the probability of zinc contamination and its effect upon the consumer are still untouched, and this report is offered for those who may be confronted with practical questions in regard to such contaminations of foods.

EXPERIMENTAL.

In attacking this problem we first made an examination of the containers in which buttermilk had been stored and found that the zinc surfaces showed evidences of having been dissolved. We next turned our attention to the buttermilk that had been stored in these containers and found from the analysis of the ash by Birckner's method that all buttermilk contained some zinc normally, but that it increased with the acidity of the milk, the length of time stored, and newness of the container. Old surfaces were much less susceptible to the attack of the weak organic acids present. In this work buttermilk that had never been in contact with metallic surfaces was obtained. Definite portions were stored in glass containers and like amounts in zinc-lined vessels for varying periods of time. Definite portions of each of these samples were then drawn off and dried in a vacuum oven and finally ashed at black heat in a muffle furnace, extracted with HCl, and reashed after dampening with nitric acid. The extracts were used and the zinc estimated by the turbidity method of Birckner. These determinations have been recently rechecked by

the improved turbidity method suggested by Fairhall. Table I gives the zinc content of a characteristic sample.

These quantities of zinc present in the buttermilk are suggestive of changes taking place. The figures are variable with samples, probably due to acidity produced by temperature, age, and origin of buttermilk.

Due to the fact that metallic surfaces might contain metals other than zinc, it appeared advisable to determine the toxicity by biological methods, using a normal growing ration, and adding definite amounts of zinc to the ration rather than the buttermilk of unknown contamination. Further, it was desired to know whether the acid radical might have some effect upon its pharmacological actions, and for that reason various salts of zinc were used. Rats were chosen as the test animals because of the ease of handling, permitting the observation of large numbers under all kinds of conditions, and because it was of interest to know not only the temporary conditions but also the effect on reproduction and sterility that might be produced by the accumulation of the metal in the body of the animal. The use of rats permitted the study of several generations in a short period of time.

TABLE I.

Zinc Content of Buttermilk Samples under Several Treatments.

Sample No.	Container.	Time of storage.	Zinc.
		hrs.	*mg. per 1000 cc.*
I	Glass.	24	5
II	Old galvanized pail.	24	15
III	" " "	48	25
IV	New zinc container.	48	33

A basal ration was used, which was known to produce satisfactory results for growth, reproduction, and rearing of young in this laboratory. Vigorous young rats were chosen and placed in cages so that each lot would be comparable so far as possible at the beginning of the experiment. One cage of these animals was used as a control lot and was fed a basal ration, while to the rations of the others were added metallic zinc and the various salts of zinc in such quantities as to contain a like amount of the zinc ion. Buttermilk was added to one set of controls and also to the one

zinc ration to see whether the protein of the milk might combine with the zinc and prevent its absorption, it being known that such proteins are often used as antidotes against certain metallic poisons due to the formation of insoluble compounds. The object in this test was to ascertain whether, due to this combination, milk that was contaminated with zinc was less dangerous than other foods so contaminated.

TABLE II.

Lot No.	Ration.	No. of males.	No. of females.	No. of litters.	Young born.	Young lived.	Young died.	Growth.*
I	Basic (B).	3	1	2	18	16	2	++++
II	B + buttermilk.	2	2	2	11	11	0	+++++
III	B + 0.25 per cent Zn dust.	2	2	6	43	36	7	++++
IV	B + 0.25 per cent Zn as $ZnCl_2$.	2	2	4	25	20	5	++++
V	B + 0.25 per cent Zn as $ZnCO_3$.	2	2	7	53	50	3	++++
VI	B + 0.25 per cent Zn as $ZnCO_3$ + buttermilk.	2	2	4	31	30	1	+++++
VII	B + 0.25 per cent Zn as $ZnSO_4$.	2	2	7	44	41	3	++++
VIII	B + 0.5 per cent Zn as ZnO.	2	3	2	13	11	2	+++
IX	B + 0.5 per cent Zn as $ZnCl_2$.	2	7	7	46	27	19	++++

*++++ represents normal growth.

The basal ration used in these tests consisted of:

	per cent		*per cent*
Yellow corn	53	NaCl	1
Ground whole wheat	20	$CaCO_3$	1
Oat	10	Alfalfa meal	5
Tankage	10		

Table II gives the ration supplements used as well as other information gathered concerning growth and reproduction when these rations are fed.

The results of the findings were unexpected and surprising for the reason that while 0.25 per cent of zinc is not a great amount of metallic ion in a ration, it is far in excess of any ordinary contamination and an amount such as would prove fatal for a toxic metal. No toxic effect was obtained; the rats grew normally. No noticeable difference could be noted between those eating the basal ration and those receiving the additions of zinc salts. Neither could we observe any great difference in the effect of the anion. Growth was normal, mating took place at the normal age, and young were born and reared in the various cages in comparable manners. The general appearance of the animals in clearness of eye, smooth-

TABLE III.

Weight of.....	Animal.	Heart.	Liver.	Kidneys.	Spleen.	Lungs.	Testicles.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Normal.....	225	0.7856	12.199	0.9513	0.6387	0.999	2.5126
25 per cent $ZnCO_3$.....	230	0.7853	10.987	1.1632	0.6989	1.0661	2.9973

TABLE IV.

	Weight of animal.	Ash of:					
		Heart.	Liver.	Kidneys.	Spleen.	Lungs.	Testicles.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Normal.....	0.225	0.0081	0.0808	0.0247	0.0300	0.0179	0.0156
$ZnCO_3$.....	0.230	0.0113	0.0831	0.0285	0.0245	0.0150	0.0140

ness of coat, and ease of movement did not indicate any deleterious effect. The offspring were continued on similar rations, and the third generation still maintains the same vigor in growth and reproduction as the animals receiving the growing rations. It was observed that the addition of buttermilk *ad libitum* did increase the vigor of both the lots receiving the basic ration as well as those containing zinc. The addition of milk to all growing rations, however, usually results in such accelerations.

After full growth had been reached, an autopsy was made and the various organs examined. So far as could be observed no lesions or other pathological conditions were evident. The organs were removed, carefully washed free of blood, measured,

weighed, and compared to the organs of normal animals. Table III presents the average findings of ten or more animals in each case.

The organs were next ashed at black heat in the muffle furnace and the ash content determined. Table IV presents the average ash content of organs in each case.

TABLE V.

Organ used.	Average weight of organ.	Average weight of zinc found.	Average concentration of zinc.
	gm.	mg.	mg. per gm.
Heart.			
Normal.....	0.7856	0.011	0.014
Zinc-fed.....	0.7853	0.014	0.018
Lungs.			
Normal.....	0.999	0.02	0.021
Zinc-fed.....	1.066	0.024	0.023
Liver.			
Normal.....	12.199	0.2439	0.02
Zinc-fed.....	10.987	0.2858	0.026
Spleen.			
Normal.....	0.6387	0.0198	0.031
Zinc-fed.....	0.6989	0.0265	0.038
Kidneys.			
Normal.....	0.9513	0.0143	0.015
Zinc-fed.....	1.1632	0.0163	0.014
Testicles.			
Normal.....	2.5126	0.032	0.013
Zinc-fed.....	2.9973	0.051	0.017

Another set of organs was ashed at black heat and the ash was extracted with 1:1 redistilled HCl, and analyzed for its zinc content by the micro method of Lutz (7) which is briefly outlined as follows: The HCl extract was treated with cupferron to precipitate the iron and copper. The filtrate is treated with sodium acetate until the mineral acids are neutralized as indicated by methyl orange. 5 cc. of dilute copper nitrate solution were added

to serve as an entrainer and hydrogen sulfide added which precipitated the zinc together with the copper. The precipitate was washed with water and hot alcohol to free it of foreign matter. This residue was dissolved in hot nitric acid and evaporated to dryness, redissolved in hydrochloric acid, and the copper again precipitated with hydrogen sulfide. The filtrate containing the zinc was evaporated just to dryness and taken up in water. Aliquots of this solution were added to Nesslerizing tubes containing alcohol. Urobilin was added, and the fluorescence obtained compared to zinc standards simultaneously prepared in a similar manner. Table V gives the zinc content of organs of normal and zinc-fed animals.

Since no noticeable deleterious effects of the zinc feeding were found through three generations of observed animals and chemical tests failed to reveal much accumulation in the organs of the

TABLE VI.

Food eaten.	No. of animals.	Time of observation.	Weight of dry feces.	Zinc.			
				Consumed.	In feces.	In urine.	Stored and unaccounted for.
gm.		*hrs.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
70	3	36	21	525.7	494	10	21.7

animals, we next turned our attention to the path of elimination of the zinc from the body. In order to gather the urine and feces uncontaminated, it was first necessary to build a metabolism cage free from all metals. This was accomplished by building upon a large shallow porcelain dish two perforated discs, the upper to serve as a floor for the cage and the lower to collect the feces but to permit the passage of the urine. These floors were made of small glass rods set in concrete rings cast so as to fit exactly the dish but still be removable. The concrete was treated to be non-porous. The sides of the cage were also made of glass tubes set in concrete rings above and below. A deep glass container protected with an upper flange was suspended in the cage permitting the rat to secure food without wasting any or contaminating the urine or feces, as well as making it possible to measure exactly the amount of food consumed. A drip tube

furnished distilled water for drinking purposes. The rats were kept in this cage over long periods of time to accustom them to their surroundings. When ready for study the amount of food eaten, the weight of the dry feces, and the weight of urine residue were noted over definite periods of time as recorded in Table VI. The figures are the average of five determinations from rats 6 months of age. The zinc was fed as zinc oxide added to the basal ration.

The samples of feed, feces, and urine were ashed, extracted, and estimated for their zinc content by the modified turbidity method suggested by Fairhall (8).

An examination of these figures indicates that zinc if absorbed, is again eliminated from the body primarily with the feces. Large storage in the internal organs was in no case evident. The urine, while showing an increased content, does not appear to furnish as important a path of excretion as do the feces. A similar observation was recently recorded by Drinker, Fehnel, and Marsh (10) in their study of the elimination of zinc in the feces of a normal man.

The feeding of commercial samples of buttermilk and zinc both to the rat and to larger animals is being continued in cooperation with members of the Dairy Department and will be reported at a later date.

SUMMARY AND CONCLUSIONS.

1. Buttermilk normally contains a small amount of zinc, the amount increasing after contact with zinc containers, and varying according to the duration of contact, acidity of the buttermilk, and the newness of the zinc surface exposed.

2. Zinc added to a normal ration either in the form of pure zinc dust, zinc oxide, or certain zinc salts, in amounts as great as ever found in contaminated foods did not interfere with growth, reproduction, and normal functions of the rat through three generations.

3. No pathological conditions were found in the organs of rats fed the rations used in this experiment.

4. The total ash content of the organs studied showed no perceptible increase.

5. Zinc is found normally in the internal organs of rats fed growing rations. Only small increase was observed in the organs of zinc-fed animals.

6. The path of excretion is primarily through the feces, though the content in the urine is slightly increased in the zinc-fed animals.

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AVAILABILITY OF TRACE ELEMENTS TO PLANTS WITH RESPECT TO SOIL-PLANT INTERACTION

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Introduction

Plants reflect, in general, the geochemical environment in which they grow. Relative ratios of elements in soil and the plant are not necessarily the same, however. Mineral elements differ markedly in their availability to plants. Moreover, plant species differ and even varieties or cultivars of some species differ in their ability or tendency to absorb specific mineral elements.

The soil system consists of three phases: solid, liquid, and gaseous. The elements are present in the solid and the liquid phases; however, the gaseous phase is critical to the metabolism of the higher plant and the soil microorganisms which play a role in availability of the mineral elements.

Many factors determine the availability of a given element e.g., relative abundance, form in which the element is present, soil pH, interaction of elements, physical condition of the soil, environmental factors of temperature and soil moisture and genetic constitution of the plant species.

Relative Abundance of Elements in the Lithosphere

Soils vary markedly in content of the major (macro) and trace (micro) elements: consequently, attempting to determine the average abundance of the elements in the lithosphere may have limited meaning. It does, however, permit one to visualize the relative ratios of the various elements.

Mineral elements essential to plant and animal life may be present in the primary minerals, the secondary minerals, as free oxides, as salts, as exchangeable ions adsorbed on the mineral and organic colloids, as ions in nonexchangeable or fixed forms, in the organic matter and in the soil solution.

The abundance in the lithosphere of certain elements of agricultural and health significance is presented in Table 1. Although it is not shown in the Table, oxygen is the most abundant element in the soil. It is a component of the primary and secondary minerals, the uncombined oxides and salts, and the organic matter. The reservoir of nitrogen in the soil is present in the organic matter, consequently no data are presented.[1]

Elements readily available are those in the soil solution, those exchangeable ions adsorbed on the colloids, and those in readily decomposable minerals. Soil tests as used in agriculture are designed to measure the elements readily available and, at present are concerned primarily with the major elements. The true measure of availability is the analysis of the plant.

Average Levels of Elements in Plants

Analyses of plants soils and rocks will reveal differences in the geochemical environment. In general, the elemental composition of the plant will reflect the composition of the soil or other growing medium. The ratios of elements in the plant will not necessarily be the same as those in the soil or even in the nutrient solution. Plants are selective, to a degree at least, in their absorption of elements.

The normal levels of a given element will vary considerably from one plant species to another or even between cultivars of a species. In the plant kingdom, we find species that tend to accumulate a particular element in extraordinary amounts and consequently are called accumulators or indicator plants. Such plants can be very helpful to the geologist prospecting for ore deposits but can also be very detrimental to the agriculturist.

Table 2 indicates the normal amount or ranges for some of the major and trace elements essential for certain fruit crops.[2] The normal level of nitrogen

Table 1

Abundance in the Lithosphere of Some of the Elements of Possible Agricultural and Health Significance[1]

Element	Abundance (ppm)	Element	Abundance (ppm)	Element	Abundance (ppm)
Si	277,200	Mg	20,900	V	150
Al	81,300	P	1,200	Zn	80
Fe	50,000	Mn	1,000	Cu	70
Ca	36,000	S	520	Co	40
Na	28,000	Cl	480	Mo	2.3
K	25,000	Sn	150	I	0.3

in leaves for various species usually does not vary as widely as that of mos other macro and micro elements. However, growth or yield is determinec more often by nitrogen availability than by other elements. Deficiencies o trace elements become a problem with the use of heavy applications of nitroger fertilizer and the "law of the minimum," as stated by Liebig,[3] becomes operative

The emphasis of this monograph is upon the trace elements; however, i is important that we consider, albeit briefly, some of the major elements, no only because of their roles in agriculture and health, but also from the stand point of their effect on the availability of the trace elements.

The levels of K, Ca and Mg in normal plants of the *Ericacae* family, a represented by the blueberry, are considerably lower than in plants of othe families. The levels required for normal growth by most other plants woulc be toxic to the blueberry. The levels of K accumulated by the banana woulc be toxic to many other plants. Citrus leaves are considerably higher in calcium than those of most other species. Magnesium is unusually high in the sweet cherry. Iron is a major component of the lithosphere, but the level in plants is relatively low. The level of Fe in the apple leaf is normally higher than in

TABLE 2

COMPARISON OF MINERAL COMPOSITION OF LEAVES OF CERTAIN PLANTS[2]

Plant Species	Concentration in Dry Matter										
	Percent					ppm					
	N	P	K	Ca	Mg	Fe	Mn	Zn	Cu	B
Apple	2.3–2.4	0.20–0.22	0.9–1.34	1.35–1.5	0.26–0.34	75–150	25–75	10–40	10–30	20–60
Banana	2.6–4.2	0.45	3.3–9.0	1.40	0.60					
Blueberry	1.8–2.6	0.07–0.32	0.4–0.8	0.3 –0.8	0.08–0.29	>60	>20	>10	>10	>10
Orange	2.5–2.7	0.12–0.16	1.2–1.7	3.0 –4.5	1.2 –1.4	50–100	25–50	25–50	5–12	36–100
Sweet Cherry	1.8–3.0	0.15–0.38	1.0–1.8	0.9 –3.0	0.3 –0.5	42–100	11–111	5–60	6–18	>15

other fruit crops. Iron deficiency in *Ericacae* plants is common in soil
a pH higher than 5, and is a serious problem with many species of
grown on alkaline soils.

Zinc deficiency is very common in the *Prunus* genus. The sweet che
representative of this genus, does not contain exceptionally high levels
Deficiency problems are due possibly to the inability of the species to
adequate amounts, particularly on alkaline or near-alkaline soils. Citru
are extremely sensitive to boron toxicity. An examination of the foliar c
of citrus indicates that the problem may be related to the tendency
genus to accumulate boron. Cane fruits such as raspberry accumulate c
ions; consequently, muriate of potash is not used as a potassium fe
because toxic levels of chloride will accumulate in the plant.[4]

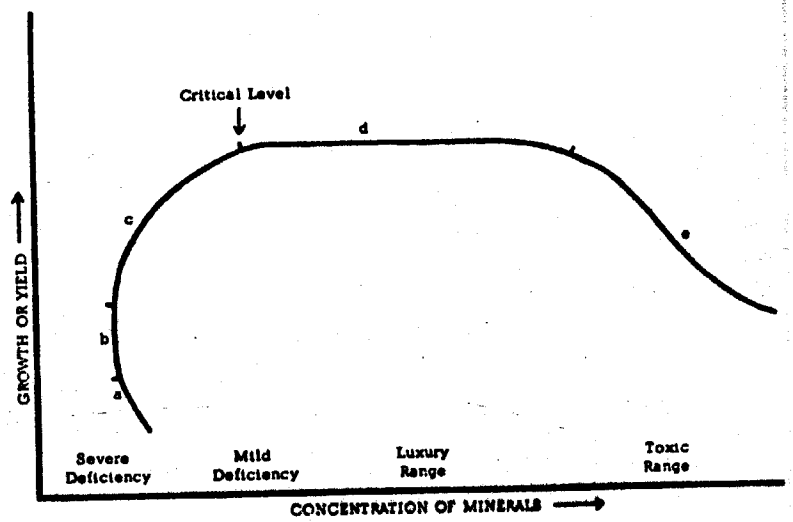

FIGURE 1. Diagram illustrating possible relationships between growth and
tration of essential minerals in leaf tissues. (From Smith.[6] Reprinted by per
of Somerset Press.)

The relation of the accumulation of essential elements to growth o
is illustrated in FIGURE 1. Zone a of the curve indicates that it is
under severe deficiency conditions to have a decrease in concentration
element with an application that results in small increase in growth
phenomenon is known as the Steenbjerg Effect.[5] With a further incr
the application, Zone b, there is an increase in growth with little incr
concentration. Zone c of the curve illustrates a simultaneous increase in
and leaf concentration of the elements. The d Zone represents an incr
concentration of the element but no corresponding increase in yield. This
sents luxury consumption, and is an area of diminishing returns from in
application of the element. With a further increase in absorption of the el
Zone e, the plant will exhibit decreased growth and possibly other
symptoms.

Factors Affecting Uptake of Mineral Elements

The uptake of elements from the soil is determined by many factors, e.g., the abundance in the lithosphere, the form of the element, soil pH, the abundance of interacting elements, the physical condition of the soil as indicated by its tilth, the temperature and moisture content of the soil and the genetic constitution of the plant species.

There are conflicting theories and several unanswered questions concerning uptake of ions by plant roots; however, it is generally agreed that uptake consists of two phases, adsorption and accumulation.[7] Adsorption is a physiochemical phenomenon: it is nonmetabolic and is predominantly concerned with cations. It is considered to be an exchange process, hydrogen ions generated by respiration being released into the culture medium in exchange for cations. Accumulation, however, is dependent upon respiration and is concerned with active uptake of both cations and anions. In the interpretation of active uptake of ions, specific carrier compounds or sites have been postulated, but their nature is still unknown, although various suggestions have been made, such as phosphorylated energy-rich nitrogen compounds and ribonucleoproteins. To what extent accumulation is dependent on adsorption is not certain.

Interactions of Mineral Elements

Interactions between ions can result in competition or competitive inhibition of uptake, whereas the interaction of other ions may result in the stimulation of uptake. It has been demonstrated that there is a competitive interaction between K, Ca and Mg.[8] As the uptake of any one of these elements increases, the uptake of the other two decreases. This is illustrated in Figure 2. With the application of increments of K, the content in the grape leaf increased while the content of Ca and Mg decreased. The yield increased with the application of the first two increments of K; with additional applications, however, there was no further increase. The plant was in a state of luxury consumption, but the toxic level of K had not been reached. From the standpoint of trace elements, possible interactions of importance are phosphorus-zinc, phosphorus-copper, iron-manganese, iron-zinc and zinc-copper.

A review of the literature reveals that a summary of interactions of ions is difficult to prepare because plant species is a factor in the response. The application of Ca in the form of limestone, however, has a marked effect on the availability of several trace elements. Both the Ca^{++} and the $CO_3^{=}$ ions play a role.

Cation and Anion-Exchange Capacity of Roots

The adsorption of ions by dead or damaged plants is called either cation-exchange or anion-exchange capacity of roots. In 1951, Drake and coworkers[9] suggested that the "differences in the ability of plants to take up cations from the soil are largely controlled by the cation-exchange capacity of the plant root and the valence of the cation." In 1963, Vose,[10] after extensive work with dead roots involving determinations of cation-exchange capacity, suggested that "some regulator other than cation-exchange capacity and Donnan

distribution laws must be postulated for the selective assimilation of monovalent and divalent cations by varieties of a species grouped within a relatively narrow range of cation-exchange capacity." It now appears that cation-exchange capacity of roots is not directly related to ion uptake.

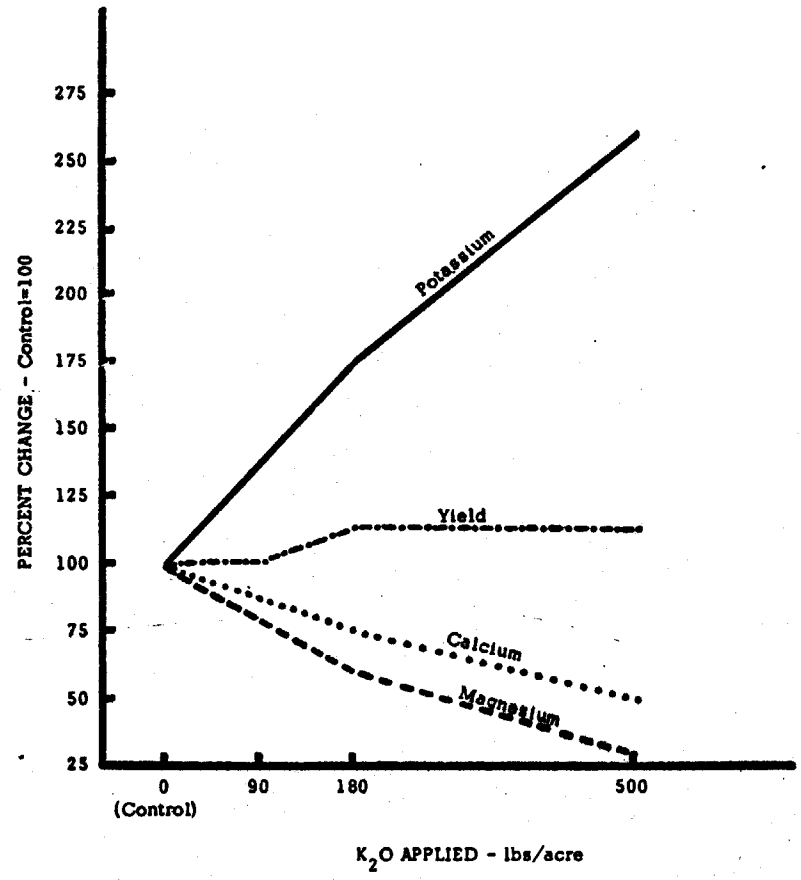

Figure 2. Effect of potash applications on yield and on potassium, calcium and magnesium content of leaf petioles (shown as percent change with control as 100). (From Larsen, *et al.*[8] Reprinted by permission of the Quarterly Bulletin of the Michigan Agricultural Experiment Station.)

Morphological Differences in Root Systems

A detailed study of the root development of field crops was published by [illegible] in 1926, and of vegetable crops by Weaver and Bruner[11] in 1927. Root systems of different plant species vary markedly in type of branching, [illegible] and depth, and some plants of the *Rosaceae* family, such as the

blueberry and cranberry, do not have root hairs. The morphological differences in the root systems of plants is an important factor in their growth and survival and undoubtedly an important factor in their ability or lack of ability to absorb mineral elements. FIGURE 3 illustrates the differences in the root system of several plants.[11]

Genetic Constitution

The inorganic nutrition of plants is a function of the environment during their growth, but obviously it is also a function of their genetic constitution. It has been suggested that a single gene determines the requirement of a given plant for one element while another gene determines its requirement for another element.[13] The tendency of a plant to accumulate nonessential elements is also undoubtedly controlled by one or more genes.

Most fruit trees are propagated by asexual methods of budding or grafting. The rootstock of such plants has a different genetic constitution than its top (scion variety). Both rootstock and scion variety affect the nutritional status of the tree to some extent.[14] In apples, it has been shown that rootstocks affected significantly the percent of P, K, Ca, Mg and Cu in the dry matter of the leaves.[15] TABLE 3 illustrates the effect of rootstock on mineral composition of foliage of Valencia orange trees.[16] Significant differences were observed for both major and trace elements. Similar effects have been reported by other workers for several other fruits.[17–19]

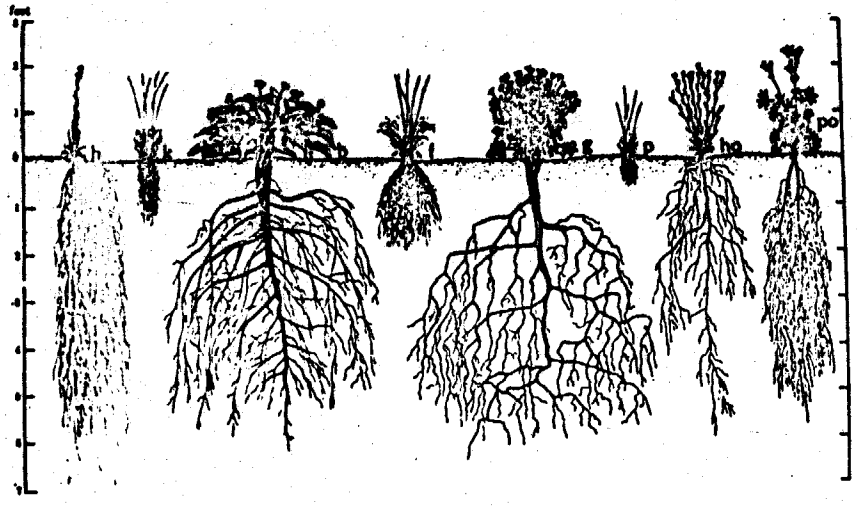

FIGURE 3. Schematic bisect showing the root and stem relationships of important prairie plants: h, *Hieracium scouleri;* k, *Koeleria cristata;* b, *Balsamorhiza sagittata;* f, *Festuca ovina ingrata;* g, *Geranium vascosissimum;* p, *Poa sandbergii;* ho, *Hoorekekia racemosa;* po, *Potentilla blaschkeana.* (From Weaver.[11] Reprinted by permission of McGraw-Hill Book Company, Inc.)

TABLE 3

EFFECT OF ROOTSTOCK ON MINERAL COMPOSITION OF FOLIAGE OF 5-YEAR-OLD VALENCIA ORANGE TREES[16]

Rootstock	Mean Percent in Dry Leaves					Mean ppm in Dry Leaves				
	N	P	K	Ca	Mg	Mn	Cu	Fe	B	Zn
Sour orange	2.55	0.136	2.11	2.26	0.49	69	5.0	75	67	17
Grapefruit	2.56	0.141	2.62	2.11	0.37	62	5.6	59	94	22
Cleopatra	2.50	0.140	1.97	2.33	0.48	88	5.3	87	81	34
Sweet orange	2.63	0.150	2.30	2.16	0.41	53	7.3	82	84	19
Rusk citrange	2.81	0.145	1.76	2.41	0.51	80	8.3	126	95	20
Rough lemon	2.74	0.141	1.98	2.44	0.51	85	5.3	102	80	20
L.S.D. at .05	0.13	0.010	0.22	0.20	0.05	15	2.2	13	13	5

ACCUMULATION OF CERTAIN HEALTH-RELATED TRACE ELEMENTS

The accumulation of health-related trace elements varies markedly between plant species and may not reflect the total amount of the element in the lithosphere. A summary of information concerning certain health-related trace elements is presented.

Chromium

Chromium is widely distributed in soils, waters and biological materials. Turkian and Kleinkopf[20] reported 0.02 ppb in Maine waters. Swaine[21] stated that chromium in soil ranges from 5–1,000 ppm. Detectable quantities are present in a wide variety of plants. Most plants contain less than 1 ppm, but the leaves of wheat may have levels of 4–6 ppm. There are no reports of beneficial effects of Cr on plants, but there are numerous reports of toxic effects. High levels of Cr are associated with witches'-broom disease in tea plants and yellow-branch disease in citrus in the Transvaal region of South Africa.[22] Chromium is accumulated in the roots at levels 20 times that in the stems, and the toxic effects are usually exerted in the roots. Toxic effects are limited, for the most part, to plants growing on serpentine soils. A heavy application of limestone will reduce or eliminate Cr toxicity.

Cobalt

Cobalt is present in most soils in the range of 1–40 ppm; however, some Hawaiian soils have been reported to have 156 ppm. The presence of cobalt in plants was demonstrated in 1841; however, it received little attention from agriculturists until 1935 when it was found to be of great importance to ruminants.[23] There is no evidence of essentiality to higher plants, although some workers have reported growth and yield responses. Cobalt has been

reported to be an essential element for blue-green algae [24, 25] and has been demonstrated to be required for symbiotic fixation of nitrogen by soybeans [26] and alfalfa.[27] Values in plants exceeding 1 ppm are rare; however, a number of plants accumulate cobalt. Kubota and coworkers [28] found up to 845 ppm in swamp black gum, *Nyssa sylvatica*. Less than 5 ppm in swamp black gum indicated an area deficient in cobalt for the needs of grazing cattle and sheep. Cobalt toxicity is not likely to occur under field conditions, although citrus shows toxicity with as low as 11 ppm in pot culture.

Copper

The copper content of soils ranges from 1–200 ppm with most in the range of 25–60. Normal plants contain from 5–20 ppm. Deficiency symptoms are likely with levels less than 5 ppm and toxic symptoms with levels above 20. Copper deficiency symptoms occur most often on peat and muck soils, leached sandy soils, calcareous sands, and leached acid sands and soils heavily fertilized with nitrogen.[29]

Exanthema (dieback) was a serious problem in citrus in Florida in early 1900s, and low copper content of forages is a serious problem in the northern European countries today, resulting in animal disease. Indicator plants for Cu deficiency are citrus, apple and apricot. Corn, beans and squash are excellent indicators for Cu excess. Cannon [30] has reported that the blue-flowered basil *Ocimum homblei* will not grow in soils containing less than 100 ppm.

Fluorine

Goldschmidt [31] listed fluorine as seventeenth in order of abundance in the earth crust, with an average of 0.03% (300 ppm). Fluorine has not been demonstrated to be essential or beneficial to plants. The usual content of plant leaves is 2–20 ppm; however, 1,900 ppm has been reported in *Camellia* and 8,000 ppm in tea. The toxic range for most plants is 100–200 ppm. Fluorine is suggested as essential for animals, but forage grasses containing 50 ppm may be detrimental to animals. Most forage grasses will, however, tolerate several times this concentration. The amount of fluorine accumulated from the soil by plants is usually not closely related to the fluoride content of the soil. Soil type, Ca and P content, and soil reaction (pH) seem to be the predominant controlling factors other than plant species.[32] Gladiolus, apricot, grape and ponderosa pine are excellent indicator plants for harmful levels in the soil. It should be recognized, however, that fluorine is readily absorbed from the atmosphere by plant foliage.

Iodine

The relation of iodine to goiter was suspected before 1900 and was definitely established during the period 1900–1910.[33]

Sea water contains 10–25 ppb, surface and well waters <10–300 ppb, soils and rocks <0.1–70 ppb, sea plants <0.001–12,500 ppm and land plants <0.1–20 ppm. Iodine has not been demonstrated to be essential for plants, although cases of increased growth have been reported. In nutrient culture experiments it has been found to be toxic at higher levels; corn is highly sensitive (8 ppm), while head lettuce is relatively tolerant (160 ppm). No cases of natural iodine toxicity to plants have been reported.[34]

Lead

Swaine [21] has reported that the earth's lithosphere contains an average of 16 ppm with a range from 2–200 ppm. Lead is not essential but is found in all plants. Many plants will tolerate high levels but some show retarded growth at 10 ppm in solution culture studies.[35] Elevated levels of lead in plants and soils are common near roadways and in areas of lead mining and manufacturing where lead is used in the process. Since Missouri is the leading lead-producing state, we are particularly concerned about possible lead contamination of the environment. Extensive studies are in progress on the possible accumulation of lead in soils and various biological materials in lead mining areas of Missouri.

It has been reported that lead is fixed largely in the root systems of plants with limited translocation to the top; however, some analyses show higher levels in the top than in the root system. Since there is considerable fallout of lead near roadways, near smelters and near certain industrial plants, more information is needed concerning the degree of absorption of lead by the foliage of different plant species.

Manganese

Manganese is widely distributed in nature and is essential for both plants and animals. Total manganese in soil ranges from 200–3,000 ppm. Normal levels in plants range from 20–500 ppm. Deficiencies occur in plants growing on calcareous soils, thin peaty soils overlying calcareous deposits and on very sandy acid mineral soils low in native manganese content. Soils with a pH above 6.5 favor oxidation of the manganous Mn to manganic Mn. Apple, cherry, citrus, oats, and sugar beets are good indicator plants for manganese deficiency, while alfalfa, cabbage, cereals, pineapple, and tomato are indicator plants for manganese excess. Total Mn is not a good measure of plant available supply.[36]

Molybdenum

Molybdenum was studied extensively for twenty-five years before it was accepted as essential to plant life. The molybdenum content of soils is usually very low, approximately 2 ppm. Normal levels in plants are 0.3–10 ppm. Levels as high as 372 ppm without injury have been reported. Toxic symptoms under field conditions are rarely observed. Indicator plants for Mo deficiency are the *Brassica* genus, tomato, lettuce, and spinach. Deficiency is readily corrected by the use of molybdenized phosphate or by liming if soil Mo level is adequately high. Toxicity in animals, "molybdenosis," is rather frequent. High Mo in soil results in low copper and high sulfate sulfur concentrations in the forage. There are no practical methods of detoxifying high molybdenum areas, but treatment of animals is effective.[37]

Selenium

Soil content of selenium varies markedly: <0.1–200 ppm. Selenium is not essential to plants but has been reported to stimulate growth in certain species. Recently, it was suggested that it plays an essential role in animal nutrition.[38] Visual symptoms of excess selenium have never been observed under field conditions. The chief interest in this element has been its toxicity to animals. Forage with 5 ppm selenium and water with 0.5 ppm are potentially hazardous.

Indicator plants such as *Astragalus* spp. and *Conopsis* spp. are very important in locating and mapping seleniferous areas, since they thrive only on soils which contain elevated levels of selenium. These plants give rise to a unique soil-plant interaction. They are capable of absorbing large quantities of selenium from soils in which the element is essentially unavailable to other plants. It is not uncommon for these plants to accumulate 10,000 ppm. The highest level recorded is 15,000 ppm.[39]

Through foliage decomposition and oxidation to inorganic salts or soluble organic compounds, toxic quantities of selenium are made readily available for absorption by cultivated crops.[40]

Vanadium

Vanadium is widely distributed in nature. Sea water contains a few ppb, and nearly all soils and plant materials contain small amounts. Soils range from 3–230 ppm, and Bertrand[41] stated that the vanadium content of 62 plant materials ranged from 0.27–4.2 ppm with an average of 1 ppm. There is no evidence that vanadium is essential for higher plants, but it has been reported to be essential for certain bacteria and algae[42] and is involved in nitrogen fixation by *Azotobacter*.[43, 44]

There are no reports of vanadium toxicity under field conditions; however, vanadium is toxic at levels as low as 10 ppm in pot culture and at levels greater than 0.5 ppm in nutrient solutions. Vanadium appears to concentrate in the roots of plants.[45] The possible significance of vanadium in health is of some concern at present.

Zinc

Zinc was not fully accepted as essential for plants until the early 1930s, although its essentiality had been investigated since the 1860s.[46] Zinc deficiency has been found in many soils throughout the world. Toxicity of zinc was recognized in the early stages of research.[47] Zinc level in soil varies from 10–300 ppm.[48] Deficiencies in fruit trees are likely to occur on soils yielding less than 2–3 ppm with various extractants and are removable by *Aspergillus niger*.[48] Normal levels of zinc in plants are 25–150 ppm. Toxicity may result at concentrations above 400 ppm.

Fruit trees, particularly citrus and peaches, are good indicators and commonly show zinc deficiency in soils where field and vegetable crops grow normally. Corn is the most sensitive of the field crops, while barley, oats, rye and wheat are relatively insensitive. Ragweed is a good indicator plant for zinc excess. It has been found growing luxuriantly in the presence of a zinc mineral outcrop when other vegetation was stunted. Horsetail was found growing on a former zinc slime pond where the total zinc of the soil was 12.5%. The zinc content of the horsetail was 5,400 ppm.[49]

Zinc in the soil is rendered less available by heavy application of nitrogen and phosphate fertilizers and by liming acid soils. Zinc deficiency may be corrected by acidification of some soils, increasing the organic matter, application of Zn fertilizer to acid soils or zinc chelates to alkaline soils or the foliar application of Zn SO_4 or Zn Bordeaux. The *Aspergillus niger* test is one of the more reliable methods of measuring zinc availability.

Cadmium

Cadmium is present in the natural environment in relatively low levels. It has not been demonstrated to be essential for the growth of plants; however, it can be readily adsorbed and, consequently, is detectable in most plants. Bowen[50] reported average levels of 0.06 ppm in soils and 0.6 ppm in terrestrial plants. Cadmium is toxic to plants at higher concentrations, but this has occurred only in cases of contamination by mining and smeltering operations. The primary interest in cadmium concerns its possible toxic effects on animals and humans in particular.

The highest levels of cadmium in soils and plants have been reported by Kobayashi[52] in Japan. The content of cadmium, zinc and lead in water, soil and agricultural products in the immediate vicinity of mines and smelters was much higher than elsewhere. Cadmium fallout from the smelters and waste water discharged directly into rivers and subsequently used for irrigation has resulted in severe contamination and toxic effects on rice and vegetables. The cadmium content of soils ranged from 23–88 ppm; of leafy vegetables, from 3.2–56 ppm; of root crops, from 1.4–17 ppm; and of fruits and grains, from .49–8.6 ppm. Persons exhibiting "itai-itai" disease were found only in those areas where food and water contained high levels of cadmium.

Mercury

Mercury is a rare element but it is widely distributed in the environment. Bowen[50] has reported a range of 30–800 ppb in typical soils and an average of 15 ppb in terrestrial plants and 30 ppb in marine plants. Accumulator plants are rare, although *Arenaria setacea* has been reported to be an indicator plant in Europe.[53] Pierce and associates[54] have reported values for typical soils in the same ranges with an average of about 100 ppb. Shacklette[54] reported that plants growing in environments with the normal small amounts of mercury seldom exceed 500 ppb, but plants in environments that have significantly larger amounts of mercury because of the natural occurrence of mercury-bearing deposits may contain 500–3,500 ppb. Although few data are available, it appears that plants reflect fairly well the mercury content of their environment.

Summary

The main source of major and trace elements for man and animals is plant materials. The adequacy or deficiency of plant materials in supplying the dietary needs of animal life depends upon several factors. The concentration of all minerals in plants depends upon the interaction of the plant species with the soil and environmental conditions during growth.

The total amount of an element in the soil is not necessarily a reliable guide to the amount that is available. Various extractants are used to determine available levels of elements; these tests may be well correlated with the uptake by certain species but are not valid for other species. Chemical extractants may not indicate the interactions of elements that influence availability and cannot indicate the influence of environmental factors of soil moisture and temperature. Moreover, the genetic constitution of the plant plays a major role in mineral element uptake, and the selenium accumulator species provide an outstanding example of this genetic effect. The ability of a plant to accumulate minerals from a specific soil can be determined only by analysis of the plant parts.

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DISCUSSION

MR. PINKERTON: Dr. Stocks, in an epidemiological investigation of stomach cancer in Great Britain, reported that zinc found in gardens in areas where stomach cancer had been reported had a value of 81 ppm of zinc soluble in half normal acetic acid, whereas, the controls had something like 51 ppm.

Do you consider these values to be rather high, and would you consider to be available to plants?

DR. HEMPHILL: It's extremely difficult to relate these values to availabili to plants. In general, the best measure of zinc availability to plants is us *Aspergillis niger* as an indicator. It's very difficult to use a chemical extract as a measure. The same thing is true of lead. We are not satisfied with so-cal available lead as a measure. We actually get a better correlation between pl uptake and what we show as total lead than with available lead. Thus, I wo not depend too much on this correlation with extractable zinc, although it n be true for some plants.

Zinc-65 Absorption and Turnover in Rats

I. A PROCEDURE TO DETERMINE ZINC-65 ABSORPTION AND THE ANTAGONISTIC EFFECT OF CALCIUM IN A PRACTICAL DIET [1,2]

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ABSTRACT Zinc-65 as chloride or glycine complex was administered to growing rats receiving a practical diet in 6 experiments. A rapid fecal excretion of Zn^{65} occurred following administration via feed or gavage. Increased dietary calcium significantly increased ($P < 0.01$) the initial rate of Zn^{65} loss following oral administration and consistently and significantly decreased the percentage remaining in the body longer than 2 days. Conversely, body loss of injected Zn^{65} decreased significantly ($P < 0.01$) with increased calcium. Comparison of retention curves at 100 to 250 hours post-administration for injected and dietary or gavage-administered Zn^{65} and their extrapolation to zero time allowed the determination of the percentage of radiozinc absorbed. Calcium significantly decreased ($P < 0.01$) the percentage of Zn^{65} absorption and increased the biological half-life (decreased turnover) of Zn^{65} beyond 100 hours post-administration. Calcium did not affect body weight. The results are explained by a decreased absorption of stable and radiozinc with increased dietary calcium. Calcium also significantly increased ($P < 0.01$) carcass Zn^{65} in the femur and decreased it in liver, kidney and muscle at 28 days post-administration.

Many investigators have demonstrated that rations high in calcium cause a marked potentiation of the zinc-deficiency syndrome in swine, poultry and dogs (1–10). The site(s) of the calcium antagonism has not been elucidated. In swine, high calcium diets were shown to decrease the zinc content of certain organs such as the liver, but not of others such as skin, pancreas or intestine (4). This led to the postulation that calcium interfered with the absorption of zinc from the intestine. Substantiating this idea were experiments conducted in vitro which demonstrated that zinc was readily removed from solution during the precipitation of calcium phosphate compounds such as might occur in the intestinal lumen (11). Phytic acid complexes have also been shown to decrease the availability of dietary zinc (12).

Attempts to demonstrate directly a calcium antagonism of zinc absorption have been largely unsuccessful. Newland et al. (13) suggested that calcium increased endogenous zinc metabolism in swine. In balance studies with rats receiving semipurified diets, Forbes and Yohe (14) concluded that increased calcium did not reduce zinc absorption. Also, Roberts and Hoekstra,[2] working with rats fed purified diets, did not observe, at 3 days post-administration, a reduced body retention of Zn^{65} administered by gavage. In an overall review of the problem, Forbes (15) speculated that the calcium antagonism occurred at the cellular level.

In view of the confusion pertaining to the site of the antagonistic action of calcium on zinc, it was decided to reinvestigate the problem more directly with rats by using Zn^{65} and a whole-animal scintillation counter. A preliminary report of this research has been published.[3] The results of these experiments, as well as results of somewhat different experiments reported subsequently by others (12, 16) indicate that under appropriate dietary conditions,

Received for publication October 21, 1964.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, Madison. Supported in part by Public Health Service Research Grant no. AM-05606 from the National Institute of Arthritis and Metabolic Diseases.

[2] Roberts, H. F., and W. G. Hoekstra, unpublished data.

[3] Preliminary reports of this research were presented at the 1963 annual meeting of American Society of Animal Science at Corvallis, Oregon, and some of the data was included in a review (1) presented at the 1964 annual meeting of the Federation of American Societies for Experimental Biology at Chicago, Illinois.

calcium does decrease zinc absorption or availability.

METHODS AND MATERIALS

Six experiments were conducted, using male albino rats of the Holtzman strain. There were 4 rats per dietary treatment group except in experiment 6 which had 8 in the groups receiving Zn^{65} in the feed or by gavage and 4 in the groups injected with Zn^{65}. The rats were obtained at 3 weeks of age and placed in individual galvanized cages with raised wire floors. They were weighed initially and at approximately weekly intervals. Distilled water was supplied ad libitum in glass water bottles.

The composition of the basal diets is given in table 1. Additional calcium at the 0.00, 0.60 or 1.76% level (also 0.30 and 0.90% in experiment 1) was provided as calcium carbonate. In each experiment a sample of the basal diet was analyzed for total calcium, phosphorus and zinc. The calcium was found to vary from 0.22 to 0.29% in air-dried samples of ration. The phosphorus content was from 0.96 to 1.01% in the first 3 experiments and 0.83 to 0.86% in experiments 4 through 6. Total zinc was determined by atomic absorption spectrophotometry and varied from 28 to 33 ppm except in experiment 1 where it was 51 ppm. Analyses of several currently available calcium carbonate sources showed none to contain over 28 ppm zinc. Of those analyzed and used in the experiments reported, none contained over 24 ppm zinc and experiments 5 and 6 utilized a source which assayed less than 5 ppm zinc.

TABLE 1

Composition of basal diet [1]

	g/kg
Ground corn	587.37
Soybean oil meal (44% protein)	287.00
Dried brewer's yeast	30.00
Alfalfa meal (17% protein)	50.00
Vitamin B_{12} (0.1% mix)	0.01
Irradiated yeast	0.12
Sodium chloride	12.50
Potassium phosphate (dibasic)	33.00 [2]
	1000.00

[1] Calcium-supplemented diets were obtained by adding calcium carbonate at the expense of the entire diet in experiments 1 through 5 and at the expense of ground corn in experiment 6.

[2] Changed to 25.00 g/kg in experiments 4, 5 and 6 with compensating increase in ground corn.

A ration of the type used, but with somewhat less soybean oil meal is often used in commercial swine feeding. Its adequacy for rats was indicated by the satisfactory growth obtained in all experiments.

A pre-experimental period varying from 13 to 21 days was allowed in all experiments to accustom the animals to their rations. During this and the experimental period, the respective rations were provided ad libitum except for a mild diet restriction just prior to administration of Zn^{65} via feed. The exact amount and duration of this restriction varied from experiment to experiment but feed intake was limited to about one-half the amount normally consumed during a 6- to 13-hour period immediately prior to administering the isotope. This restriction served to stimulate the appetite, thus insuring adequate isotope ingestion. Animals to receive the isotope via gavage or intramuscular injection were not subjected to any diet restriction.

In experiment 1, high specific activity Zn^{65} [4] was administered as its chloride in dilute HCl solution. Prior to administration in the other experiments the Zn^{65} chloride was complexed by adding glycine at a 4:1 (glycine:zinc) molar ratio and adjusting the pH to 7.4 with sodium hydroxide. Radioactive feed was prepared by adding an aliquot of the Zn^{65} solution to a sample of each ration. The samples were dried and thoroughly mixed, and each animal was offered a similar amount of radioactive feed varying in different experiments from 2.5 to 7.5 g. In the experiments where the isotope was administered by gavage, the animals were lightly anesthetized to facilitate insertion of the stomach tube. Animals injected with Zn^{65}-glycine intramuscularly in the rear leg received no special pre-administration treatment. Each animal received from 100,000 to 300,000 counts per minute (about 0.5 to 1.5 μc of Zn^{65}) irrespective of administration method. The exact amount was determined at the time of the initial measure of animal radioactivity.

[4] The radiozinc was obtained as zinc-65 chloride in HCl from Oak Ridge National Laboratory, Oak Ridge, Tennessee.

After allowing the animals to eat their radioactive feed for a 4- to 5- hour period, they were counted in a whole-animal gamma-scintillation detector [5] to determine the administered dose. Thereafter all rats received their normal rations for the duration of the experiment which varied from 250 to 1100 hours. During this period the animals were counted at intervals ranging from 12 hours to several days. A Zn^{65} standard was measured regularly and all values were corrected for background, isotope decay and any minor changes in counter efficiency. A 4- to 5-hour delay between administration and first counting was also allowed when the isotope was given via gavage or intramuscular injection. It was shown separately that essentially no loss of body radioactivity occurred during this time.

RESULTS AND DISCUSSION

Growth was not influenced during the duration of the experimental periods by the addition of calcium (table 2). However, level of calcium in the ration had a significant effect on the retention of ingested Zn^{65} (fig. 1). This figure illustrates the results from experiment 3, which are similar to those obtained in all other experiments. For each increase of calcium in the diet, the percentage of the ingested oral Zn^{65} which was retained decreased at

[5] Armac scintillation detector model 440 manufactured by Packard Instrument Company, Inc., La-Grange, Illinois.

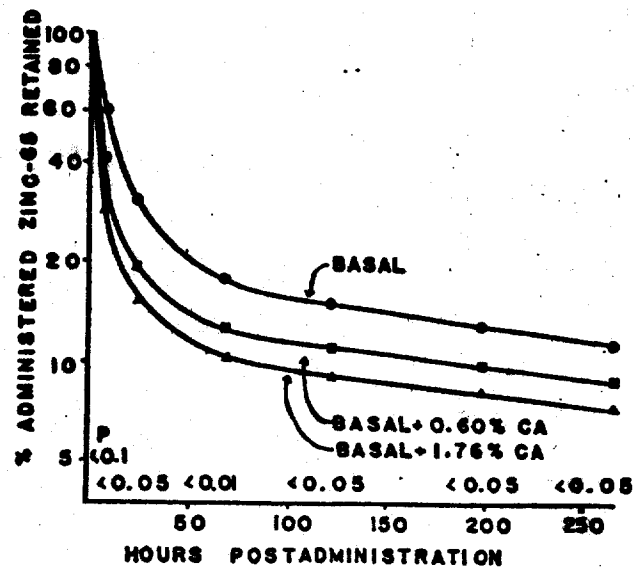

Fig. 1 Effect of dietary calcium on retention of Zn^{65} administered in the feed (exp. 3).

TABLE 2

Effect of dietary calcium on body weight

Exp.	Days fed experimental diet	Mean [1] final [2] body weight			Significance due to calcium
		Basal	+ 0.60% Ca	+ 1.76% Ca	
		g	g	g	
1 [3]	50	258	280	276	P > 0.1
2	24	149	160	150	P > 0.1
3	35	238	244	233	P > 0.1
4	34	220	219	231	P > 0.1
5	46	305	311	314	P > 0.1
6	49	301	299	296	P > 0.1

[1] Treatment means (dietary, injected and gavage Zn^{65} treatments combined where applicable).
[2] Initial body weights within an experiment were similar between treatments.
[3] Intermediate calcium levels omitted, but omitted values correspond to those listed.

each post-administration measurement of radioactivity. Early in the experiment (zero to about 50 hours post-administration) there was a rapid loss of body Zn^{65}. This represents primarily the passage of isotope through the intestine and its subsequent loss in the feces. This early loss of Zn^{65} was increased by increasing dietary calcium. After 50 hours, the loss of Zn^{65} was at a much slower rate representing excretion of Zn^{65} which had been absorbed from the intestine into the body proper. The later rate of loss was also influenced by calcium, but in the opposite direction from the effect on early Zn^{65} loss, as evidenced by converging of the retention curves with increasing time (the animals were counted for a longer post-administration time than illustrated in fig. 1 which, if plotted, would show converging). It appeared, therefore, that increased dietary calcium both increased the early loss of Zn^{65} by decreasing Zn^{65} absorption and decreased the rate of turnover of body Zn^{65}.

This effect of calcium on zinc turnover was substantiated when Zn^{65} was injected rather than fed. Increased calcium very significantly increased the retention of the injected Zn^{65} at all time periods (fig. 2). This is explained on the basis that increased calcium also reduced the absorption of stable dietary zinc thereby causing the Zn^{65} which was in the body to be replaced at a slower rate. This effect occurred regardless of whether the Zn^{65} had entered the body via injection or by absorption from the intestine.

TABLE 3

Effect of dietary calcium on fecal excretion of injected Zn^{65} (exp. 2)

Treatment	Body Zn^{65} lost/g dry feces [1]
	%
Basal	1.51
Basal + 0.60% Ca	1.11
Basal + 1.76% Ca	0.84
Significance due to Ca	$P < 0.01$

[1] Feces collected for 12 hours starting at 157 hours post-administration. Growth and feed intakes were similar between treatments.

The effect of calcium on the loss of body Zn^{65} was reflected by altered fecal excretion of Zn^{65} (table 3). This was expected because numerous workers have demonstrated that body zinc excretion via the urine is extremely small compared with fecal excretion, and any effect on urinary excretion could not greatly affect total body excretion of zinc.

When Zn^{65} retention was plotted on a logarithmic scale against time on a linear scale (fig. 1), the retention curves were essentially linear in all experiments between 100 and 250 hours post-administration. Furthermore, the slopes of these lines were the same whether Zn^{65} was injected or administered orally. It was thought, therefore, that by comparing the retention curves for the 2 methods of administration a procedure might be developed for calculating the actual percentage absorption of

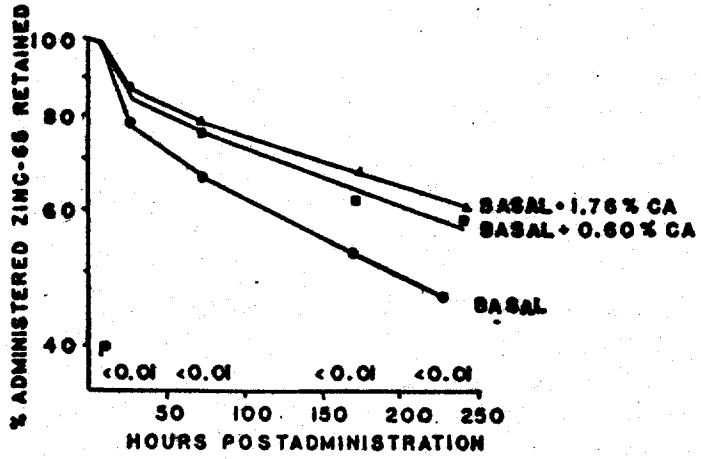

Fig. 2 Effect of dietary calcium on retention of intramuscularly injected Zn^{65} (exp. 2).

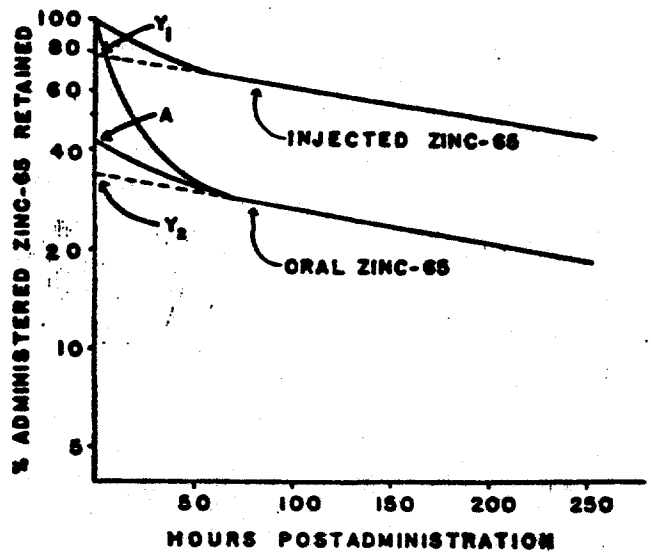

Fig. 3 A comparison of retention curves for Zn^{65} administered in the feed and by intramuscular injection (exp. 2). Percentage absorption (A) of oral Zn^{65} is calculated by dividing Y_2 (the y intercept of the extrapolated retention curve for oral Zn^{65}) by Y_1 (the y intercept of the extrapolated retention curve for injected Zn^{65}) and multiplying by 100.

orally administered isotope. Figure 3 shows the separate retention curves for Zn^{65} when it was administered orally and injected. At a single level of calcium, both methods of administration produced linear retention curves 100 to 250 hours postadministration whose slopes did not differ significantly in any of several experiments.

The procedure and rationale for calculating percentage Zn^{65} absorption (fig. 3) was as follows. 1) The retention curve of the injected isotope showed an initial rapid decrease but followed a simple exponential curve during the period 100 to 250 hours. Extrapolation of this linear portion of the injected isotope curve to zero time gave 79% of the injected dose. 2) The retention curve for orally administered isotope was similar except for the much greater early loss of body radioactivity (prior to 100 hours). Extrapolation of the linear portion of the oral isotope retention curve (100 to 250 hours) to zero time gave a value of 34% of the dose. 3) Therefore, comparison of the retention curves for oral versus injected isotope indicated that the y intercept value of 34% of the dose for the oral dose represented 79% of the isotope actually entering the body (the y intercept for the injected dose). 4) Division of 34% by 79% and multiplication by 100 gave 43% which represents that portion of the oral Zn^{65} which was absorbed from the intestine (i.e., that portion which was handled in the body similarly to an injected dose). Since the y intercept values for injected Zn^{65} were influenced somewhat by calcium, it was desirable to use similar calcium levels for oral and injected Zn^{65} in determining the percentage of Zn^{65} absorbed. Cotzias et al. (17) proposed a similar method to determine the absorption of Zn^{65} in mice. However, their method did not utilize any injected animals and individual variation was found to be excessive with 6 mice. This was not the case with rats fed a practical diet in the present study.

Curves as described above were developed for each animal with every level of calcium in each of the 6 experiments. Experiments 1, 3 and 4 included only oral Zn^{65} treatments. Since the above method was developed after experiment 4 was completed, data from the injected rats of experiment 2 were used to determine the percentage absorption in the other 3 experiments. Experiments 5 and 6 were conducted to verify the procedure used to determine the percentage Zn^{65} absorption.

The Zn^{65} absorption data from the 6 experiments calculated as described are presented in table 4. Increased dietary calcium significantly reduced the percentage absorption of Zn^{65} in all experiments except for isotope administered via feed in experiment 5 in which the trend was the same, but the differences were not statistically significant. Variations in the percentage zinc absorption in the different experiments were noted. When 100 ppm of stable zinc were added to the diet, the percentage absorption of oral Zn^{65} was markedly reduced, but the effect of calcium was still significant ($P < 0.05$). This observation is not in agreement with Berry et al. (18) who reported that the addition of calcium to a swine ration containing 100 ppm supplemental zinc increased the retention of oral Zn^{65}. Calcium significantly reduced Zn^{65} absorption whether it was administered in the feed or by gavage; however, there was a greater percentage absorption of Zn^{65} administered by gavage. The magnitude of the effect of calcium was greater between the low and middle calcium levels than between middle and high levels regardless of administration method.

The retention curves for Zn^{65} through 250 hours post-admistration could be resolved into 2 linear components as described by Comar (19) and the biological half-lives were calculated. Also, experiments extending beyond 250 hours showed additional logarithmic components of zinc turnover representing zinc compartments in the body which had slow turnover rates.

Table 5 shows the biological half-lives of the 3 different components in experiment 6. There was a significant decrease in the half-life of the earliest component (zero to 60 hours) due to calcium. (These values were obtained after correcting for the sec-

TABLE 4

Effect of calcium on absorption of Zn^{65}

Exp.	Method of Zn^{65} administration	Administered Zn^{65} absorbed			Significance due to calcium
		Basal	+ 0.60% Ca	+ 1.76% Ca	
		%	%	%	
1 [1]	Diet	28.2 A [2]	20.6 B [2]	18.1 B [2]	$P < 0.01$
2	Diet	42.8 A	20.6 B	15.7 C	$P < 0.01$
3	Diet	24.1 A	15.4 B	12.6 B	$P < 0.01$
3 [3]	Diet	8.7 A	7.8 AB	6.3 B	$P < 0.05$
4	Diet	28.9 A	19.2 B	13.3 C	$P < 0.01$
5	Diet	29.8 A	21.8 A	21.8 A	$P > 0.1$
6	Diet	30.9 A	23.1 B	20.3 B	$P < 0.01$
5	Gavage	36.3 A	30.6 B	27.3 C	$P < 0.01$
6	Gavage	36.7 A	27.2 B	25.6 B	$P < 0.01$

[1] Intermediate calcium levels omitted, but omitted values fall between those listed.
[2] Treatment means. Values not followed by same letter within the same row are significantly different ($P < 0.05$).
[3] Diets supplemented with $ZnCO_3$ to provide 100 ppm added zinc.

TABLE 5

Influence of dietary calcium on zinc turnover (exp. 6)

Period post-administration	Method of Zn^{65} administration	Biological half-life of Zn^{65}			Significance due to calcium
		Basal	+ 0.60% Ca	+ 1.76% Ca	
hours		*hours*	*hours*	*hours*	
0–60	Diet	8.8 A [1]	7.2 B [1]	6.3 B [1]	$P < 0.01$
	Gavage	8.4 A	7.8 A	5.7 B	$P < 0.01$
100–250	Diet	316 A	432 B	492 C	$P < 0.01$
	Intramuscular injection	325 A	434 B	500 B	$P < 0.01$
	Gavage	338 A	428 B	483 C	$P < 0.01$
300–661	Diet	721 A	1042 B	1053 B	$P < 0.01$
	Gavage	713 A	992 B	1129 C	$P < 0.01$

[1] Treatment means. Values not followed by same letter within the same row are significantly different ($P < 0.05$).

and component.) This occurred whether the Zn^{65} was administered in the feed or by gavage. Graphic analyses of the data from the first 4 experiments substantiated this observation. Calcium significantly increased the half-life or body Zn^{65} during the period 100 to 250 hours post-administration regardless of whether the isotope had been administered orally, by injection or by gavage. When all methods of isotope administration were considered, there was only one case in the 6 experiments in which the half-life of Zn^{65} during this period was not significantly increased by adding 0.60% calcium to the basal diet. In this isolated case (injected isotope in experiment 5) it was necessary to add 1.76% calcium to statistically increase the half-life of body Zn^{65}. In most cases adding 1.76% calcium further increased the Zn^{65} half-life over 0.60% added calcium, but many of these differences were not statistically significant. These results substantiate those illustrated in figure 2. Calcium was also shown to increase the half-life of Zn^{65} which remained in the body longer than 250 hours.

Table 5 also shows that at a single level of calcium there was no difference in the 100 to 250 hour half-life regardless of whether the Zn^{65} was administered in the diet, by intramuscular injection or by gavage. A separate experiment showed that the intramuscularly injected Zn^{65}-glycine moved rapidly away from the site of injection and only about 8% of the administered dose remained at or near the injection site by 24 hours post-administration. This had decreased to 4% by 4 days. These observations are important in justifying the previously described procedure for determining the percentage absorption of oral Zn^{65}.

Dietary calcium not only affected Zn^{65} absorption and turnover, but also altered the distribution of Zn^{65} in the carcass at 28 days post-administration as shown in figure 4. Calcium decreased the Zn^{65} content of the soft tissues as represented by liver, kidney and muscle and increased it in the bones as represented by the femur. This was true regardless of whether Zn^{65} was expressed per organ or per gram of dry organ, although not all the soft tissue differences on a concentration basis were statistically significant.

The mechanism by which calcium acts to reduce zinc absorption is unknown. Calcium may effect zinc-binding in the intestinal contents or some other type of competition between calcium and zinc may exist either in the intestine or elsewhere in the body. The observation that increased calcium decreased the half-life of orally administered Zn^{65} during the period zero to 60 hours post-administration indicates an accelerated fecal loss of intestinal Zn^{65}. An explanation of this observation might be that calcium is simply increasing the rate of passage of zinc through the intes-

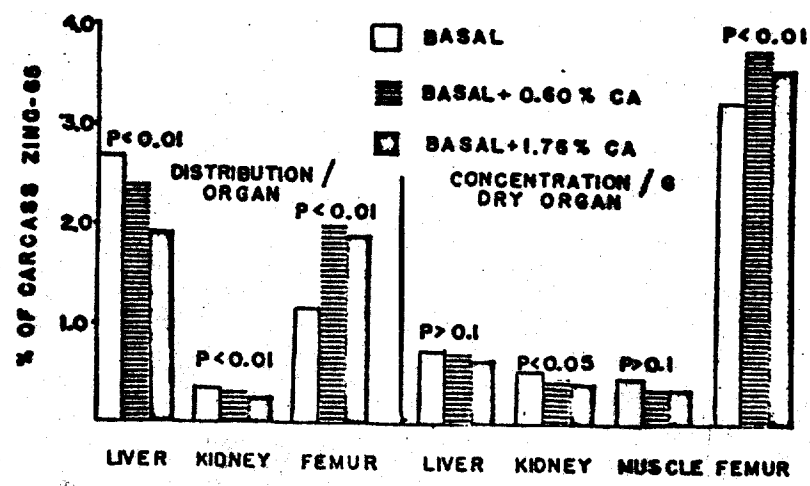

Fig. 4 Effect of dietary calcium on distribution of carcass Zn^{65} at 28 days post-administration (exp. 5).

tine and thus decreasing zinc absorption per se. Another explanation might be that calcium allows normal absorption of zinc, but accelerates the rapid recycling of recently absorbed zinc back into the intestine (possibly via the liver and pancreas) thus decreasing the body retention which would result in a decreased net absorption. Either of these conditions would result in a decreased biological half-life during the early period post-administration. The effect of calcium in decreasing Zn^{65} turnover at extended periods post-administration probably relates to the continued action of calcium on the absorption of stable dietary zinc.

The factor in the corn-soybean diets which interferes with zinc absorption in the presence of calcium has been speculated to be phytic acid (12). However, under our experimental conditions we have not been able to potentiate a zinc deficiency in chicks or decrease the absorption of oral Zn^{65} in rats by the addition of phytic acid to purified diets containing animal proteins or protein hydrolysates. In the present studies phytic acid and vitamin D were constant within each experiment. Our results on the effect of added calcium on Zn^{65} absorption with purified diets have been inconsistent. However, under the experimental conditions employed with the practical diet, increased dietary calcium consistently and significantly reduced absorption of oral Zn^{65} and decreased its turnover in the body.

ACKNOWLEDGMENTS

The authors are indebeted to Donald Willet, Feed and Fertilizer Section, General Laboratory Division, Wisconsin Department of Agriculture, for his analyses of calcium and phosphorus in the rations. The authors are also indebted to Wayne Becker for his assistance in preparing the electronic computor program which was used in the analysis of a portion of the data.

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J. Biol. Chem. 134 : 425-42 (1940)

THE EFFECT OF ZINC ON ALKALINE PHOSPHATASES*

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(Received for publication, March 27, 1940)

Zinc has been shown to be an element essential to the life and growth of rats, but as yet there is no clear indication of what its function may be in metabolism. There are several reports of the occurrence of zinc in pure biological compounds. Scott and Fisher (1) have shown zinc to be necessary for the crystallization of insulin, although other metals were also effective. Keilin and Mann (2) report that carbonic anhydrase contains 0.32 per cent zinc. However, it has been well established that this enzyme is inhibited by small amounts of added zinc (Meldrum and Roughton (3), and unpublished data from this laboratory). In connection with the rôle of this red blood cell enzyme it may be significant that a zinc-porphyrin compound is excreted in the urine of humans (4). A zinc-porphyrin has also been isolated from diptherial toxic broth (5). Foster and Waksman (6) have shown zinc to be essential for the growth and fumaric acid production of *Rhizopus nigricans*. Recently Holmberg (7) has found that a purified uricase preparation contains 0.13 per cent zinc.

In previous work from this laboratory (8, 9) we have been able to find no detectable difference in the carbohydrate metabolism of zinc-deficient rats. However, a disturbance of the nitrogen metabolism was observed. This involved a decreased blood non-protein nitrogen, a marked delay in the rate of absorption of amino acids fed by mouth, an increase in fecal nitrogen excretion, a decrease in the daily urinary creatinine excretion, and a marked

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

Supported in part by a grant from the Wisconsin Alumni Research Foundation.

reduction in the pancreatic trypsin content. It was also shown that there is no appreciable decrease in the zinc content of most of the soft tissues of the zinc-deficient animal. However, bone, teeth, and to a lesser degree small intestines do show a decreased concentration of this element. Because of this change our attention was attracted to the possible effect of zinc ions on the activity of phosphatases of the bone and other tissues.

A survey of the literature reveals that Erdtman (10) found a 0.004 mM concentration of zinc ions to produce an 18 per cent inhibition of dialyzed kidney phosphatase. Jenner and Kay (11) state that zinc has no, or slight inhibitory, effect on phosphatases, although they give no experimental details or data. However, Pett and Wynne (12) observed a stimulation of bacterial pyrophosphatases by zinc in small amounts and inhibition at higher levels. Other bacterial phosphatases were inhibited by all concentrations of zinc used.

EXPERIMENTAL

Bone phosphatase preparations were made by grinding the cleaned fresh bone (rat femurs), making up to a 5 per cent suspension in water, and allowing to autolyze for 24 hours at 37° in the presence of chloroform. This mixture was then filtered through a Whatman No. 42 paper, producing a water-clear enzyme preparation containing about 4 mg. of dry matter per cc. The crude intestinal enzyme was prepared in the same way except that the washed, whole, rat intestine was minced and made up to a 10 per cent suspension. After the filtration this crude intestinal phosphatase solution had about 14 mg. of dry matter per cc. When this was dialyzed at pH 6.1 for 48 hours, the dry matter content was reduced to 2 mg. per cc. The enzyme solution took on a non-filtrable cloudiness during dialysis which precipitated after a few days in the refrigerator. Toluene was used as a preservative during the dialysis, and a mixture of toluene and chloroform afterwards.

The substrate used in a majority of the experiments to be reported has been sodium β-glycerophosphate, although disodium phenyl phosphate, sodium hexosediphosphate, and sodium pyrophosphate have also been used. These phosphate esters were made up as a 4.8 mM solution (1 cc. = 0.15 mg. of P) in a 50

mM veronal buffer. The final pH was adjusted to 9.2. For each determination 10 cc. of the substrate solution and 0.5 cc. of the enzyme solution in a total volume of 11.5 cc. were incubated at 37°. The reaction was stopped with 2 cc. of a 10 per cent trichloroacetic acid solution, filtered if necessary, and the liberated inorganic phosphorus determined by the customary Fiske-Subbarow method. Blanks were run with every experiment. Any substance added to the reaction mixture was tested for its effect on the substrate alone, under the identical time and temperature conditions of the reaction.

Effect of Zinc on Phosphatases in Vitro—In the preliminary experiments on the effect of zinc on the activity of animal alkaline phosphatases *in vitro* the standard King-Armstrong method was used. The substrate for this method is disodium phenyl phosphate, and the incubation time is half an hour at 37.5°. It was found that the activity of crude bone phosphatase was progressively inhibited by increasing concentrations of zinc ion. The effect of zinc on the action of crude kidney phosphatase was also inhibitory, but only to a small degree. However, when crude intestinal phosphatase was tested, zinc was found to have no effect at all.

These preliminary results appeared to indicate a difference in the phosphatases from the three sources, so the work was extended to other substrates. The effect of zinc on the hydrolysis *in vitro* of β-glycerophosphate (21 hours incubation) by the crude bone and kidney phosphatases was more or less markedly inhibitory, thus agreeing with the first results obtained with the phenyl phosphate substrate. However, the effect of zinc on the hydrolysis of glycerophosphate by the crude intestinal phosphatase was markedly activating. Zinc also was found to activate the hydrolysis of sodium hexosediphosphate and sodium pyrophosphate by the crude intestinal phosphatase. These results are shown in Fig. 1. During the last 12 months this stimulating effect of zinc on the hydrolysis of β-glycerophosphate by crude rat intestinal phosphatase has been repeated on approximately 60 to 70 individual preparations, the degree of activation varying between 40 and 110 per cent. Zinc chloride, sulfate, and nitrate are equally effective. The pH of the reaction mixture is not altered by the amounts of zinc salt used in these experiments.

The time course of the hydrolysis of β-glycerophosphate by the crude intestinal phosphatase alone, and with optimum zinc, optimum magnesium, and a combination of optimum amounts of zinc and magnesium, is shown in Fig. 2. From the curves in this chart it can be seen that both of the metal ions undergo an augmentation in their percentage activation effect with time. This has previously been observed for the magnesium ion by Bodansky (13) and Holmberg (14). It should be noticed that the percent-

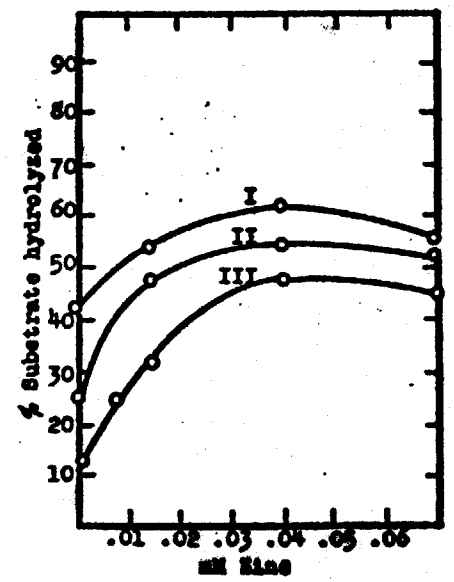

FIG. 1. The effect of zinc on crude intestinal phosphatase activity *in vitro*. 21 hour incubation period at 37°, pH 9.2. Substrates, Curve I, hexosediphosphate; Curve II, glycerophosphate; Curve III, sodium pyrophosphate.

age activation due to zinc at ½ hour's incubation is slight, amounting to only 5 per cent. This furnishes a possible explanation of the lack of zinc activation of the hydrolysis of the phenyl phosphate substrate by the crude intestinal phosphatase, since here the incubation was only continued for a half hour; and in fact, it has been found that with a longer incubation period zinc does activate the hydrolysis of this substrate. Since the metal ions, zinc and magnesium, together produce an effect greater than

either alone (Fig. 2), it seems possible that the mechanism of activation by these ions is, in part at least, different and independent.

Dialysis of the crude intestinal phosphatase resulted in a complete loss of the ability of zinc ions to activate the enzyme. In fact the intestinal enzyme after dialysis was inhibited by zinc; this observation brings a uniformity into the action of zinc ions on phosphatases from the three sources—bone, intestine, and kidney. The degree of the zinc inhibition of intestinal phosphatase increases slowly with the length of dialysis, as is shown in Fig. 3. From the curves shown in Fig. 3, it is evident that the ability of zinc to stimulate the magnesium-activated dialyzing enzyme outlasts the ability of zinc to stimulate the dialyzing enzyme alone. However, this property also is lost when dialysis is sufficiently complete, as is shown in Preparations 2 and 3 of Table I. Also from Table I it is evident that the dialysate, when again added to the dialyzed intestinal enzyme, can restore the zinc

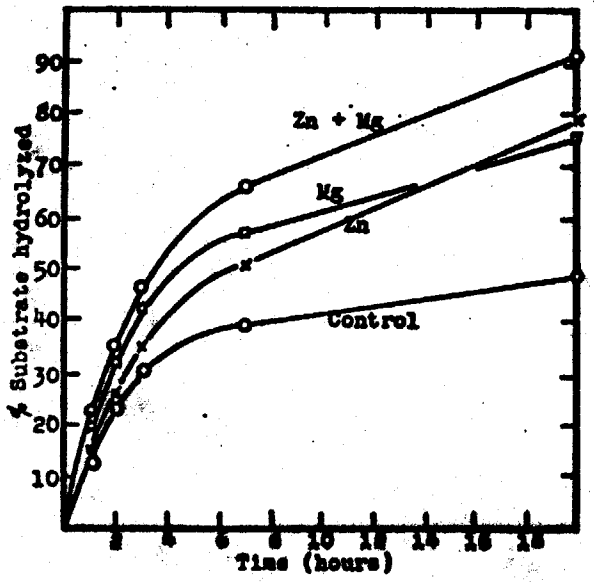

FIG. 2. The time course of the hydrolysis of β-glycerophosphate by crude intestinal phosphatase. Concentration of zinc, 0.03 mM; of magnesium, 0.1 mM.

activatability of the enzyme. The dialysate alone, or in the presence of zinc or magnesium or both, had absolutely no effect on the substrate.

These results indicate that there is present in crude intestinal preparations, but seemingly not in bone or kidney preparations, a dialyzable substance which, together with zinc ion, is able to activate phosphatases. This dialyzable zinc coactivator is not destroyed by taking the dialysate to dryness at 100°. It is destroyed by ashing. It is soluble in water, partially soluble in absolute ethyl alcohol, and completely insoluble in pyridine, acetone, and absolute butyl alcohol. It is soluble in dilute acids and bases, although it seems to be partially destroyed by being taken to dryness in the presence of concentrated ammonia or NaOH. A boiled tissue extract from a fresh intestine has none of this substance. A boiled tissue extract from a portion of the same intestine after a 24 hour autolysis at 37° has high activity.

Since the zinc coactivator appeared to be a product of autolysis,

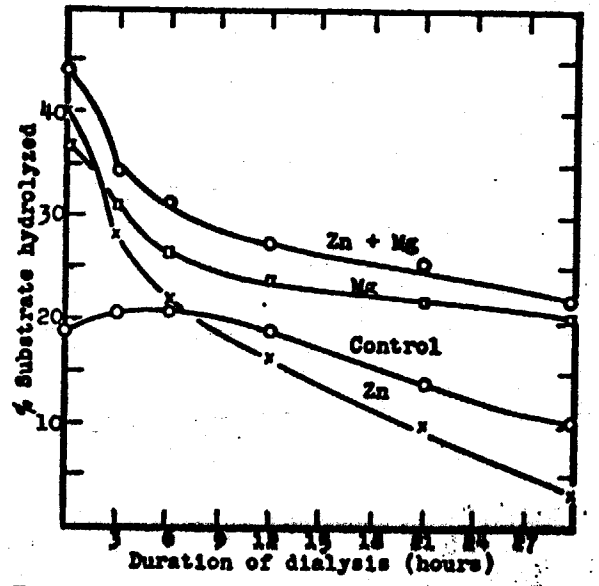

FIG. 3. The change of metal ion action on intestinal phosphatase activity with increasing time of dialysis. Substrate, β-glycerophosphate; incubation period, 21 hours; concentration of zinc, 0.03 mM; of magnesium, 0.2 mM.

a casein hydrolysate was tried and found to have a high activity, as is shown in Table II. Following this observation, a group of pure amino acids was tested (the pH of the amino acid solutions having been adjusted before use). As is evident from Table II, all of the α-amino acids had a more or less strong zinc-coactivating property. Their effect seemed to a degree to be inversely related to the length of the chain. The corresponding β-amino acids, keto acids, hydroxy acids, or aliphatic acids had little or no activity. This is likewise true of such N compounds as choline and cholamine. Glutathione had a relatively high activity. This high activity may have been a result of the combined activity of each of the three α-amino groups in the molecule, indicating that an entirely free NH_2 group is not necessary, or it may be related in some way to its —SH group. It should be noticed, however, that the high percentage activation due to zinc in the case of glutathione is due ultimately to the very marked inhibition pro-

TABLE I

Effect of Dialysis for 36 Hours and of Metal Ions on Intestinal Phosphatase Preparations

Substrate, β-glycerophosphate; incubation period, 21 hours at 37°; final concentration of added metal ions, Zn 0.05 mM, Mg 0.2 mM.

Preparation No.	Metal ion added	Non-dialyzed		Dialyzed		Dialyzed + dialysate	
		P liberated	Change due to metal	P liberated	Change	P liberated	Change
		mg.	per cent	mg.	per cent	mg.	per cent
1	None	0.790		0.587		0.700	
	Mg	0.950	+25	0.644	+10	0.903	+24
	Zn	1.14	+44	0.477	−19	1.04	+48
	Mg + Zn	1.18	+50	0.732	+25	1.06	+49
2	None	0.420		0.249		0.279	
	Mg	0.695	+65	0.464	+86	0.549	+97
	Zn	0.520	+24	0.063	−75	0.448	+61
	Mg + Zn	0.770	+84	0.272	+9	0.804	+187
3	None	0.366		0.216		0.320	
	Mg	0.649	+77	0.403	+88	0.545	+70
	Zn	0.636	+74	0.054	−75	0.470	+47
	Mg + Zn	0.724	+101	0.200	−7	0.611	+92

duced by the GSH alone and not to a higher final activity brought about by the addition of zinc to the glutathione.

TABLE II

Action of Amino Acids and Other Organic Compounds As Zinc Coactivators for Dialyzed Intestinal Phosphatase*

Substrate, β-glycerophosphate; incubation period, 21 hours at 37°; final concentration of zinc, when added, 0.05 mM.

	Final concentration of organic compound	Hydrolysis of substrate		Activation due to Zn
		No Zn	Plus Zn	
	mg. per cc.	per cent	per cent	per cent
Casein hydrolysate	0	13.6	10.3	
	0.3	13.0	27.0	108
	1.0	11.6	33.5	190
	mM			
Glycine	1.5	10.5	26.1	148
dl-Alanine	1.5	13.0	25.8	100
Aspartate	1.5	12.1	20.8	72
Glutamate	1.5	13.1	19.0	46
Valine	1.5	12.2	20.0	63
Lysine	1.5	11.2	21.0	88
Arginine	1.5	12.0	18.9	57
Cystine	1.0	12.5	24.3	94
GSH	1.5	6.7	21.3	220
β-Alanine	1.5	13.7	15.1	9
Choline	1.0	13.7	14.8	8
Cholamine	1.5	13.6	15.2	10
Lactate	1.5	14.8	12.5	
Pyruvate	1.5	13.9	13.7	
Glutarate	1.5	13.7	13.7	
Nicotinate	1.5	13.6	12.7	
Ascorbate	1.5	12.8	12.1	

* The organic compounds were adjusted to pH 8.5 before use.

From Fig. 4 it is evident that neither zinc nor the amino acid alanine had any effect on the pH optimum of the intestinal phosphatase.

The next point to establish was the optimum concentration of the amino acids in their action as zinc coactivators. The work with the amino acid glycine will be reported in detail. From Table III the following points are apparent. (*a*) The optimum

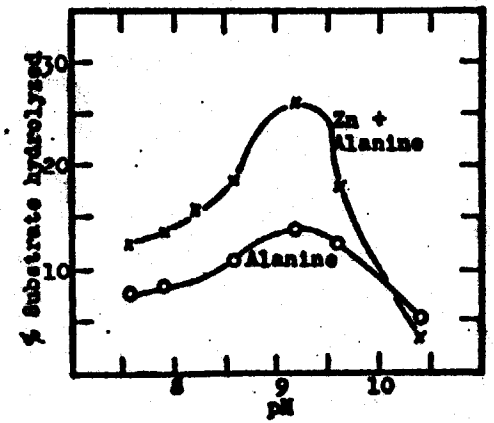

FIG. 4. The pH-activity curve of dialyzed intestinal phosphatase in the presence of alanine (1 mM) and alanine plus zinc (0.03 mM). Substrate, β-glycerophosphate; incubation period, 18 hours.

TABLE III

Optimum Concentration of Zinc and Glycine for Activation of Dialyzed Intestinal Phosphatase

Substrate, β-glycerophosphate; pH, 9.2. The figures are the percentage hydrolysis of the substrate.

Time incubated	Zn concentration	Glycine concentration				
		0 mM	0.5 mM	1.0 mM	2.0 mM	4.0 mM
hrs.	mM					
2	0	4.3	4.5	5.0	5.9	3.9
	0.0015	4.8	5.9	7.9	7.7	5.7
	0.006	4.5	6.7	8.5	9.5	9.3
	0.015	3.8	4.8	7.5	10.0	9.2
	0.035	2.5	3.6	6.7	9.9	10.4
		0 mM	0.9 mM	1.8 mM	4.5 mM	8.7 mM
24	0	32.1	25.7	22.0	15.0	12.5
	0.008	38.0	48.0	46.5	38.1	28.0
	0.035	30.9	45.2	52.0	47.1	37.5
	0.075	28.5	36.5	47.0	50.8	47.3
	0.150	26.0	32.4	37.2	45.0	50.0

The bold-faced figures represent the highest values found.

zinc and optimum glycine concentrations are interrelated and depend on each other. A rough proportionality of the concentrations for optimum stimulations is 1 molecule of zinc for every 75 to 100 molecules of glycine; (*b*) the final optimum reached appears to be about the same for all absolute levels of the zinc-glycine optimum concentrations; (*c*) the optimum range becomes much broader and flatter when the higher levels of glycine are used. The same general relations apply to the action of zinc and glycine on the β-glycerophosphatase of the kidney (Table IV) and bone (Table V) and the hexosediphosphatase and pyrophosphatase of the dialyzed intestinal preparations (data not shown).

TABLE IV

Effect of Zinc and of Glycine on Hydrolysis of β-Glycerophosphate by Kidney Phosphatase Preparation

The figures are the percentage hydrolysis of the substrate in 21 hours.

Zn concentration	Non-dialyzed enzyme	Dialyzed enzyme			
		Glycine concentration			
		0 mM	1.0 mM	3.0 mM	6.0 mM
mM					
0	25.0	13.5	12.8		10.9
0.0015	28.0	16.8	18.7	15.9	13.5
0.005	27.7	10.5	22.0	17.1	16.1
0.015	17.2	8.0		20.0	
0.050	9.5	5.6	14.1	16.3	17.5

As has been shown by Bodansky (13), the degree of activation of phosphatases by the magnesium ion is augmented by the presence of an amino acid. This is also evident from the magnesium curve of Fig. 3. A shifting of the optimum magnesium concentration with the amount of glycine in the reaction mixture is shown in Table VI.

The additions of combinations of zinc and magnesium ions or zinc and manganese ions to dialyzed phosphatases have produced some interesting results. As has been shown in Fig. 3 and Table I, the ability of zinc ion to stimulate intestinal phosphatase activity disappears on dialysis, but the ability of this element to stimulate the same dialyzing phosphatase when it is

activated by an optimum magnesium concentration does not disappear with anything like the same rapidity. With the completely dialyzed enzyme preparation the strong inhibitory

TABLE V

Action of Zinc and Glycine on Degree of Hydrolysis of β-Glycerophosphate by Bone Phosphatase Preparations at pH 9.8

The figures are the percentage hydrolysis of the substrate.

Time incubated	Zn concentration	Non-dialyzed enzyme	Dialyzed enzyme			
			Glycine concentration			
			0 mM	1.0 mM	3.0 mM	6.0 mM
hrs.	mM					
2	0	11.3	**8.8**	9.0	8.5	7.8
	0.0015	14.6	**8.3**	**9.8**	8.7	**8.8**
	0.0045	11.2	6.7	9.6	10.0	9.2
	0.015	6.5	4.5	7.0	9.7	10.0
	0.030	4.4	3.0	5.0	8.7	9.3
21	0		**34.0**	30.5	25.6	23.0
	0.003		31.7	**36.6**	34.0	29.8
	0.005		14.5	29.0	**34.4**	31.0
	0.025		6.4	14.7	26.2	**32.8**

The bold-faced figures represent the highest values found.

TABLE VI

Effect of Glycine on Magnesium Activation of Dialyzed Intestinal Enzyme

Substrate, β-glycerophosphate; incubation period, 18 hours at 37°. The figures are the percentage hydrolysis of the substrate.

Mg concentration	Glycine concentration		
	0 mM	1.0 mM	3.0 mM
mM			
0	19.8	19.8	18.4
0.04	26.6	28.6	28.0
0.10	28.6	31.3	33.6
0.40	28.8	32.8	36.3

effect of zinc and the activating effect of magnesium tend to cancel each other (Table I).

In Figs. 5 and 6, the effect of combinations of zinc and manganese upon crude bone phosphatase is shown. It is apparent

that zinc alone inhibits in the concentrations used, and that manganese alone has a slight activating effect. However, when the two ions are added together at a minimum ratio of Mn:Zn of about 10:1, the activity of the phosphatase is brought to a point higher than by Mn alone. Thus the crude bone enzyme reacts to the two metals in a way analogous to the response observed in the incompletely dialyzed intestinal enzyme. This peculiar relation of the metal ions to phosphatase activity was used to establish the reversibility of the zinc inhibition. The addition

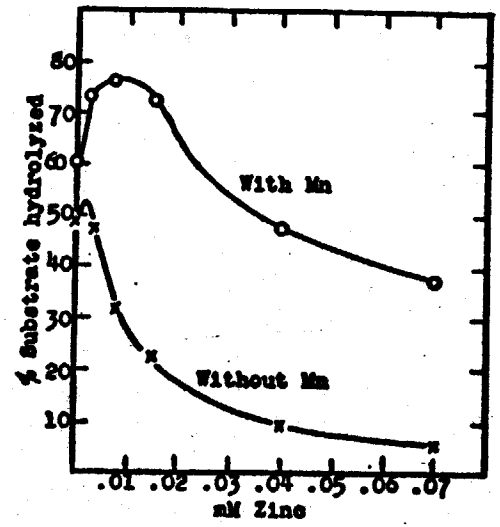

FIG. 5. The effect of increasing amounts of zinc alone, and in the presence of a constant amount of Mn (0.08 mM), on the activity of crude bone phosphatase. Substrate, β-glycerophosphate; incubation period, 21 hours.

of the stimulating ion (Mg or Mn) to the zinc-inhibited reaction mixture after a certain period of incubation resulted in the complete restoration of activity to that attained when the two ions were added from the beginning of the incubation period. These results are shown in Table VII. From this, it is evident that the responses of the incompletely dialyzed intestinal phosphatase and the crude bone enzyme are similar.

Relation of Zinc to Phosphatases in Vivo—The phosphatases of the bone and intestines of zinc-deficient animals (prepared as

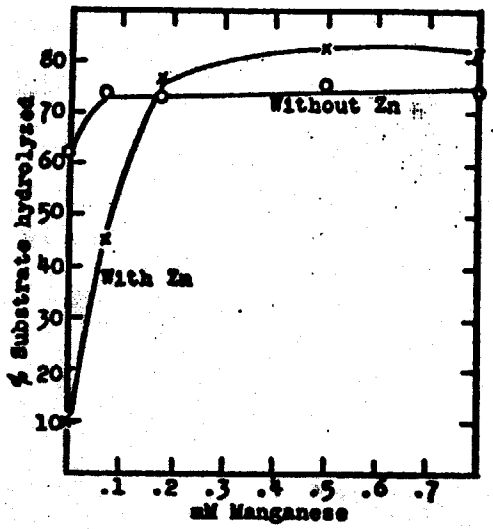

FIG. 6. The effect of increasing amounts of Mn alone, and in the presence of a constant amount of Zn (0.04 mM), on the activity of crude bone phosphatase. Substrate, β-glycerophosphate; incubation period, 21 hours.

TABLE VII

Reversibility of Zinc Inhibition of Phosphatases

Substrate, β-glycerophosphate; final concentration of ions, when added, Zn 0.05 mM, Mg 0.2 mM, Mn 0.1 mM; Enzyme A, 24 hour dialyzed intestinal phosphatase, Enzyme B, non-dialyzed bone phosphatase. The figures are the percentage hydrolysis of the substrate.

Enzyme	Metals added	Time incubated			
		0.5 hr.	1.0 hr.	2.0 hrs.	3.0 hrs.
A	None	21.3	33.0	43.1	47.5
	Zn	16.5	22.0	28.5	32.0
			Mg added	50.0	62.1
	Mg	29.5	39.1	54.1	60.2
	Zn + Mg	29.1	43.1	58.2	64.5
		10 hrs.	20 hrs.	40 hrs.	70 hrs.
B	None	38.0	48.8	62.8	70.0
	Zn	6.7	8.7	11.3	14.0
			Mn added	57.0	77.5
	Mn	39.8	51.1	65.0	72.5
	Zn + Mn	43.2	54.0	70.0	78.0

described by Hove, Elvehjem, and Hart (9)) and of rats on a high (toxic) zinc diet have been determined. The crude preparations were used throughout.

Zinc Deficiency—From the data in Table VIII it can be seen that the intestinal phosphatase of the zinc-deficient rats is considerably lower than that in normal controls. There was also a slight, but possibly insignificant, lowering of the bone phosphatases. When optimum zinc is added *in vitro* to these crude intestinal preparation reaction mixtures, the response of the preparations from the zinc-deficient rats is much greater than that of preparations from the normal rats. This difference in the reaction to zinc *in vitro* cannot be due to a lower zinc content in the preparations from the deficient rats, since an entirely analogous difference exists with respect to the addition of mag-

TABLE VIII

Intestinal Phosphatase Content of Zinc-Deficient and Control Rats

Substrate, glycerophosphate; enzyme, crude intestinal preparations; incubation period, 21 hours at 37°.

Group	Per cent hydrolysis of substrate				
		Plus 0.06 mM Zn	Per cent increase in activity due to Zn	Plus 0.2 mM Mg	Per cent increase in activity due to Mg
Zinc-deficient rats	32.1	56.5	75.5		
	18.5	47.0	154	43.8	105
	33.0	61.5	87.3	56.4	51.6
	21.6	43.7	102	45.2	107
	16.4	34.0	108		
	32.8	63.0	92.5		
	37.5	74.0	96.0	57.1	58.0
Average	27.4	54.3	102	50.6	80.4
Normal control rats	46.6	85.0	82.5		
	48.0	71.0	48.1	63.0	25.0
	44.5	63.2	41.5	61.2	38.0
	27.5	51.4	94.0	49.5	76.6
	60.0	73.2	22.0	72.0	19.8
Average	45.3	68.7	57.6	61.4	39.8

nesium ion, *in vitro*, as is shown in Table VIII. The possible explanations are that the crude preparations from the zinc-deficient rats have a lower amino acid content, or that there is a less abundant formation of intestinal phosphatase in the zinc-deficient animal. If the latter is the case, then the greater percentage activation by the metal ions may, perhaps, be related to the greater dilution of these enzyme solutions.

Rats on High Zinc Diet—Seven adult male rats were placed upon a stock ration and seven similar rats on the stock ration to which had been added 0.5 per cent of zinc (as zinc sulfate).

TABLE IX

Effect of Feeding High Zinc Diet (0.5 Per Cent Zinc As Zinc Sulfate) to Normal Adult Male Rats for 4 Weeks

	7 control rats		7 rats on high zinc diet	
	Range	Average	Range	Average
Bone ash, %.....	59.6 -63.0	61.3	54.8 -59.0	56.4
Zn of dry bone, *mg. per gm*....	0.20- 0.27	0.23	0.76- 1.25	0.97
Bone phosphatase, % *hydrolysis of substrate in 21 hrs*.....	28.0 -34.0	31.0	46.1 -55.4	50.0
Intestinal phosphatase, % *hydrolysis of substrate in 21 hrs*.....	13.5 -27.0	21.4	16.7 -41.2	28.7
Intestinal phosphatase after dithizone extraction.....	13.5 -25.0	20.3	12.9 -30.0	21.5
Loss of activity due to dithizone extraction, %.....		5.0		25.8

Bone ash, zinc content of the bones, and bone and intestinal phosphatase were determined (Table IX). In confirmation of the work of Waltner (15) a decreased bone ash was noticed when the high zinc diet was fed. Ionic zinc was removed from the intestinal phosphatase preparations by shaking with dithizone (in CCl_4) followed by pure CCl_4 to remove any adsorbed dithizone. The activity of the intestinal phosphatases after this treatment was considerably less for the rats on the high zinc diet and only slightly less for the rats on the basal diet. This indicates that the higher level of phosphatase activity in the rats on the high zinc diet was actually due to the higher water-extractable zinc content in their intestinal walls.

DISCUSSION

The marked activation of crude intestinal phosphatase by minute amounts of zinc ion has been shown to occur only in the presence of a dialyzable, organic substance which is present in the crude intestinal preparations but seemingly present to a much less degree in kidney and bone enzyme preparations. There is no evidence that this compound is in any way related to the co-phosphatase of Albers (16). It has been shown that several pure amino acids have an action equal to that of the zinc coactivator present in the crude intestinal preparation. However, it has not been established that the amino acids liberated by the autolysis of the intestinal mucosa are the only compounds with a zinc-coactivating property present in the crude preparation. In fact, the lability of a portion of the natural coactivator to strong alkali suggests the possible rôle of —SH groups. The pure amino acids do not show this strong alkali lability.

It has been consistently observed that the crude intestinal enzyme preparation gives no visible precipitate when trichloroacetic acid is added, while the crude bone and kidney enzyme preparations do. This indicates that the hydrolysis of the tissue proteins during the autolysis is more complete in the case of the intestinal preparations and strengthens the assumption that the natural coactivator is the liberated amino acids. The explanation for this greater degree of autolysis of the intestinal preparations may be related to the adherence of bits of the pancreas, or pancreatic juice itself, to the intestinal wall, thus furnishing tryptic enzymes to aid the breakdown of proteins. The effect of these tryptic enzymes could not be very great in the autolyzing or stored preparations, since the pH is too low; but during the incubation period of the phosphatase runs these tryptic enzymes can be expected, owing to the now alkaline pH, to be active and produce a certain amount of amino acids from the proteins in solution. Such an amino acid production may be a factor in the marked increase in the percentage activation by zinc and magnesium ions of phosphatase activity with time. It is also possible that the cells of the mucosa may be loaded with amino acids or protein fragments from food digestion.

Both the carboxy group and the NH_2 group in the α position are essential for zinc coactivation. Yet the wide difference in

the concentration of the zinc and the amino acid for optimum effect makes it difficult to conceive of a final zinc-amino acid complex as being the actual activator. The fact that the zinc ion has a very broad and flat optimum when the amino acid concentration is near that of the substrate concentration suggests a possible rôle of the amino acid as a phosphate transporter or carrier in the dephosphorylation.

Lohmann and Kossel (17) have shown that zinc in a concentration of about 3×10^{-3} mM stimulates the decarboxylation of pyruvic acid by yeast maceration juice plus thiamine pyrophosphate. When they used washed yeast as the source of carboxylase, zinc had only an inhibitory effect. Since in the preparation of the maceration juice the yeast was presumably allowed to autolyze before being ground, it undoubtedly contained a much higher free amino acid content than the washed yeast. It seems a possibility, therefore, that the same relation between zinc and amino acids holds for the activation of this reaction, as we have demonstrated here for the alkaline phosphatases.

The lowered intestinal phosphatase content (or activity) of zinc-deficient rats may be related ultimately to the previously established decrease in pancreatic tryptic activity during the zinc deficiency (9). It had also been shown previously (8) that the zinc deficiency is characterized by a lowered blood non-protein nitrogen and by an increase in the time required for an amino acid given by mouth to pass from the gastrointestinal tract to the blood stream. This delay in the absorption of amino acids may be explained on the basis of intestinal stasis resulting from the decreased pancreatic enzymes, or, if amino acid absorption should be shown to involve phosphorylation the slower amino acid absorption in zinc-deficient rats may be explained on the lowered intestinal phosphatase of these rats. Thus, the *in vitro* relations shown in this paper among zinc, amino acids, and organic phosphoric esters may indicate an *in vivo* relation between these substances and the reactions governing them.

SUMMARY

1. Crude intestinal phosphatase activity is increased 40 to 100 per cent by the addition of zinc ions *in vitro*. Crude kidney and bone phosphatase activities are progressively inhibited by concentrations of zinc of 4×10^{-3} to 70×10^{-3} mM. This difference in response to zinc cannot be considered as an indication that intestinal phosphatase is different from the others, since after dialysis all three enzymes show a marked inhibition by zinc.

2. The readdition of the dialysate from intestinal phosphatase preparations restores the ability of zinc to activate the dialyzed enzyme.

3. The dialyzable zinc coactivator of intestinal phosphatase preparations is a product of mucosal tissue autolysis. All of the pure α-amino acids tried have the same coactivating property as the dialysate. β-Amino, keto, hydroxy, or aliphatic acids, or organic amines have little or no activity.

4. The ratio of the concentrations of a pure amino acid (glycine) to zinc ion is about 100:1 for maximum activation. At high glycine concentrations the zinc optimum is broad and flat. At lower glycine concentrations the zinc optimum is sharp.

5. The intestinal phosphatase of zinc-deficient rats is considerably lower than that of normal controls. These results are discussed in relation to previously established disturbances in the nitrogen metabolism in the zinc-deficient rats.

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THE LANCET 7605: 1069-1071, 1969.

ORAL ZINC SULPHATE IN LEG ULCERS

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Summary A hundred and four inpatients of various ages with ulcers of the lower limb were included in a controlled trial of oral zinc sulphate. Identical capsules containing 220 mg. of lactose (placebo) or zinc sulphate were given three times a day to equal numbers of controls and treated patients. Ulcers healed more quickly in the treatment group. The ulcers of the treatment group healed in 32 on average, as against 77 days for the control group. There was no significant dependence on age or sex. Mild diarrhœa in three patients was the only toxic effect of zinc-sulphate therapy. Oral zinc sulphate would seem to have a place in the treatment of chronic leg ulcers which do not respond to topical treatment. Prolonged or repeated

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courses should, however, be avoided since not enough is known about the side-effects of systemic zinc salts.

Introduction

THERAPEUTIC agents recommended for topical use in the treatment of chronic leg ulcers are legion, and this itself suggests that none is entirely satisfactory. Many workers (Wright 1931, Bisgaard 1948, Anning 1954, Fergusson and Logan 1961) agree that bed rest and attention to the underlying vascular lesion are essential in the management of such cases. Most leg ulcers take around 11 weeks to heal completely, irrespective of treatment (Fergusson and Logan 1961, Cockrane 1961, Lorch 1961, Haegar 1964, Munro-Ashman and Wells 1968).

Pories et al. (1967a, b) found that oral zinc sulphate accelerated wound healing after the excision of pilonidal sinuses, and this stimulated interest in the application of this therapy to leg ulcers of various types. The results of a pilot investigation in sixteen cases of chronic ulcers of the lower limb treated with systemic zinc sulphate seemed to warrant a controlled trial.

Patients and Methods

One hundred and four patients, of various ages, with ulcers of the lower limb were admitted to the dermatological wards. Each patient received the same form of topical therapy depending on the severity of the ulcer (i.e., 12·5–25% aqueous solution of sodium sulphate, oily lotion of calamine, or eusol dressings) during their stay in the hospital. Although it was impossible to standardise bed rest for each patient, every effort was made to

TABLE I—DISTRIBUTION OF PATIENTS ACCORDING TO AGE AND SEX

Age (yr.)	Control			Treatment		
	M	F	Total	M	F	Total
31–40	0	1	1	1	5	6
41–50	3	2	5	1	6	7
51–60	4	12	16	2	8	10
61–70	4	17	21	2	15	17
71–80	2	7	9	1	11	12
Total	13	39	52	7	45	52

ensure that maximum bed rest was achieved. Every effort was made to ensure that ulcers of similar sizes and appearance fell in approximately equal numbers in the treated and control groups.

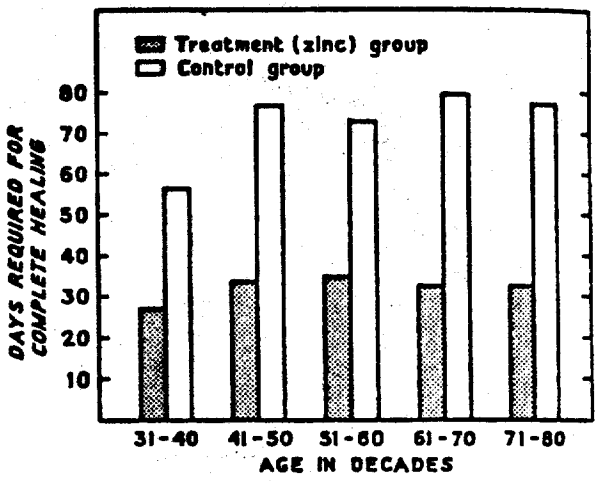
Capsules containing 220 mg. lactose (placebo) or 220 mg. zinc sulphate *B.P.* were prepared by the pharmacy departments. These had been labelled A or B, but I did not know which was which.

Equal numbers of patients were allocated to treatment or control groups (table I). The average age of the control group was 62·05 years; the average age of the treatment group was 60·34 years.

The dosage was one capsule three times daily, half an hour after meals.

Two methods were used in the weekly assessment of the ulcers. In the early stages, when the ulcers had some depth, their volume was measured by the amount of hydrocolloid gel ('Tissutex') required to fill them (Pories et al. 1967a, b). As the ulcers began to heal and became shallower, and also in the large superficial ulcers, this method was unsatisfactory, so a second method was used. The ulcer outline was traced on to transparent paper, and the tracing was transferred to graph paper. The areas was then measured in sq. mm. by counting the small squares (Fergusson and Logan 1961). The ulcer was considered to be healed when there was no scab formation.

Samples of venous blood were taken for the estimation of serum-zinc before treatment, after 2 weeks during the treatment, and a week after the ulcer had healed. Unfortunately, the trial ceased to be double-blind after 10 patients had been treated; it became clear that capsule B contained zinc sulphate because



Time to complete healing in relation to age.

the patients who were receiving it showed a striking rise in serum-zinc at the second estimation during treatment. Serum-zinc levels were estimated during treatment to ensure that the patients were taking the capsules regularly.

Results

The ulcers of the treated group healed more quickly than those of the control group. This clinically significant increase in wound healing produced by zinc sulphate was substantiated by the statistical analysis. The ulcers of the treated group healed in days ± S.E. 32·3 (±1·662) days (mean) and those of the control group took days ± S.E. 77·19 (±4·321) days. This was extremely significant on Mann-Whitney V test ($P<0·001$). The ulcers of patients who were on zinc sulphate healed 44·89 days sooner than the patients in the control group. The ranges for the total number of days required for healing were 12–71 days for the treatment group and 35–232 days for the controls. Out of 104 patients (52 patients in control group) with ulcers only 3 took more than 150 days to heal completely.

Time to complete healing was independent of age (see figure), and there was no significant difference between the rate of healing of males and females for either preparation (controls, t 0·055, D.F. 50; treated, t 0·998, D.F. 50) (table II).

The mean serum-zinc estimation in the pilot-study cases was µg./ml. ± S.E. 1·015 (±0·0406) µg. per ml. at the first estimation, µg./ml. ± S.E. 1·886 (±0·2405) µg. per ml. during treatment, and µg./ml. 1·282 (±0·1149) µg. per ml. after treatment.

The average surface area of the ulcers was 4290 sq. mm. in the control group and 4830 sq. mm. in the treatment group, and the healing-rates were 90 sq. mm. per day and 421·8 sq. mm. per day, respectively.

TABLE II—AVERAGE NUMBER OF DAYS REQUIRED FOR COMPLETE HEALING IN MALES AND FEMALES

Sex	Control		Zinc (treatment)	
	No. of patients	Average no. of days required for healing	No. of patients	Average no. of days required for healing
Male	13	76·7	7	28·1
Female	39	77·4	45	33·0
Total	52	77·19	52	32·3

Discussion

"Leg ulcers present a difficult therapeutic problem and both patients and doctors tend to accept the presence of an ulcerated leg with a grim stoicism born of years of unsuccessful treatment" (Rivlin 1958). If this attitude prevails, the chronically disabled patient may not seek treatment again unless given the stimulus of new hope. Topical antibiotics and other preparations have been used in the treatment of leg ulcers, as have eusol soaks (Fergusson and Logan 1961), silver spray plus aluminium foils (Haeger 1965), and firm pressure bandages or soft medicated bandages. Diuretics to reduce the œdema were advocated by Fox (1962) and grafting and "disobliteration" was recommended by Irvine et al. (1965) for arterial ulcers. However, it is difficult to assess the effects of these many forms of treatment on the healing-rate of leg ulcers due to their multifactorial ætiology and the difference in size and depth of these ulcers.

Zinc is an essential element in nutrition and is a constituent of most foods. Zinc salts are sometimes given by mouth for their properties as reflex emetics but they are mainly used topically as mild astringent and disinfectants (*Extra Pharmacopœia* 1967), and several topical preparations containing zinc salts are used for the treatment of leg ulcers.

Radioisotope studies have shown that zinc-65 becomes incorporated into the red blood-cells, where it is closely associated with metalloenzymes (Tucker and Salmon 1955, Vallee 1959, Underwood 1962). Zinc is preferentially concentrated in healing tissue (Savlov et al. 1962) probably due to a shift of zinc from plasma into the formed elements of blood (Ross et al. 1958, Graig and Siegel 1960, Rubini et al. 1961, Spencer et al. 1966).

Although the clinical appearance of the ulcers varied from patient to patient initially, it was quite clear that, after 2–3 weeks' treatment with local therapy combined with zinc sulphate systemically, the ulcers were cleaner and showed healthy granulation tissue. There was also a striking reduction in the purulent exudate. On the other hand, the control group showed only slight improvement, granulation tissue was not quite so evident, and a purulent exudate was invariably present. During the ensuing weeks, up to the time of complete healing, the zinc-sulphate group seemed clinically to show more rapid epithelialisation than the control group.

This trial was done on inpatients because not enough is known about the side-effects of zinc salts given by mouth. This point was carefully explained to all the patients before the trial started. However, apart from three patients who had mild diarrhœa, which quickly cleared up on discontinuing the treatment, I saw no clinical evidence of drug toxicity. Nevertheless the period of treatment in some patients was short, and a cumulative toxic effect in patients on prolonged or repeated courses of zinc sulphate cannot be excluded.

Although bed rest combined with topical therapy will, in most cases, result in healing of chronic leg ulcers (Fergusson and Logan 1961), I believe that systemic therapy with zinc sulphate has a place in the management of these patients. In this trial, bed rest and topical therapy were used, in addition to oral zinc, so it is difficult to predict the effect of zinc sulphate on ambulant patients, but at least treatment with zinc sulphate can be considered a useful adjuvant therapy in the management of chronic leg ulcers. Until more information is available, however, prolonged therapy and repeated courses for recurrent ulceration should be avoided.

I thank Prof. J. A. Milne and Dr. W. B. McKenna for encouragement and advice, Dr. R. L. Cormie and Dr. W. N. Morley for allowing me to include their cases, Dr. W. H. Lyle, Dista Laboratories, Liverpool, for the estimation of serum-zinc, Mr. R. A. Elton for statistical analysis, and my colleagues and the nursing staff for their cooperation.

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Am. J. Vet. Res., Vol. 27, No. 116, 1966

Effects of Sublethal Doses of Certain Minerals on Pregnant Ewes and Fetal Development

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SUMMARY

Seven groups of 4 yearling ewes each were fed salts of antimony, arsenic, bismuth, copper, lead, tellurium, and zinc. In each group, 2 ewes were given a mineral daily for the first 45 days of gestation, and the other 2 ewes were given the mineral throughout gestation. The 2 ewes in each group given the mineral for 45 days were kept through parturition for observation and then killed. The 2 ewes given the mineral throughout their gestation periods were killed with their lambs at parturition. Tissues samples were taken from ewes and lambs for analysis.

Copper and zinc were the only elements that accumulated in body tissues of the treated ewes and their lambs to levels detectable by X-ray spectrographic method. No analyses were made for lead in liver and kidneys of the ewes which died of lead poisoning.

One of the ewes given bismuth for 45 days aborted, whereas the other gave birth to a deformed lamb.

Lead acetate given at the rate of 9 mg./kg. of body weight, caused the death of the 2 ewes.

An experiment was conducted to determine the effects of sublethal doses of certain mineral elements upon pregnant ewes and their developing fetuses. This information is needed for the diagnosis of malformation in lambs, abortion, and certain other reproductive problems in sheep. Antimony, arsenic, bismuth, copper, lead, tellurium, and zinc were selected for experimental investigation since they may occur in relatively great amounts in certain parts of rangeland areas in western United States.

Received for publication Jan. 18, 1965.

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Materials and Methods

Twenty-eight yearling ewes were divided into 7 groups of 4 ewes each and administered certain mineral salts (Table 1). The schedule was started on the 1st day of gestation in a given ewe—the 1st day being determined by using rams equipped with a ewe-marking harness. The rams were left with the ewes throughout the experiment. Alfalfa hay, salt, and water were fed ad lib throughout the experiment.

The mineral salts were given daily for the periods shown (Table 1). The daily dose of a mineral salt was administered in a gelatin capsule via a balling gun. This dose was based on body

Group No.	Mineral	Dose (mg./kg. of body wt.)	Days given mineral	Lamb at birth
1	Antimony potassium tartrate	2	45	Normal
		2	45	Normal
		2	154	Normal
		2	155	Normal
2	Potassium arsenate	0.75*	18	Very small
		0.50	45	Normal
		0.50	140	Normal
		0.50	147	Normal
3	Bismuth tartrate	5	45	Deformed
		5	45	(Abortion)
		5	146	Normal
		5	145	Normal
4	Copper sulfate	10	45	Normal
		10	45	Normal
		10	146	Normal
		10	142	(Abortion)
5	Lead acetate	5	45	Normal
		5	45	Normal
		9**	59†	(Abortion)
		9**	106†	(Abortion)
6	Potassium tellurate	2	45	Normal
		2	45	Normal
		2	145	Normal
		2	151	Normal
7	Zinc sulfate	5	45	Normal
		5	45	Normal
		5	152	Normal
		5	147	Normal

* Dose of 0.75 mg./kg. of body weight was toxic; dose was reduced to 0.50 mg./kg. of body weight.
** Dose of 9.0 mg./kg. of body weight was toxic; dose was reduced to 5.0 mg./kg. of body weight for the other 2 ewes. † Died because of severe emaciation due to lead poisoning.

weight and was adjusted to changes in body weight of the ewe at biweekly intervals during the first 56 days. The mineral salt doses were not changed after that time.

Blood samples were collected, and the following blood analyses were made weekly throughout the experimental period: packed cell volume, hemoglobin, plasma urea nitrogen, serum glucose, and serum protein. The sulfobromophthalein (BSP) test of liver function was performed once each week to determine any liver dysfunction.

The 2 ewes of each group given the mineral salt throughout their gestation were killed with their lambs at parturition. Samples of bone from the ilium and metacarpus, and soft tissue from the brain, kidney, liver, and muscle tissues were collected from the ewes and lambs and were analyzed for mineral elements given. The 2 ewes scheduled to be given lead throughout gestation died; analyses were not made of the tissues.

The 2 ewes of each group given mineral salt for the first 45 days of gestation were kept until parturition in order to permit observations of the lambs. Animals not administered a given mineral were considered controls for that mineral.

All tissue samples were dried in a freeze-drying apparatus; the iliac and metacarpal samples were also ether-extracted. The samples were then ground to pass a 20-mesh stainless steel sieve.

X-ray spectrographic readings were made on 0.5-Gm. subsamples, using the method described by Brandt and Lazar.[1] Readings were obtained on each sample at the K *alpha*, the L *alpha*, or the L *beta* line for each mineral of interest. Additional readings were taken at angles that were relatively free of lines to establish counting rates at the backgrounds. Ratios of the counting rates at line of interest to the counting rates at background were then determined.

Since study of ratios for the different minerals indicated that only copper and zinc had been accumulated in amounts detectable by this method in the tissues of animals given these mineral salts, no effort was made to determine the true concentrations of the other minerals.

Working curves for copper and zinc were established by obtaining X-ray spectrographic data on synthetic reference samples; such samples were prepared by adding measured increments of copper or zinc in aqueous solutions to weighed portions of the tissue being analyzed. Slopes of these curves were established from the relationship of the ratios to the increments of added copper or zinc. Intercepts of these curves were obtained from scans made of the synthetic reference samples over the angles of interest. The scans were smoothed through the K *alpha* lines of copper and zinc to simulate samples free of these elements. Count-

ing rates were estimated from the smoothed scans for K *alpha* lines of copper and zinc and for the angle chosen for background. Ratios for the synthetic reference samples simulating samples free of copper and zinc were then calculated and averaged. These average ratios were used as intercepts of the respective curves. Concentrations of the copper and the zinc in the tissues being analyzed were then calculated from the equations of the respective working curves.

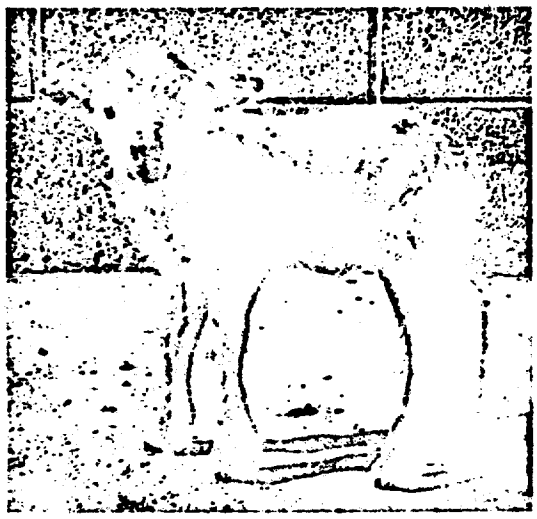

Fig. 1—Lamb from a ewe given 0.75 mg. of potassium arsenate per kilogram of body weight for first 18 days of gestation.

Results and Discussion

Antimony and Tellurium.—All ewes fed antimony or tellurium gave birth to normal full-term lambs (Table 1). No adverse effects were observed in the ewes. These 2 minerals did not accumulate in detectable amounts in tissues of the ewes or the lambs.

Arsenic.—Since the 1st ewe started on arsenic developed signs of toxicosis after 18 days, the mineral administration was discontinued. The tongue and gums of the ewe changed from pink to black. [The other 3 ewes assigned to the group were

Fig. 2—Lamb from a ewe fed 5 mg. of bismuth tartrate per kilogram of body weight for first 30 days of gestation.

therefore given reduced dosages (Table 1).] This ewe gave birth to a full-term but hairy lamb that weighed only 1.6 kg. (Fig. 1). The other 3 ewes given reduced doses of arsenic gave birth to normal lambs.

Arsenic did not accumulate in detectable amounts in the tissues in the ewes fed through gestation or the lambs obtained from the 2 ewes on the reduced treatment.

Bismuth.—One of the 2 ewes given bismuth for 45 days gave birth to a malformed lamb at the end of gestation (Fig. 2). It lacked normal covering of wool except a small amount on lower parts of the legs; the lamb was exophthalmic, and the ears were inflexible. The joints of the legs also lacked normal flexibility. The other ewe aborted after the feeding period was completed.

The 2 ewes given bismuth throughout gestation gave birth to normal lambs. The ewes had no adverse effects from their ingestion of bismuth. Bismuth had not accumulated in detectable amounts in tissues of the ewes or the lambs.

Copper.—Cunningham,[2] reporting on the copper contents of livers of 27 newborn lambs, gave the average normal as 168 p.p.m. with a range of 74 to 430 p.p.m. The

TABLE 2—Average Copper Concentrations in Dry Tissues of Ewes and Lambs

Treatment	Composition of group	Concentration in tissues (p.p.m.)					
		Ilium	Metacarpus	Brain	Kidney	Liver	Muscle
Copper sulfate	2 ewes	5	16	20	23	1725	10
	1 lamb	6	9	14	9	510	38
Control	10 ewes	5	6	15	19	180	8
	10 lambs	5	10	12	18	191	14

Treatment	Composition of group	Concentration in tissues (p.p.m.)					
		Ilium	Metacarpus	Brain	Kidney	Liver	Muscle
Zinc sulfate	2 ewes	61	50	58	86	193	161
	2 lambs	99	94	74	88	365	65
Control	10 ewes	55	48	55	84	144	153
	9 lambs	76	69	68	75	171	66

average copper concentration in livers of the 10 control lambs in this experiment was 191 p.p.m. with a range of 120 to 305 p.p.m.

A marked accumulation of copper was noticed in livers of all the ewes given copper. One of the 2 ewes being given copper throughout the gestation period aborted prior to the end of the feeding period. The liver of the lamb from the other ewe contained 510 p.p.m. of copper, indicating an accumulation of copper in the fetal lamb. This result was opposite to the findings of Allcroft and Uvorov [3] who reported that copper in the liver of the newborn lamb could not be increased above normal values by feeding additional copper to the dam during gestation.

Lead.—The 2 ewes that were to be given lead throughout gestation became severely emaciated, aborted, and died after 59 and 106 days, respectively. Thus, the dose was decreased for the 45-day ewes, and they gave birth to full-term normal lambs. Tissue samples were not taken for analysis.

Zinc.—Data defining normal zinc content of tissues are meager. Widdowson and Spray [4] found little zinc storage in human fetal development. The normal zinc content of tissues of sheep is higher than that given for corresponding tissues of humans, rats, and pigs.[5] In this experiment, zinc apparently accumulated in the liver and possibly in the bone of the fetal lamb but not in the other lamb tissues studied (Table 3). Muscle tissues from both the control and the zinc-fed ewes had more zinc than comparable samples of the lambs.

Blood and Liver Studies.—No deviations from normal values were noticed in packed cell volume, hemoglobin, serum protein, blood glucose, or plasma urea nitrogen in any ewes in the groups. The ewes given lead throughout gestation had a marked retention of the BSP dye in the blood serum, indicating some degree of liver dysfunction. The results of the liver tests on the other groups of ewes showed no treatment effects.

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The Tolerance of Growing Chickens for Dietary Zinc

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(Received for publication on May 8, 1961)

IT WAS found in an earlier experiment, conducted at the Lime Crest Research Laboratory (Mehring, Brumbaugh and Titus, 1956), that young growing chickens tolerate as much as 778 p.p.m. of zinc (in the form of zinc oxide) added to a corn-soybean oil meal diet without their growth or efficiency of feed utilization being affected. Previously, Insko, Lyons, and Martin (1938) had observed at the Kentucky Agricultural Experiment Station that the addition to the diet of as small a quantity as 30 p.p.m., each, of zinc and aluminum increased the incidence of perosis. The diet fed in their experiments was based on corn and dried skim milk; it was low in both calcium and phosphorus, deficient in manganese, and probably contained only about 3.5 p.p.m. of copper.

That chickens tolerate relatively large quantities of zinc in their diet was confirmed by Klussendorf and Pensack (1958) who reported that 1,000 p.p.m. of zinc in the diet is tolerated, but that 2,000 p.p.m. and 3,000 p.p.m. of zinc retard growth. Subsequently, Roberson and Schaible (1960) demonstrated that 1,000 p.p.m. of zinc is tolerated by the growing chicken, but that 1,500 p.p.m. or more depresses growth and adversely affects feed conversion. They compared the effects of the carbonate, sulfate, and oxide of zinc and concluded that, at the

higher levels of zinc supplementation, the carbonate depressed growth more than the sulfate, and the sulfate, more than the oxide.

The findings of Brink *et al.* (1959) suggest that the tolerance of swine for zinc is about the same as that of the chicken. These workers observed that gain in live weight, feed consumption, and efficiecny of feed utilization of growing pigs were depressed by 2,000 p.p.m. or more of zinc included in the feed.

Grant-Frost and Underwood (1958) reported that the inclusion of 5,000 p.p.m. of zinc in the diet of rats reduced growth, feed consumption, and the concentration of hemoglobin in the blood. The concentration of copper was reduced in the blood, liver, kidney, and the remaining portion of the carcass. Magee and Matrone (1960) reported that a level of 7,500 p.p.m. of zinc in the diet of rats reduced not only the content of copper in their livers but the content of iron as well.

EXPERIMENTAL

Two experiments were conducted; and in each, rapidly-growing, cross-bred, broiler chicks were used: a sex-linked cross in experiment 1 and a white-feathered cross in experiment 2.

In the first experiment each diet was fed to a single lot of chicks, but in the second experiment each diet was fed to duplicate lots of chicks. The first experiment was conducted to ascertain how much zinc in the form of zinc oxide is tolerated by growing chickens. The second experiment was conducted to obtain data on the effect of graduated quantities of zinc in the diet on (1) growth and efficiency of feed utilization; (2) the concentration of zinc, copper, molybdenum, and sulfate in the liver and spleen; and (3) the recovery of growing chicks, from the effects of growth-depressing quantities of zinc, when fed the basal diet (which did not contain added zinc).

TABLE 1.—*Formula of the basal diet*

Ingredient	Parts
Ground yellow corn	61.57
Soybean oil meal (44% protein)	31.00
Fish meal (Menhaden)	3.00
Dicalcium phosphate (21% phosphorus)	1.90
Calcite flour	1.68
Salt	.45
Choline chloride supplement[1]	.12
Trace-mineral pre-mix (Delamix[2])	.10
Vitamin B_{12} and antibiotic supplement (Pro-Pen "2:3"[3])	.10
Vitamin A supplement (Nopcay "10"[4])	.04
Vitamin E supplement (Myvamix[5])	.0315
Vitamin D_3 supplement (Bo Dee MS3,000[6])	.0125
Niacin	.0030
Menadione sodium bisulfite	.00055
Calcium pantothenate	.00050
Riboflavin	.00038
Total	100.00843

[1] Dry mixture containing 25% of choline chloride.
[2] Contained 6% manganese, 0.12% iodine, 2% iron, 0.2% copper, 0.02% cobalt and 0.01% zinc.
[3] Contained 2 grams of procaine penicillin and 3 milligrams of vitamin B_{12} per pound.
[4] Contained 10,000 I.U. vitamin A per gram.
[5] Contained 20,000 I.U. vitamin E per pound.
[6] Contained 3,000 I.C.U. vitamin D_3 per gram.

For each experiment, a few more chicks than the required number were hatched, and the extremely light and heavy chicks were discarded. The sex of each chick was ascertained; and then the chicks of each sex were distributed at random in such a way that each lot contained ten male and ten female chicks, and the live weights of the chicks in each lot were as nearly as possible the same as those of the chicks in each of the other lots. There was a total of eight lots of chickens in experiment 1 and twelve lots in experiment 2.

For the first four weeks, the chicks were kept in metal starting batteries in a room in which the temperature was maintained at 75° ± 2°F. and the humidity was held at 50 ± 5 percent. At the end of the fourth week the chicks were transferred to other metal batteries in another room in which the temperature was held at 70° ± 2°F. and the humidity, at 50 ± 5 percent, and were kept there until they were sixteen weeks old. Both rooms were without windows and were artificially illuminated for fourteen hours of each day. Throughout the experiments, the chicks were supplied with distilled water in stainless-steel waterpans, and their feed was provided in stainless-steel feed troughs.

A practical-type, corn-soybean oil meal, all-mash, basal diet was used. The formula of this diet is given in Table 1. The content of zinc in four different mixes of the basal diet was 45, 38, 57, and 47 p.p.m. The concentration of copper in the four mixes of basal diet was 50, 19, 19, and 22 p.p.m. The additions of zinc were made to the basal diet in the form of zinc oxide which, on analysis, was found to contain 79.5 percent of zinc. The quantities of zinc that were added to the diets of the chickens are shown in Table 2. The largest quantity of zinc added to the feed in experiment 1 was 3,200 p.p.m. and in experiment 2 it was 5,000 p.p.m.

In experiment 2, when the chicks were ten weeks old, two males and two females were selected at random from each lot. Their livers and spleens were removed. The four livers from each lot and the four spleens from each lot were combined into separate composite samples. Each sample was analyzed for its content of zinc by the method of Shirley *et al.* (1949), copper by the A.O.A.C. method (1955), molybdenum by the method of Sandell (1959), and sulfate by precipitation with barium chloride.

In both experiments, after the chickens

TABLE 2.—*Average live weight, feed efficiency, and mortality of the chickens receiving graduated levels of zinc in their diet for the first ten weeks, but no added zinc thereafter*

Zinc added to the feed 0–10 wks.	Zinc in the feed by analysis	Average live weight[1] at age of				Feed efficiency[2] for period between		Mortality in first 10 weeks
		10 weeks	12 weeks	14 weeks	16 weeks	0–10 weeks	10–16 weeks	
p.p.m.	p.p.m.	grams	grams	grams	grams			percent
				EXPERIMENT 1[3]				
0	45	1,680	2,058	2,433	2,705	.390	.201	0
540	—	1,680	2,077	2,416	2,723	.368	.186	5
732	—	1,690	1,998	2,383	2,626	.386	.172	0
988	—	1,620	2,035	2,337	2,543	.360	.181	0
1,329	—	1,682	2,039	2,419	2,706	.392	.198	5
1,784	—	1,602	2,004	2,346	2,641	.389	.197	0
2,391	—	1,522	1,889	2,295	2,579	.378	.204	5
3,200	—	1,432	1,904	2,221	2,459	.372	.205	0
				EXPERIMENT 2[4]				
0	57	1,776	2,179	2,632	2,933	.388	.214	0
1,000	979	1,806	2,206	2,629	2,913	.388	.194	2.5
2,000	1,974	1,715	2,020	2,482	2,826	.384	.208	2.5
3,000	2,918	1,596	1,988	2,330	2,707	.389	.210	2.5
4,000	3,875	1,252	1,687	2,132	2,501	.366	.231	10.0
5,000	4,941	1,164	1,479	1,892	2,192	.360	.225	15.0
Standard Deviation[6]		55	107	109	110	.008	.009	—

[1] Average of the average for the males and the average for the females.
[2] Unit gain in live weight per unit of feed consumed.
[3] Single lot of chickens on each diet.
[4] Duplicate lots of chickens on each diet.
[6] Square root of error mean square from an analysis of variance.

were ten weeks old, all lots were fed the basal diet (without additons of zinc) until they were sixteen weeks old. This was done in order to find out whether or not the chickens could recover from the toxic effects of the high levels of zinc. In experiment 2, when the chickens were sixteen weeks old, samples of livers and spleens were again taken from each lot of chickens, in the same manner as before, and were analyzed for their content of zinc, copper, molybdenum, and sulfate.

RESULTS AND DISCUSSION

A summary of the data on live weight, feed efficiency, and mortality for each experiment is given in Table 2. These data indicate that the chicken can tolerate in its diet a level of zinc, in the form of zinc oxide, greater than 1,000 p.p.m. In the pilot study (experiment 1), a marked depression of growth did not occur except in the case of the lots fed the diets containing 2,391 p.p.m. and 3,200 p.p.m., respectively, of added zinc. The results of this experiment did not indicate any great change in efficiency of feed utilization as the quantity of added zinc was increased to 3,200 p.p.m. As had been anticipated from the results of experiment 1, the live weight of the chickens that received 1,000 p.p.m. of added zinc in their diet in experiment 2 was not adversely affected. The live weight of the chickens that received 2,000 p.p.m. of added zinc in their diet was, however, slightly depressed at ten weeks of age. With each additional increment of zinc above 2,000 p.p.m., the live weight of the chickens was further decreased.

The live weights of the chickens at ten weeks of age and the gain in live weight between the ages of ten and sixteen weeks are compared in Table 3. It is apparent that all lots of chickens made about the same average gain in this period. Thus

TABLE 3.—*Average live weight at ten weeks of age and average gain in live weight from ten to sixteen weeks of age*

Zinc added to the feed 0–10 weeks	Average live weight[1] at the age of 10 weeks	Average gain[1] from the age of 10 to 16 weeks
p.p.m.	grams	grams
	EXPERIMENT 1[2]	
0	1,680	1,025
540	1,680	1,043
732	1,690	936
988	1,620	923
1,329	1,682	1,024
1,784	1,602	1,039
2,391	1,522	1,057
3,200	1,432	1,027
	EXPERIMENT 2[3]	
0	1,776	1,157
1,000	1,806	1,107
2,000	1,715	1,122
3,000	1,596	1,110
4,000	1,252	1,249
5,000	1,164	1,028
Standard Deviation[4]	55	81

[1] Average of the average for the males and the average for the females.
[2] Single lot of chickens on each diet.
[3] Duplicate lots of chickens on each diet.
[4] Square root of error mean square from an analysis of variance.

the chickens, the live weights of which were depressed by the feeding of zinc, made about the same gain in weight in the recovery period as those chickens that had received the basal diet during the entire sixteen-week feeding experiment.

In experiment 1, efficiency of feed utilization was not affected by the addition of zinc to the diet. In experiment 2, in which larger quantities of zinc were added to the diet, a reduction of efficiency of feed utilization was apparent when 4,000 and 5,000 p.p.m. of zinc were added to the diet.

There were no significant differences in efficiency of feed utilization in the period between ten and sixteen weeks, although there was a tendency for the efficiency to be greater for those chickens that had received the highest levels of zinc than for the others (Table 2). This greater efficiency of feed utilization probably was due to the fact that the birds on the diets containing the two largest quantities of zinc weighed less at ten weeks than the birds on the diets containing the lesser quantities of zinc, and, therefore, had a smaller maintenance requirement.

Mortality was not an important factor in these experiments. In experiment 2, however, mortality tended to be greater in the lots of chickens fed the diets containing the highest levels of zinc. Nevertheless, only six of the forty chickens that were fed the diet containing 5,000 p.p.m. of added zinc died.

The results of the chemical analyses of the livers and spleens are presented in Table 4. There was a highly significant increase in concentration of zinc in the liver of the chickens with the increase in the level of zinc in the diet. The quantity of zinc in the dried substance of the livers increased from 145 p.p.m. in the case of the chickens fed the basal diet, to 580 p.p.m. in the case of the chickens fed the diet containing 5,000 p.p.m. of zinc. An appreciable portion of the zinc that had been stored in the liver up to the age of ten weeks was lost between the ages of ten and sixteen weeks, while the chickens were fed the basal diet without added zinc.

The zinc content of the spleens of the chickens was about the same at sixteen weeks of age as it had been at ten weeks of age. The average zinc content of the dry matter of the spleens of the twelve lots of chickens was 103 p.p.m. at ten weeks of age. At sixteen weeks, the zinc content of the dry matter of the spleens was 97 p.p.m.

It is noteworthy that the concentration of copper in the livers of the chickens was not affected by the several graded levels of added zinc, up to and including 4,000 p.p.m. The concentration of copper in the

TABLE 4.—*Content of zinc, copper, molybdenum, and sulfate in composite samples[1] of livers and of spleens taken from the chickens at ten and sixteen weeks of age—Experiment 2*

Zinc added to the feed	Zinc content[2]				Copper content[2]				Molybdenum content[2]				Sulfate content[2]			
	Liver		Spleen		Liver		Spleen		Liver		Spleen		Liver		Spleen	
	10 wks.	16 wks.	10 wks.	16 wks.	10 wks.	16 wks.	10 wks.	16 wks.	10 wks.	16 wks.	10 wks.	16 wks.	10 wks.	16 wks.	10 wks.	16 wks.
p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	Amount of tissue too small to detect sulfate.
0	145	90	100	90	4.4	9.0	9.1	15.0	3.3	3.0	0.9	0.4	5.3	6.0		
1,000	146	87	90	92	4.4	10.2	20.2	8.0	3.1	3.6	1.4	0.3	9.2	5.9		
2,000	238	81	94	90	4.3	12.0	20.4	11.3	3.5	3.3	0.7	0.5	14.8	6.1		
3,000	416	88	101	89	5.0	9.8	19.6	10.2	3.8	3.0	2.2	0.3	5.6	6.0		
4,000	481	123	120	108	4.8	12.6	36.7	16.2	3.4	4.1	1.2	0.8	4.7	11.2		
5,000	580	82	114	112	2.6	13.4	22.8	19.5	3.6	4.7	1.3	1.2	14.6	11.4		
Standard Deviation[3]	49	23	12	14	0.3	1.3	10.1	7.3	0.3	1.0	0.9	0.2	4.1	2.5		

[1] A composite sample consisted of livers or spleens from two males and two females selected at random.
[2] Dry matter basis.
[3] Square root of error mean square from an analysis of variance.

livers of the chickens fed 5,000 p.p.m. of zinc, however, was significantly reduced. A similar decrease of copper in the tissues of the rat was noted by Grant-Frost and Underwood (1958) and Magee and Matrone (1960). In their experiments only extremely large quantities of zinc were added to the diet. The quantity of zinc required to change the concentration of copper in the liver was larger than the quantity of zinc required to depress growth. At the end of sixteen weeks, all lots of chickens had about the same concentration of copper in their livers; the average content was 11.2 p.p.m. in the dry matter. The concentration of copper in the dry matter of the spleens was quite variable; the average concentration for all lots of chickens was 21.4 and 13.8 p.p.m. at the ages of ten and sixteen weeks, respectively.

Zinc supplementation had no effect on the concentration of molybdenum in the livers. The average concentration of molybdenum in the liver was 3.4 p.p.m. and 3.6 p.p.m. when the chickens were ten and sixteen weeks of age, respectively. The average concentration of molybdenum in the spleens of all lots of chickens at ten weeks of age was 1.3 p.p.m. When the chickens were sixteen weeks old, the content of molybdenum in their spleens was lower than when the chickens were ten weeks of age; and the spleens of the chickens that had been fed the diets containing the two largest quantities of zinc contained significantly more molybdenum than the spleens of the chickens that had been fed the diets containing the smaller amounts of zinc. There was little or no effect of zinc on the sulfate content of the livers. Not enough sample was available to permit an estimate of the sulfate content of the spleens.

SUMMARY

The results of two experiments indicate that chickens tolerate at least 1,000 p.p.m. of zinc added to a practical, corn-soybean oil meal diet. In the first experiment, growth was not markedly depressed on the diets containing less than 2,391 p.p.m. of added zinc. In the second experiment there was a slight reduction in growth on the diet containing 2,000 p.p.m. of added zinc, and on the diets containing 3,000, 4,000, and 5,000 p.p.m., there were significant reductions in growth. The addition to the diet of less than 4,000 p.p.m. of zinc, as zinc oxide, did not affect efficiency of feed utilization.

The chickens, the growth of which was depressed by the added zinc in their diet during the first ten weeks of the experiment, gained in weight about as much from ten to sixteen weeks as the chickens that had received the basal diet during the entire sixteen weeks of the experiment.

Determinations were made of the zinc, copper, molybdenum, and sulfate in samples of the livers and spleens taken from the chickens when they were ten and sixteen weeks old. Zinc was stored in the liver when zinc was included in the diet; and an appreciable portion of the stored zinc was lost from the liver when zinc was removed from the diet. The concentration of copper was significantly reduced in the livers of the chickens that received the diet containing 5,000 p.p.m. of added zinc. When the chickens were sixteen weeks old, the concentration of molybdenum in the spleens of those chickens that had received the diets containing 4,000 and 5,000 p.p.m. of added zinc in their diet to the age of ten weeks was significantly greater than that in the spleens of those that had received diets containing the smaller quantities of added zinc to the age of ten weeks.

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THE EFFECT OF ORALLY ADMINISTERED ZINC SULFATE ($ZNSO_4$) ON HEALING INCISED WOUNDS

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IT IS KNOWN THAT ZINC plays an active role in the healing of granulating wounds in both man and animals. Of major interest to surgeons is the healing of musculofascial sutured wounds of the abdominal wall. Previous studies from our laboratory showed that large doses of locally applied zinc had no effect on the sutured musculofascial wound in the rat [1]. Sandstead and Shepard demonstrated diminished skin healing in zinc-deficient animals [4]. O'Rian, Copenhagen, and Calman, again utilizing skin incisions in the rat, showed no significant effect with large intraperitoneal doses of zinc [2].

Since to date the only beneficial effect noted with any type of wound has been from large oral doses of zinc (Pories) [3], we wished to see if similar amounts of excess oral zinc (in the range of 10 times the normal daily intake) would benefit the healing of a sutured musculofascial wound.

METHODS

Male albino Wistar rats weighing an average of 248 grams were housed in individual cases and maintained on a standard laboratory diet (Purina Chow). The laboratory chow contains 58 mg. of zinc in 1 kg. of chow and the rats took an average of 1 mg. of zinc per day from chow.

Under intraperitoneally administered pentobarbital anesthesia, the rats were clipped and wounded under sterile conditions. The skin and subcutaneous tissue of the upper part of the abdomen were incised at the midline from the xyphoid process to the infraumbilical area, reflected, and a 4-cm. left paramedian incision was made through the musculofascial layer and peritoneum. The musculofascial layer and peritoneum were closed as one layer with seven interrupted sutures of atraumatic No. 4-0 silk beginning 5 mm. from the apex of the incision and placed 5 mm. apart. The subcutaneous tissue and skin were closed as one separate layer with a continuous No. 4-0 silk suture. The wounds were sprayed with aero-plast. The rats were then returned to their cages for observation.

Two groups of rats were studied. On postoperative day 7 the rats that lost weight, showed signs of infection, hematoma, and wound dehiscence were discarded from this study. The control groups gained an average of 23 grams and the experimental group 18 grams.

Group 1—Controls. These 16 rats served as

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Submitted for publication May 28, 1970.

controls. After wounding and suturing, they were simply observed until they were killed.

Group 2—Zinc sulfate solution. These 15 rats were given extra zinc sulfate solution in their water bottle (150 mg. of zinc sulfate in 450 cc. of water) starting 3 days prior to wounding and continued until they were killed. They took an average of 5–10 mg. of zinc sulfate per day.

Wound healing was evaluated in the following manner: All rats were killed on postoperative day 7 with intraperitoneally administered pentobarbital. The skin with the attached subcutaneous tissue of the abdominal wall was removed and discarded, and the entire remaining abdominal wall excised. Three transverse strips were cut; each strip was 1 cm. wide and included two of the seven sutures; the most caudal suture was not included. The tensile strength of each strip was determined by suspending it in a motor-driven tensiometer which recorded the breaking force in grams.

RESULTS

Wounds in the control rats showed a mean tensile strength of 1,299 ± 169 grams, with a range of 900–1573 grams. The wounds of the rats in the experimental group showed a mean tensile strength of 1281 ± 163 grams, with a range of 1020–1620 grams.

By applying a test of the pooled variances with 29 degrees of freedom, the strength of the wounds in the control rats was found to be no greater than that for the experimental group (zinc sulfate group), $t = 0.293$ ($PO\ 8 = 0.256$, $PO\ 7 = 0.389$).

DISCUSSION

The wounds in majority of animals in both groups healed cleanly and without infection. There was no evidence of systemic toxicity in the animals treated with zinc. Tensile strength determination at day 7 were almost identical in both groups, and this was statistically valid.

Zinc is known to be an essential factor in many enzyme systems. For example, it is the essential metal in alkaline-phosphatase, carbonic anhydrase, and carboxypeptidase. In microorganisms it has been shown to promote synthesis of protein and nucleic acids. In man and animals it exists in high concentration in the hair and skin. Normal zinc intake in man is in the range of 10–15 mg. daily. Of this 1 mg. is excreted in the urine and 10 mg are excreted in the feces. In Pories et al's experiments approximately 10 times this daily dose was given and well tolerated. If this is increased to 20 times the daily normal intake weakness and diarrhea occur. In animal experiments the normal daily intake in rats is in the range of 1 mg. per day. Ten times this dose may be given without systemic toxic effects.

In animal experiments zinc deficiency slows the healing of granulating wounds and produces a surrounding parakeratosis; the process is reversed by the administration of oral zinc. In laboratory animals maintained on diets with normal zinc content Strain, Pories, and Hinshaw speeded the healing of granulating wounds by doubling the intake of oral zinc [5]. They demonstrated a similar beneficial effect in humans utilizing 10 times the normal daily intake of zinc; the control group in the experiment was maintained on a diet containing normal amounts of zinc [3]. As mentioned earlier, studies from our laboratory utilizing zinc applied locally to the incised musculofascial wound failed to alter tensile strength. However, since it is possible that this lack of effect was based on the nonabsorption of the locally applied zinc, further studies were necessary utilizing an oral route of administration. In the present study relatively large doses of orally administered zinc (5–10 times the amount given to control animals) failed to stimulate the healing of incised wounds. This dose was in same range as that given to human volunteers in Pories et al's experiments. Hence, appears that zinc, either locally applied or orally administered, does not play a significant role in the healing of incised musculofascial wounds in the experimental animal.

SUMMARY

The effect of orally administered zinc sulphate was studied in sutured musculofascial wounds of normal rats. When tested on postoperative day 7, wound tensile strength of the treated animals was not significantly different from that of the controls. Both groups of animals thrived and gained similar amounts of weight. Thus it appears that orally administered zinc does not significantly affect the sutured musculofascial wound of the experimental animal.

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Newer Aspects of Zinc Metabolism

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ZINC IS generally recognized as an essential element in the metabolism of all vertebrates[1] and of many plants.[2] The earlier view was that neither deficiency nor toxicity occurred in farm animals fed normal rations and maintained under usual conditions.[10] Deficiency rations for mice were described as early as 1935.[8]

In 1953, it was suggested that swine parakeratosis was a nutritional deficiency disease[5] and, in 1955, that apparently zinc and calcium were involved; the amount of calcium present in the feed seemed to determine, or to relate to, the availability of zinc.[3,9]

Just recently, an informal discussion[4] among a group of research workers indicated that zinc may be a factor in a number of abnormalities observed in chickens.

SWINE TESTS

Until a few years ago, it was assumed that it would be exceedingly difficult to formulate a ration for farm animals capable of producing manifestations of zinc deficiency. The usual ingredients of the rations of pigs contain zinc in appreciable amount—upward of 30 parts per million (p.p.m.) of total ration.

When swine parakeratosis appeared,[5] several other possibilities were considered before the importance of zinc was established.[3,9] Since then, the metabolism of zinc and the interactions between zinc and various other minerals — major and minor — have been studied. The subject has been reviewed[6,7] and will not be repeated here.

The effect of supplemental zinc on pigs was tested at varying dosage levels, using rations with and without antibiotics. This latter variable was introduced because of the development of an antibiotic feed supplement (Baciferm*), in which the antibiotic bacitracin is stabilized by precipitating with zinc, and additional zinc required to insure complete precipitation is chemically bound to various protein fractions to form complex organic compounds.

Field tests showed that parakeratosis, which occurred spontaneously under farm conditions, responded equally well whether zinc was supplied in the antibiotic feed supplement or as zinc chloride. When either form (100 p.p.m. of total feed) was added to the deficiency ration, response was rapid and even dramatic.

Initial tests were designed to show that the antibiotic, as a zinc complex, is biologically available to chickens, turkeys, and pigs.

TABLE 1—Effect of Low Levels of Zinc

(Gm.) Antibiotic	Zinc* (p.p.m.)	No. pigs	Final weight	Daily gain	Feed per lb. gain
0	0	20	207	1.49	4.75
6	0	20	204	1.46	4.64
6	15	20	215	1.55	4.26
10	0	22	214	1.54	4.22
10	30	21	215	1.56	4.10

*In all instances, this represents supplemental zinc, the basic ration being formulated from natural feeds probably containing about 35 p.p.m. of zinc. Calcium levels were held at 0.83 per cent.

TABLE 2—Zinc Content of Pork Tissues

Antibiotic (Gm.)	Added zinc in feed (p.p.m.)	Zinc (µg./Gm.) Liver	Zinc (µg./Gm.) Muscle
0	0	83	20.0
6	0	357	19.0
6	15	320	20.1
10	0	296	19.2
10	30	No sample	31.2
1,000	3,000	315	17.1

A typical test provides the following data (table 1) in which no significant variations occur, except in the improved efficiency of feed utilization in the group fed the ration containing 10 Gm. of antibiotic and 30 p.p.m. of zinc. This indicates that the antibiotic was active and continued to exert the usual biological effect even though present as the stable zinc salt.

Liver and muscle were collected for zinc assay when the pigs had attained market weight. An unexpected and unexplained result was the high zinc content of the livers of all pigs fed antibiotics, regardless of zinc supplementation, and failure of the

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Presented at the National Mineral Feeds Association, annual convention, Sept. 19, 1957, Chicago, Ill.

*Baciferm, containing zinc bacitracin, feed grade, is a product of Commercial Solvents Corp., Terre Haute, Ind.

TABLE 3—Effect of Supplemental Zinc

Antibiotic (Gm.)	Zinc added (p.p.m.)	No. pigs	Final weight	Daily gain	Feed per lb. gain
0	0	8	174.8	1.12	4.62
10	0	8	199.7	1.33	4.40
10	0	8	185.9	1.21	4.86
10	9	7	220.4	1.51	4.26
10	18	8	196.1	1.30	4.44
300	546	8	185.8	1.21	4.55

TABLE 5—Effect of Zinc on Swine Growth

Antibiotic (Gm.)	Zinc added (p.p.m.)	No. pigs	Average weight	Daily gain	Feed per lb. gain
0	0	6	207.8	1.48	4.55
10	0	6	204.5	1.45	6.04
10	0	6	207.7	1.48	5.30
10	0	6	200.8	1.42	4.62
10	15	6	221.1	1.61	4.34
300	450	6	210.7	1.52	4.88

muscles to follow this pattern (table 2). Even the amounts of zinc found in the livers were regarded as being well within the range of safety for human consumption.

In another test, a similar series of data were collected but were amplified by examination of the blood and by zinc assay of the tissues at the time of slaughter. The findings show that the bacitracin was available, although present in the feed as the zinc salt (table 3). Again, no value is shown for added zinc, since no deficiency existed.

Blood films were prepared from each pig, at the time of slaughter, and showed a normal number of leukocytes of each type on a differential count. For each group of pigs, the variations within the group were within the normal range (table 4).

Still another test was conducted, using six groups of 6 pigs each. Three antibiotics were used, of which only one was zinc-stabilized bacitracin. It was fed at low (10 Gm.) level, and at high (300 Gm.) level; the high level was no more effective than the low. However, both groups fed the zinc-containing antibiotic feed supplement grew exceptionally rapidly and used feed efficiently (table 5).

Samples of blood, liver, muscle, and bone were collected when the pigs reached market weight. Only bone appeared to store zinc in this series of animals. None of the edible tissues, not even the liver, contained more than the normal amount of zinc (table 6). We have no explanation for the wide difference in amount of zinc in the livers of pigs in the first and second tests.

TABLE 4—Differential Leukocyte Count in Pigs

Antibiotic (Gm.)	Zinc added (p.p.m.)	Lymph.* (%)	Mono.* (%)	Baso.* (%)	Eosin.* (%)	Neut.* (%)
0	0	0.88	3.06	33.69	60.44	1.56
10	0	0.69	4.13	21.31	73.13	0.88
10	0	0.81	4.44	23.00	69.30	0.56
10	9	0.86	4.21	33.07	60.79	0.29
10	18	1.00	5.19	31.69	60.56	1.25
300	546	1.31	4.00	30.31	64.44	0.19

*Baso. = basophils; eosin. = eosinophils; neut. = neutrophils; lymph. = lymphocytes; mono. = monocytes.

SUMMARY OF SWINE TESTS

1) Zinc was biologically available when supplied in an antibiotic feed supplement in which some zinc was chemically bound to the antibiotic and some was present in complex organic compounds.

TABLE 6—Effect of Zinc on Pork Tissues

Antibiotic (Gm.)	Zinc added in feed (p.p.m.)	Zinc (μg./Gm.) Blood	Bone	Liver	Muscle
0	0	11.0	44.25	28.45	79.56
10	0	12.46	43.70	28.82	65.22
10	0	10.46	37.50	27.20	59.56
10	0	12.45	41.88	26.88	77.18
10	15	11.46	52.70	25.31	87.41
300	450	11.20	68.45	26.32	76.11

2) Zinc was not stored in edible pork tissues, even when supplemental amounts were fed to supply 450 p.p.m. of total ration.

3) Zinc supplementation had no effect upon the blood picture, insofar as this was mirrored by a differential leukocyte count.

4) This feed supplement, in which the antibiotic bacitracin was stabilized by precipitating with zinc, when fed at moderate levels appeared to have a favorable effect upon the efficiency of feed utilization and upon rate of growth of swine. In other words, the response to the antibiotic was not lost because it was chemically bound with zinc.

POULTRY TESTS

The effect of supplemental zinc on poultry was tested at varying levels. Again, an antibiotic was used in rations with and without zinc.

The early tests were designed to determine the effect of increasing amounts of supplemental zinc on growth. The antibiotic feed supplement was used as a source of zinc, except in the final group,

TABLE 7—Effect of Zinc on Growth of Chickens

Antibiotic (Gm.)	Zinc added (p.p.m.)	Wt. (Gm.) at 69 days	Feed per lb. gain	Response over controls (%)
0	0	1,394	2.45	0
25	30	1,466	2.34	5.2
50	60	1,436	2.40	3.0
100	120	1,462	2.36	4.9
200	240	1,444	2.40	3.6
400	480	1,461	2.38	4.8
500	600	1,451	2.36	4.0
750	900	1,429	2.42	2.5
1,000	1,200	1,428	2.49	2.4
1,000	88	1,461	2.36	4.8

TABLE 8—Effect of Zinc on Growth of Chickens

Antibiotic (Gm.)	Zinc added (p.p.m.)	Wt. (Gm.) at 70 days	Feed per lb. gain.
0	0	1,384	2.61
25	46	1,426	2.50
50	91	1,445	2.57
100	182	1,426	2.46
200	365	1,443	2.53
400	729	1,448	2.42
500	911	1,489	2.42
750	1,367	1,424	2.51
1,000	1,823	1,296	2.61
1,000 (pharm. grade)	88	1,429	2.54

where a pharmaceutical grade of zinc bacitracin was used. Every supplemented group surpassed the controls, with a trend indicating that full growth response was attained until the zinc content approached 1,000 p.p.m. The data (table 7) suggest that the antibiotic was available to the chickens, while the zinc showed no detrimental tendency up to about 900 p.p.m.

A subsequent test, similar in nature, included an experimental antibiotic feed supplement having a somewhat greater zinc content. As with pigs, a trend was observed (table 8) toward more efficient feed utilization and more rapid growth, indicating that the antibiotic present was available and that zinc was not harmful until the ration was supplemented with approximately 900 p.p.m. of zinc.

When the zinc bacitracin (feed grade) was fed to a group of commercial broilers, the antibiotic response was equally favorable (table 9) until the zinc supplementation reached 240 p.p.m.

White Rock hens in three pens were fed and observed for 11 months. The hens in all pens had the same basal ration, but one group was given unsupplemented feed, another had 100 Gm. of antibiotic in a feed supplement, and the third had 100 Gm. of the zinc-precipitated antibiotic feed supplement, which supplied 180 p.p.m. of zinc.

Differential leukocyte counts were made, and all types remained within the normal range (table 10).

Eggs were collected and assayed for zinc, but showed no significant alteration from the normal in zinc content of the albumen, the yolk, or the shell, even after feeding for 182 days (table 11).

All eggs not used for zinc assay were collected for hatching, and the results of this test (table 12) show no significant effect from either antibiotic or zinc.

Feces collected under the dropping boards were also assayed for zinc (table 13). Although it is difficult to draw an accurate comparison between zinc ingested in the feed and that excreted in the feces, it would appear that essentially all of the supplemental zinc appeared in the feces.

Upon completion of the laying season, tissues were gathered at necropsy to study the zinc content. Blood, liver, muscle, and bone were ashed and assayed. Again, as with swine (table 6), an unexpected and unexplained result was a bone assay in the antibiotic-fed group which parallels that of the zinc-supplemented group (table 14). Here the difference is much less pronounced and, in fact, lies within the normal range.

TABLE 9—Effect of Zinc on Commercial Broilers

Antibiotic (Gm.)	Zinc added (p.p.m.)	No. birds	Average Wt. (lb.)	Feed per lb. gain
4	0	3,569	2.94	2.74
50	60	2,450	3.13	2.68
100	120	3,570	2.99	2.67
200	240	2,640	3.04	2.82

Two tests were conducted with chickens to determine if zinc, supplied as part of a complex organic compound, differed in its effect from zinc supplied as the chloride. In the first test (table 15), at

TABLE 10—Differential Leukocyte Counts in Hens

Antibiotic	Zinc added (p.p.m.)	Basophils		Eosinophils		Heterophils		Lymphocytes		Monocytes	
		Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.
0	0	2-5	3.0	0-10	3.25	26-39	33.0	50-57	53	4-8	5.5
100	0	1-4	2.6	1-10	4.25	20-33	26.8	54-65	59	5-9	7.4
100	180	2-4	2.5	2-10	6.00	16-49	29.5	43-63	55	1-11	5.6

TABLE 11—Zinc Content of Eggs

Zinc added in ration (p.p.m.)	Zinc (μg./Gm.)		
	Albumen	Yolk	Shell
0	0.0	1.9	2.0
180	0.4	1.1	2.0

3,000 p.p.m. of the total ration, both forms of zinc caused a regression in growth, as compared with the control birds. The regression was much more marked in the zinc chloride group. Palatability of the feed appeared to be an important factor, especially with the ration containing zinc chloride.

In the second test (table 16), zinc was added to the ration at the rate of 1,000 and 2,000 p.p.m., using proteinate, carbonate, and acetate. The antibiotic levels in all except control rations were kept constant.

TABLE 13—Zinc Content of Feces of Chickens

Ration		Feces
Antibiotic (Gm.)	Zinc added (p.p.m.)	Zinc (μg./Gm.*)
0	0	170
100	0	129
100	180	316

*The figures are not based on a full day's excrement.

The results indicate no significant differences between the effect of zinc proteinate, zinc carbonate, and zinc acetate.

SUMMARY OF POULTRY TESTS

1) The antibiotic, bacitracin, is biologically available to chickens when added to the ration as a feed supplement in which the bacitracin has been stabilized with zinc.

2) Zinc is not concentrated in the eggs or in the edible tissues of the body, but excessive amounts are eliminated in the feces.

3) Zinc supplementation has no recognizable effect upon the differential leukocyte count.

4) Zinc supplementation of practical

TABLE 15—Zinc Proteinate Versus Zinc Chloride in Chickens

Zinc added (p.p.m.)		Weight (Gm.)	Feed per lb. gain	Response over controls (%)
Proteinate	Chloride			
0	0	1,470	2.65	....
30	0	1,519	2.60	3.3
300	0	1,557	2.33	5.9
3,000	0	1,215	2.72	−17.3
0	3,000	1,026	2.90	−30.2

TABLE 12—Effect of Zinc on Eggs

Ration		Egg production (%)	Hatchability (%)	
Antibiotic (Gm.)	Zinc added (p.p.m.)		Fertile	All eggs
0	0	53.7	87.4	76.1
100	0	47.3	89.4	79.6
100	180	51.0	88.2	75.1

rations at moderate levels with zinc bacitracin, feed grade, appears to have a favorable effect upon the efficiency of feed utilization and upon rate of growth, suggesting that the antibiotic present is biologically available. Both advantages are lost when the rate of supplementation approaches 1,000 p.p.m. of total ration.

POULTRY DEFICIENCIES

At the 1957 meeting of the Poultry Science Association, a group of workers discussed zinc deficiencies in birds con-

TABLE 14—Zinc Content of Tissues

Ration		Zinc (μg./Gm.)			
Antibiotic (Gm.)	Zinc added (p.p.m.)	Blood	Bone	Liver	Muscle
0	0	24.4	106.1	40.1	11.1
100	0	19.4	135.1	43.0	19.7
100	180	26.1	130.6	37.4	15.0

suming rations presumed to be normal and complete. Attention has been focused on this subject by the more precise analytical methods available and the basic realization that zinc is essential for the proper functioning of several enzyme systems; and that these, in turn, determine the efficient utilization of nutrients by the body.

A number of abnormalities were mentioned, and attention was directed toward an interaction between zinc and several other minerals—major and minor. The possible or probable effects on the functioning of specific enzyme systems was not discussed; neither was the manner or pathway through which a deficiency of zinc might interfere with the utilization

TABLE 16—Zinc Proteinate Versus Zinc Salts in Chickens

Added zinc (p.p.m.)			Sexed wt. (Gm.) at 71 days	Response over control (%)
Acetate	Carbonate	Proteinate		
0	0	0	1,626	....
1,000	0	0	1,639	0.8
0	1,000	0	1,600	−1.6
0	0	1,000	1,536	−5.5
2,000	0	0	1,535	−5.6
0	2,000	0	1,548	−4.8
0	0	2,000	1,505	−7.5

of nutrient substances provided by the feed.

A tendency for the long bones—especially the femur and tibia—to become short, thick, and flat, rather than retain their normal long, slender, round, and firm characteristics, had been observed in chickens fed rations containing as little as 15 p.p.m. of zinc. Supplemental zinc seemed to reduce the incidence or prevent the condition.

The feathers of certain lots of chickens and turkeys were straggly and unkempt. Their rations proved to be low in zinc. Microscopic examination of the feathers showed that the barbs developing on each side of the shaft or quill to form the vane were abnormal. They did not develop the tiny barbules which normally interlock to keep the feathers orderly and smooth. When supplemental zinc was added to the ration, the incidence of the condition was reduced, or it disappeared completely. Whether this is related to the hairy-turkey condition sometimes observed was not established.

Hock enlargement in turkeys was produced consistently when a ration containing 2 per cent calcium was fed. The swelling was readily observed at the age of 1 week. At 2 weeks, some birds had recovered while others were more severely affected. This trend persisted—some birds recovered with age while others were affected with more prominent lesions. Zinc supplemented at the rate of 0.58 per cent (5,800 p.p.m.) did not completely correct the condition. The interaction of manganese in this connection was not discussed.

Dermatitis developed on the feet and legs of turkeys. This sometimes responded to supplemental zinc and consistently responded when wheat was added to the formula to constitute 15 to 20 per cent of the ration. The question of a concurrent selenium imbalance is raised.

The zinc content in normal feed ingredients varies widely, so that results and conclusions from previously reported tests should be re-examined. Even the purified proteins may contain as much as 27 p.p.m. of zinc, of which only 12 p.p.m. remains after careful washing.

The type of equipment used to house, feed, and water the birds can be highly important—the use of plastic or stainless steel throughout may result in a response which varies widely from that obtained when the usual galvanized metal cages, feeders, and fountains are used with birds from the same hatch and eating the same ration.

Supplemental zinc resulted in a favorable response when used with a ration devoid of fish proteins, but failed when the same type of ration contained fish. This raises the question of zinc being at least a part of the inorganic or ash unidentified growth factor.

Zinc toxicity also deserves attention. One experimental group is working with levels of zinc which are recognizably toxic to swine. Other groups may be conducting similar experiments.

Many questions remain unanswered, but the growing importance of zinc and other micro-minerals in rations is recognized, and definitive answers are developing.

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Proc. Soc. Exptl. Biol. Med. 84:487-491, 1953.

Copper, Molybendum and Zinc Interrelationships in Rats and Swine.* (20687)

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It is generally recognized that naturally occurring molybdenum in pasture plants of certain areas produces a severe disease of cattle and sheep which is apparently cured or prevented by supplemental copper. The literature on molybdenum in relation to the health of farm animals has been reviewed to 1944 by Russell(1) and the influence of molybdenum on the copper metabolism of animals has been reviewed to 1952 by Marston(2). Most of this experimental work has been done with rats, sheep and cattle. While chronic molybdenum toxicity symptoms are similar to those of copper deficiency, there does not seem to be a simple stoichiometric relationship between the two elements, and the existence of other variables is indicated. The role of molybdenum and copper in various flavin enzyme systems(3-5) may need to be considered in any explanation of toxic effects and antagonisms. Of particular interest are the findings that under some conditions molybdenum causes a depletion in copper reserves, whereas under others, there occurs a tissue accumulation of copper coincidental with symptoms of copper deficiency(2). There has been some indication of a relationship between zinc and copper in that some of the effects of high dietary zinc in rats were overcome by copper supplementation(6,7). However, this relationship has not been well established. This study shows the effects of high levels of molybdenum, zinc and copper on the tissue storage of these elements and also upon the distribution of radioactive copper. Rats and swine were used, the latter because there is little or no information in regard to copper-molybdenum relationships in this species.

Methods. Weanling rats from a highly inbred Wistar strain and 10-week-old Hampshire pigs were each lotted into 6 groups and maintained on a basal ration to which copper, molybdenum, zinc or combinations were added at levels as indicated in Tables I and II. Molybdenum was supplied as sodium molybdate, and copper and zinc as the sulfate. The swine basal ration consisted of 61.3% ground yellow corn, 30% soybean oil meal (solvent process), 7% alfalfa meal, 1% sodium chloride and 0.7% calcium carbonate. It was found to contain by analysis 8.9, 2.1 and 39.2 p.p.m. of copper, molybdenum and zinc respectively. One group of the rats received a ration identical to that fed the swine, and the second group received a synthetic ration previously described(8) to which the copper, molybdenum and zinc supplements were added. The growth response and tissue analyses of the rats for the two groups were similar, and for brevity only results on the natural ration will be discussed. A third short term study with old and young rats involved administration of a molybdenum solution by stomach tube for 8 consecutive days to determine the effect of such supplementation upon tissue uptake of radiocopper intramuscularly administered and these results are presented in the discussion.

Swine were maintained for 27 weeks and rats for 14 weeks on the experimental rations. They were then dosed orally with radiocopper and placed in metabolism units for 24 hours previous to sacrifice for tissue distribution studies and tissue analyses(9-11). Each rat received 320 micrograms of labeled copper with an initial activity of about 100 microcuries and the swine received 75-100 mg of copper as the chloride containing an initial

* Published with the approval of the Directors of the Tenn. and Fla. Agric. Exp. Stations. Taken in part from a thesis submitted by R. Kulwich in partial fulfillment of degree of Doctor of Philosophy in the Graduate School of the University of Florida. The radioactive copper was obtained from the Oak Ridge National Laboratory on allocation from the U. S. Atomic Energy Commission.

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TABLE I. Effects of Dietary Levels upon Accumulation of Copper, Molybdenum and Zinc in Rat Tissues and upon Uptake of Copper 64.*

	Basal ration	Basal + 1000 p.p.m. Mo	Basal + 1000 p.p.m. Zn	Basal + 200 p.p.m. Cu	Basal + Mo and Cu	Basal + Zn and Cu
Wt gain, g/8 wk	115	38	105	119	40	107
Copper (p.p.m.)						
Liver	14	48	13	17	415	13
Kidney	20	152	21	21	131	24
Molybdenum (p.p.m.)						
Liver	2.1	33	2.6	1.6	36	2.2
Kidney	1.0	146	1.7	<1	57	<1
Femur	<1	19	1.7	<1	19	1.8
Zinc (p.p.m.)						
Liver	65	101	100	77	127	71
Kidney	97	99	96	100	102	91
Femur	149	120	177	144	129	229
Copper 64†						
Blood	29	186	10	5	36	7
Liver	49	164	61	79	164	94
Kidney	257	397	298	233	136	273
Spleen	19	66	12	49	38	9
Muscle	2.1	16.5	1.2	1.8	4.0	1.7
Skin	6.0	22.9	8.2	5.4	13.7	3.6
Red bone marrow	395	650	86	125	158	151

* Values represent averages of individual tissues from 3-5 animals except for kidneys which were pooled. Femurs calculated on fresh wt basis, all others on dry wt.

† Reported as % of ingested dose $\times 10^6$/g of fresh tissue corrected to body wt of 275 g.

activity of 25 to 30 millicuries. Samples for radiocopper assay were prepared by wet-ashing in concentrated nitric acid and diluting to a volume of 10 ml(12). Samples with sufficient radioactivity were transferred to a 60 mm Petri dish and counted directly with a thin mica end-window Geiger counter. With samples containing low levels of Cu 64, a one milliliter aliquot was pipetted into a tared stainless steel planchet and evaporated to dryness under an infra-red lamp. The dried samples were then counted in a restricted atmosphere proportional counter. Self-absorption corrections were applied as determined from an empirical self-absorption curve. Samples which were to be analyzed for total copper, molybdenum or zinc in addition to radioactivity measurements, were treated after radioassay, according to the method of Lindner and Harley(13) to yield a completely oxidized sample suitable for the chemical determinations. The carbamate method(14) was used for copper determinations, using isoamyl alcohol as the solvent. The thiocyanate-stannous chloride method, essentially as described by Perrin(15) was used for molybdenum determinations. Zinc was determined by the dithizone method(16).

Results with rats. To conserve space only average values are given. While there was considerable variation between individuals, the differences observed were so large that there is little question as to significance. As noted in Table I, the addition of 200 p.p.m. of copper or 1000 p.p.m. of zinc had no apparent effect upon growth rate of the rats whereas the addition of 1000 p.p.m. of molybdenum resulted in a reduced growth rate. The supplement of 200 p.p.m. of copper did not alter the effects on growth of the high molybdenum intake. When the rats were drenched daily with molybdenum the growth was also retarded and the effect was greater in weanlings than in 24-week-old animals.

Analyses of livers, kidneys, and femurs of the rats indicated that supplementary zinc or copper resulted in little increase in the concentration of copper, zinc or molybdenum in tissues after 14 weeks on the ration. However, rats receiving supplementary molybdenum either with or without copper showed appreciably higher copper concentrations in the liver and kidney, ranging from a 3-fold to a 30-fold increase. High dietary molybdenum

TABLE II. Effects of Dietary Levels upon Accumulation of Copper, Molybdenum and Zinc in Swine Tissues and upon Uptake of Copper 64.*

	Basal ration	Basal + 1000 p.p.m. Mo	Basal + 1000 p.p.m. Zn	Basal + 200 p.p.m. Cu	Basal + Mo and Cu	Basal + Zn and Cu
Daily gain (lb)	1.1	.95	1.0	.98	.96	1.0
Feed/100 lb of gain (lb)	423	462	415	397	417	444
Copper (p.p.m.)						
Liver	29	149	27	60	239	32
Kidney	40	144	47	59	60	43
Spleen	6.6	25	3.1	4.4	6.9	4.8
Plasma	238	376	260	231	330	247
Molybdenum (p.p.m.)						
Liver	5.0	67	5.6	4.6	32	5.0
Kidney	4.4	75	3.7	3.2	25	4.9
Spleen	1.1	21	.9	.4	8.8	.5
Femur shaft	1.9	32	.2	1.5	22	1.1
Copper 64†						
Blood	14	19	10	10	10	10
Liver	66	137	48	42	40	46
Kidney	75	80	59	50	37	67
Spleen	4.9	7.7	3.1	2.3	2.9	2.9
Muscle	2.4	2.2	1.3	2.1	1.5	1.2
Skin	2.6	2.7	4.9	3.9	2.7	4.1
Red bone marrow	1.2	3.4	1.3	.4	2.9	1.3

* Values represent averages of individual tissues from 3 animals in each lot. Plasma calculated as $\mu g/100$ ml, all other analytical results as parts per million on a dry matter basis.

† Values reported as % of ingested dose $\times 10^5$/g fresh wt corrected to a body wt of 200 lb. The No. of animals per lot were as follows: 2, basal; 1, basal + Mo; 3, basal + Zn; 2, basal + Cu; 3, basal + Mo and Cu; 3, basal + Zn and Cu.

was also found to increase the molybdenum content of the liver, kidney and femur.

The high molybdenum rats on the purified rations, the individual values for which are omitted, showed a 3-fold increase in skin copper concentration at the basal copper level and an even greater, though less uniform increase at the high copper level. Skin samples were taken from the back without removing the hair. It is of interest that Hundley and Ing(17) reported a 4-fold increase in copper concentration of skin samples of pantothenic acid-deficient black rats which showed a graying of the hair coat. These authors suggested that gray hair production in pantothenic acid deficiency may involve interference with copper utilization. Singer and Davis(18) reported graying of hair coat in piebald rats on a copper deficient ration which was corrected either by additional dietary copper or calcium pantothenate. These findings support the viewpoint that the high copper concentrations in skins of rats showing graying of the hair coat, after being fed rations deficient in pantothenic acid but not in copper, may reflect the presence of physiologically unavailable copper. In the light of these findings it is plausible to assume that the high copper concentrations found in the skin and other tissues of rats maintained on high levels of dietary molybdenum may also represent copper that is physiologically unavailable.

Results obtained from the tissue distribution of radiocopper (Table I) provide further evidence of increased copper retention by animals fed supplementary molybdenum. At the basal copper level, uptake of copper 64 by blood, liver, kidney, spleen, muscle, skin and red bone marrow was increased in those rats receiving high levels of molybdenum, although there was considerable variability within groups. At 200 p.p.m. dietary copper, the high molybdenum groups showed increased radiocopper uptake by all tissues examined except kidney and spleen. The groups receiving the high level of dietary zinc at the basal copper level showed evidence of lowered blood and red bone marrow copper 64 accumulation, but this did not occur at the high level of copper intake.

Drenching with molybdenum for 8 consecu-

tive days was likewise shown to increase the uptake of Cu 64 administered intramuscularly to 24-week-old rats. The blood showed a 3-fold increase; spleen, skin and liver showed smaller increases but the kidney value was lowered. A somewhat similar pattern was noted with weanling rats. Copper 64 uptake by the blood in the molybdenum groups was increased over 3 times that found in the control group. The skin and liver showed a slight increase in Cu 64 uptake for the molybdenum-drenched group, while kidney and spleen values were lower than those for controls.

Results with pigs. Growth rate and feed consumption data for the various lots of swine (Table II) indicated that the addition of 200 p.p.m. of copper or 1000 p.p.m. of molybdenum or zinc to a natural ration did not result in any marked growth retardation nor did it influence the feed utilization efficiency. Pigs fed 1000 p.p.m. of zinc in this experiment showed no ill effects although they received a lower level of zinc, on a dry matter basis, than did those in the experiments reported by Grimmett *et al.*(19), in which 1000 p.p.m. of zinc as the lactate was added to whole milk with pigs becoming lame and unthrifty within two months. Apparently zinc accumulation in the present experiment had not reached the toxic level.

Histological examination of sections taken from the cerebrum, cerebellum, cervical cord, thoracic cord and lumbar cord of pigs from all lots showed no degenerative changes and were believed to represent normal tissues. Leg joints and muscles taken from the gluteal region showed no pathological change.

Analytical data for copper, molybdenum and radiocopper are also presented in Table II. Ingestion of 200 p.p.m. of supplementary copper did not cause any increase in spleen or plasma copper levels when compared to the basal group, although kidney and liver values were somewhat higher. Supplementary zinc did not appear to affect copper accumulation in these tissues studied. Dietary supplementation with molybdenum, on the other hand, appeared to markedly increase tissue copper accumulation.

At the basal copper level, pigs receiving supplementary molybdenum showed a 5-fold increase in liver and about a 4-fold increase in spleen and kidney copper concentration. Plasma copper levels were elevated about 58%. An increase in tissue copper concentration by molybdenum supplementation was also found at the high copper level; there was a 4-fold increase in liver copper concentration and a 43% increase in plasma copper concentration observed in the control group. However, when high dietary copper levels were fed, supplementary molybdenum did not appear to materially affect kidney or spleen copper concentration.

Ingestion of 1000 p.p.m. of molybdenum resulted in high molybdenum values for liver, kidney, femur and spleen. Molybdenum concentration in these tissues from groups receiving supplementary copper in addition to molybdenum, however, were considerably lower than corresponding values for groups receiving only supplementary molybdenum. This indicates that the excess copper intake lowered the accumulation of dietary molybdenum. Accumulation of molybdenum was apparently unaffected by the high zinc level.

Unfortunately, copper 64 uptake data were obtained for only one pig of the lot receiving supplementary molybdenum at the basal copper level. The data for this animal indicated an increased concentration in liver, spleen and red bone marrow of pigs on the basal ration plus high molybdenum. At the high copper level, however, the lot receiving supplementary molybdenum showed a definite increase in copper 64 uptake only in the red bone marrow. High dietary level of zinc did not appear to have any appreciable effect upon the uptake of labeled copper.

The increased tissue copper accumulation observed in swine fed high dietary molybdenum levels is in agreement with the rat data and the results of Marston with rats who reported that supplementation with 1 mg of molybdenum daily resulted in increased copper accumulation.

Summary and conclusions. Substantial increases in tissue copper concentrations and radiocopper accumulation resulted when a level of 1000 p.p.m. of molybdenum as sodium molybdate was fed to rats and swine on a

natural ration. This increase was especially pronounced in the kidneys and livers and is in accord with the hypothesis that excess copper, which is not physiologically effective, may be accumulated when a high level of molybdenum is fed. A dietary level of 1000 p.p.m. of zinc did not appear to have any appreciable effect upon copper metabolism. Growth depression in rats produced by 1000 p.p.m. of added molybdenum to a natural or synthetic ration was not counteracted by the addition of 200 p.p.m. dietary copper. Swine appeared to be more resistant to molybdenum toxicity than did rats. No degenerative changes were observed in the spinal cord, leg joints or muscle tissues of swine fed levels of 1000 p.p.m. of molybdenum or 1000 p.p.m. zinc for a 7-month experimental period. With the criteria of these experiments it appears that dietary zinc was not involved in copper-molybdenum imbalance.

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Received October 26, 1953. P.S.E.B.M., 1953, v84.

Arch. Biochem. Biophys. 31: 251-261 (1951)

The Effect of Zinc on the Oxidation of Krebs Cycle Intermediates by Rat Liver and Kidney Homogenates

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Received November 30, 1950

Introduction

The inhibition of enzymes by small concentrations of heavy-metal ions is a well known phenomenon and has been described and cited by a large number of workers. These studies, however, have chiefly been on the effect of heavy-metal ions on the action of a single enzyme in more or less purified conditions. The effect of these ions on multienzyme systems has not been extensively investigated.

Evidence that zinc ions inhibit enzyme action in a manner typical of heavy-metal ions has been obtained in the case of the inactivation or inhibition of urease (1), prolidase (2), phosphoglucomutase (3), and the succinoxidase system (4). Krebs and Eggleston (5) reported that traces of zinc (10^{-6} M) inhibited the respiration of minced pigeon breast muscle. We have studied the effect of zinc ions, as a type of heavy metal ions, on the enzyme systems of rat kidney and liver which oxidize intermediates of the Krebs citric acid cycle in an effort to extend the knowledge of the mechanism of heavy-metal-ion inhibition on multienzyme respiratory systems. The results of these experiments are the subject of this paper.

Experimental

All experiments were performed at 25°C. with a Warburg apparatus shaken at 150 strokes of 3.0 cm. length/min. Tissue homogenates were prepared from tissues obtained from mature rats of the Sprague-Dawley strain maintained on a diet of commercial dog pellets. The animals were stunned by a blow on the head and were bled and the desired tissues were removed immediately. After thoroughly chilling the tissue, a portion was weighed and homogenized in 9 vol. of alkaline isotonic KCl

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solution (1.13% potassium chloride in 0.01 M potassium phosphate buffer, pH 7.3) or distilled water in a Potter-Elvehjem glass homogeniser (6).

Reaction components were varied for the different experiments and are shown in the legends of the tabulated or graphically represented data. All components were kept cold until the flasks were placed in the 25°C. bath. Oxygen uptake was measured in 10-min. intervals with zero time taken at the moment the flasks were placed in the bath. Where controlled pressures of CO_2 were used, the oxygen uptake was measured by the method described by Pardee (7).

Citric acid and sodium succinate were obtained from General Biochemical Company. α-Ketoglutaric acid was purchased from Nutritional Biochemicals Corporation. Malic acid was recrystallized from a product obtained from the Eastman Kodak Company. The pyruvate was prepared by diluting 1 N acid and neutralizing with $KHCO_3$. The 1 N acid was prepared by distillation of Eastman pyruvic acid under reduced pressure, with immediate dilution. Adenosine 5-phosphate (muscle adenylate) was obtained from Ernest Bischoff Company. Adenosine triphosphate (ATP) was prepared by the method described by Dounce *et al.* (8). The crystalline zinc insulin (0.61% zinc) and the amorphous insulin were generous gifts of Eli Lilly and Company through the courtesy of Dr. F. B. Peck.

Results

Zinc Inhibition of Succinoxidase

Because of the stability of the succinoxidase system, it can be studied under a variety of conditions which would inactivate the systems oxidizing other intermediates of the Krebs cycle. By the use of water homogenates and the addition of $AlCl_3$ and $CaCl_2$, as in the succinoxidase assay method, the succinate is oxidized quantitatively to fumarate and malate (9). Thus the oxidation of succinate may be essentially isolated, since malate, oxalacetate, or pyruvate are not oxidised under these conditions.

In preliminary studies it was noted that when zinc sulfate in the order of 1–4 × 10^{-5} M is added to the system, the oxidation of succinate by a water homogenate of rat liver is essentially "irreversibly" inhibited. In a typical experiment, 2 × 10^{-5} M Zn ion caused an average reduction in oxygen uptake during a 20-min. period of 78 μl./flask with 0.4 ml. liver homogenate and 80 μl. with 0.6 ml. of the homogenate. A given amount of zinc ions inhibited the same amount of succinoxidase activity although the concentration of the enzyme was varied. Ackermann and Potter (10) have described this type of inhibition which is essentially the outcome of an "irreversible" reaction between the enzyme and inhibitor, so that the per cent inhibition depends upon the enzyme concentration as well as the inhibitor concentration. Rat kidney homogenates present similar results, but a greater zinc concentration is required to obtain the same amount of inhibition. While 2 × 10^{-5} M Zn ion reduced the oxygen uptake in the first 10 min. after equilibration from 78 to 39 μl./flask with 0.4 ml. liver homogenate, the oxidation by a comparable amount of kidney was decreased from 96 μl. to only 87 μl./flask.

To extend these studies, an experiment was designed so that zinc, crystalline zinc insulin, and copper were tipped in from the side arm of the flask sometime after the temperature equilibration period and the addition of the substrate. The results are shown in Fig. 1. Although the substrate was present, the addition of zinc sulfate to give a final concentration of 2 × 10^{-5} M produced a substantial immediate fall in activity to a rate which did not change much during the experimental period. Three times as much cupric sulfate, 6 × 10^{-5} M, produced only

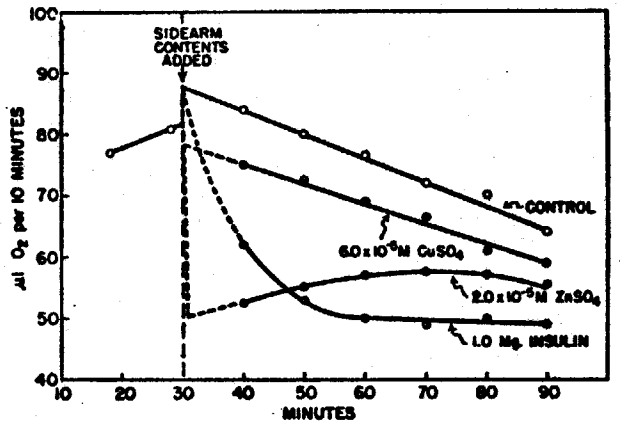

FIG. 1. Comparison of the inhibition of liver succinoxidase by copper, zinc, and crystalline zinc insulin. System contained water to make a final volume of 3.0 ml. and final concentrations of 1.3 × 10^{-5} M cytochrome c, 0.05 M Na succinate, 0.016 M K phosphate (pH 7.3), 4 × 10^{-4} M $CaCl_2$, 4 × 10^{-4} M $AlCl_3$, and 0.4 ml. water homogenate of rat liver. Six-tenths ml. water (for control data), $CuSO_4$, and $ZnSO_4$ solutions to give final molarities as indicated, or crystalline insulin suspension was tipped in as indicated.

about one-third the inhibition that the zinc did. Although cupric ions, in addition to combining with the enzyme, may inhibit because they are an oxidizing agent, they have only one-tenth of the toxicity shown by zinc ions, which are not an oxidizing agent. The addition of 1.0 mg. crystalline zinc insulin per flask resulted in a rapid decrease in activity. This decrease was not immediate as was that caused by the zinc sulfate, but it containued for 30 min. after the addition of the insulin. This probably reflects the dissociation of the zinc insulin and the subsequent formation of a zinc–enzyme complex which is less dissociable than the

zinc insulin. This is also substantiated by the observation that amorphous insulin does not protect the succinoxidase system from zinc inhibition.

Reversal of Zinc Inhibition of Succinoxidase

If conditions which allow oxidative phosphorylation, namely, the isotonic KCl homogenate, fortified with muscle adenylate or ATP and magnesium ions, are used in the oxidation of succinate, a reaction to zinc is obtained which is different from that of the assay type system. Data from a representative experiment with a rat liver homogenate are shown in Fig. 2. When succinate is added to this phosphorylating

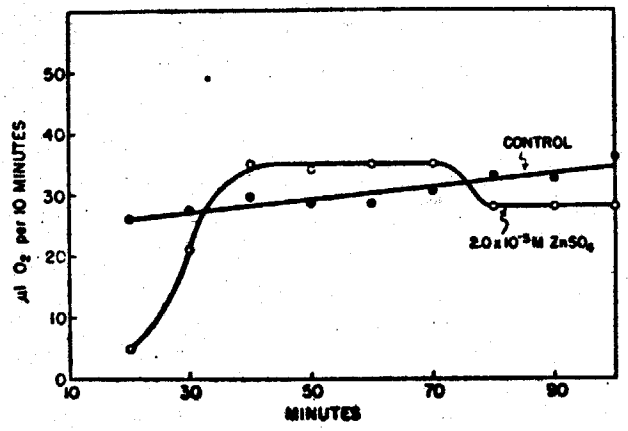

FIG. 2. Transitory inhibition of the oxidation of succinate by zinc sulfate in isotonic KCl liver homogenate system containing ATP and Mg. The system included water to make 3.0 ml., 1.3×10^{-4} *M* cytochrome c, 0.05 *M* Na succinate, 0.0033 *M* $MgCl_2$, 1.6×10^{-3} *M* potassium ATP, 0.016 *M* potassium phosphate (pH 7.3), 0.4 ml. isotonic KCl homogenate, and $ZnSO_4$ as indicated. Temperature, 25°C.

system, the rate slowly increases in a linear fashion. When 2×10^{-5} *M* zinc sulfate is present there is almost complete inhibition for 20 min., after which the rate of oxidation rapidly rises to a level even higher than the control. This level is maintained with little change until between 60 and 80 min., when a significant decline in oxidation is observed. This is followed by a long period of oxidation during which the rate very gradually diminishes in a linear fashion. A smaller level of zinc produces less initial inhibition and a longer time between recovery from inhibition and the drop to the lower level of activity.

From these data, it appears that zinc inactivates or inhibits succinoxidase by forming a complex with some enzyme in the system. This complex is essentially nondissociated in the system used for assay of the enzyme. In the "phosphorylating" system, the zinc–enzyme complex appears to dissociate or else more enzyme starts to oxidize succinate. Although the activity of the KCl homogenate as used in the "phosphorylating" system was somewhat less than the water homogenate system, the latter premise seems unlikely since a range of levels of zinc from 0 to 4×10^{-5} *M* did not produce any significant differences in the maximum oxygen uptake by the system.

It does not appear that the reversal of this inhibition is due to the formation of an insoluble compound with adenylic acid or ATP. These substances were not protective in any other system which was studied, and in an experiment in which the ATP or adenylic acid was not added, a similar reversal of inhibition was obtained. The secondary decrease in oxygen uptake may be due to the inhibition of other oxidative systems which operate to some extent under these conditions. The data supporting this are given below.

Zinc Inhibition of α-Ketoglutarate Oxidation

The pattern of oxidation of α-ketoglutarate by rat liver and kidney homogenates is somewhat different from that of the oxidation of succinate. When α-ketoglutarate is added to the rat liver or kidney homogenate systems capable of oxidizing this compound, the rate slowly declines in a linear fashion only after an extended period of relative stability. When small amounts of zinc (to give a final concentration of 2×10^{-6} *M* $ZnSO_4$ or less) are added to this system, the initial rate of oxidation is not greatly affected, but the duration of oxidative activity is markedly shortened. Figure 3 shows data which are representative of this phenomenon. When larger amounts of zinc are present (3.3×10^{-5} *M* $ZnSO_4$), the maximum rate of oxidation is lowered and the duration is markedly reduced.

Experiments have shown that the rate of any multienzyme system is governed by one enzyme which may be termed the limiting enzyme (11). In the succinoxidase system, even the smallest amounts of zinc ions used appear to inhibit the enzyme which limits the maximum rate of the system, that is, the succinic dehydrogenase (12). In contrast, the initial rate of oxidation of α-ketoglutarate is not appreciably affected

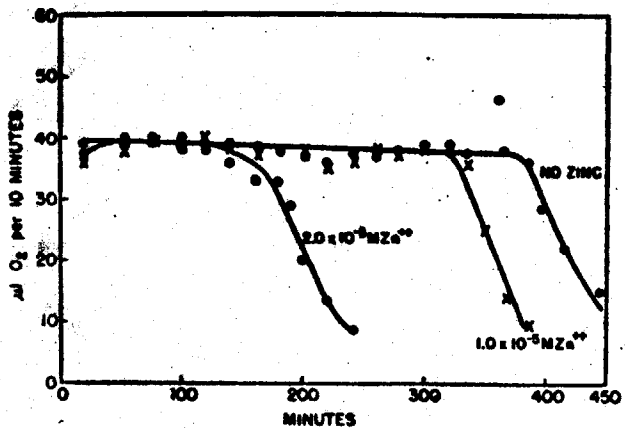

FIG. 3. The effect of zinc ions on the course of oxidation of α-ketoglutarate by rat liver homogenates. Each flask contained water to make 3.0 ml., 1.3×10^{-4} *M* cytochrome c, 1.0×10^{-3} *M* K adenylate, 0.017 *M* K phosphate (pH 7.3), 0.003 *M* $MgCl_2$, 0.01 *M* K α-ketoglutarate with 0.4 ml. of 10% isotonic KCl homogenate of liver. KCl was added to each flask to give a final concentration of 0.055 *M*. Zinc sulfate added as indicated.

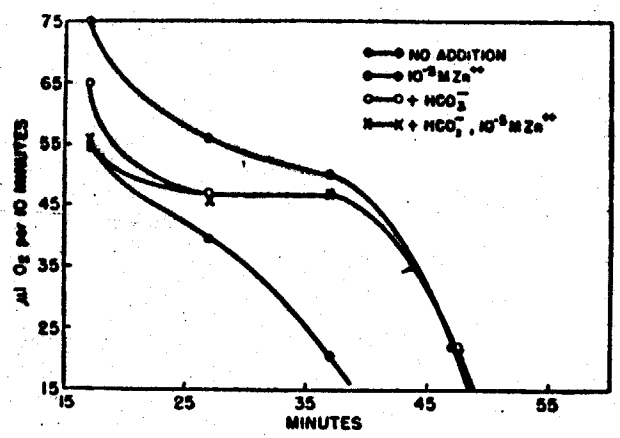

FIG. 4. Prevention of zinc inhibition of malate oxidation by bicarbonate and CO_2. Each flask contained water to make 3.0 ml., 1.3×10^{-4} *M* cytochrome c, 1.7×10^{-3} *M* K adenylate, 0.017 *M* K phosphate (pH 7.0), 0.003 *M* $MgCl_2$, 0.016 *M* K malate, 0.003 *M* K pyruvate, 0.8 ml. isotonic KCl homogenate of kidney, and when used, 0.013 *M* $KHCO_3$. Zinc sulfate was added as indicated. One per cent CO_2 maintained in the gas phase over systems containing $KHCO_3$.

by levels of zinc of 2×10^{-5} *M* or less. Zinc here does not appear to inhibit the initial "limiting" enzyme in this system, but inhibits another enzyme in the system which eventually becomes the limiting enzyme.

Zinc Inhibition of Malate Oxidation

Potter and LePage (13) were able to show that the oxidation of malate was irreversibly inhibited by cupric, cadmium, and mercuric ions. Isotonic KCl homogenates fortified with ATP and magnesium ions were used by these workers. In such a system, zinc ions behave in an identical fashion. It is to be noted that conditions which may be assumed to allow concomitant phosphorylation had no effect in reversing the inhibition. Maintaining a constant atmosphere of 1% CO_2 by the method described by Pardee (7), however, markedly reduces the inhibitory action of zinc upon this system. These data are presented in Fig. 4. This diminution of inhibition is apparently due to the formation of insoluble zinc bicarbonate.

Inhibition of Citrate Oxidation by Zinc and Insulin Preparations

The pattern of zinc inhibition of citrate (isocitrate) oxidation is similar to that of α-ketoglutarate oxidation. The inhibition is primarily evident in that the duration of activity is shortened and the initial rate of oxidation is lowered to some extent. These data are given in Fig. 5 and Table I.

Proteins such as insulin have a protective action in the zinc inhibition of phosphoglucomutase (3). Experiments in this laboratory have shown that insulin and bovine albumin protect crystalline urease from zinc poisoning. Amorphous insulin, however, has no protective effect in zinc inhibition of citrate or succinate oxidation. It can be seen from the data presented in Fig. 5 that crystalline zinc insulin has the same inhibitory action on citrate oxidation as zinc sulfate when each source is added in amounts containing equal quantities of zinc. On the other hand, if amorphous insulin in levels of 400–500 μg./flask is added to the rat kidney system, it causes a slight decrease in the initial rate, but a more rapid falling off in activity than in the control systems is evident. This phenomenon is reproducible and therefore cannot be considered as an artifact. Liver homogenates do not show such a response to the presence of insulin. Shorr and Barker (14) have described a similar

TABLE I

Effect of Zinc Ions on the Oxidation of Citrate, α-Ketoglutarate and Malate

Each flask contained water to make 3.0 ml., 1.3×10^{-4} *M* cytochrome c, 1.0×10^{-3} *M* potassium adenylate, 0.017 *M* potassium phosphate (pH 7.3 for citrate and α-ketoglutarate systems, pH 7.0 for the malate system), 0.0033 *M* $MgCl_2$, and the potassium salt of substrate and zinc sulfate as indicated, with various levels of isotonic KCl homogenates of rat liver or kidney. Gas phase, air; temperature 25°C. Potassium chloride was added in each case to give a final concentration of 0.06 *M*. Duration was determined as the number of minutes required until the rate of oxygen uptake fell to one-half the maximum rate of the flask.

Substrate	Tissue and volume of homogenate	Zinc addition	Maximum O_2 uptake	Change in maximum O_2 uptake	Duration
		($\times 10^{-5}$ *M*)	*μl./10 min.*	*μl./10 min.*	*min.*
Citrate, 0.01 *M*	Kidney, 0.8 ml.	None	77	—	180
		0.7	60	−8	150
		3.0	34	−43	40
	Liver, 0.8 ml.	None	55	—	220
		0.7	56	1	235
		1.0	50	−5	207
		2.0	50	−5	160
		None[a]	56	1	210
	Liver, 0.4 ml.	None	31	—	250
		1.0	23	−8	200
α-Ketoglutarate, 0.01 *M*	Kidney, 0.8 ml.	None	107	—	137
		1.0	98	−9	137
	Kidney, 0.5 ml.	None	63	—	200
		1.0	46	−17	77
	Liver, 0.4 ml.	None	41	—	455
		2.0	41	0	330
		3.3	29	−12	95
Malate, 0.013 *M* Pyruvate, 0.003 *M*	Kidney, 0.8 ml.	None	76	—	43
		0.5	66	−10	40
		1.0	54	−22	34
	Liver, 0.8 ml.	None	42	—	305
		1.0	38	−6	240
		2.0	30	−12	90
	Liver, 0.4 ml.	None	23	—	240
		1.0	12	−11	28
		2.0	0	−23	<8

[a] Amorphous insulin 400 μg. added.

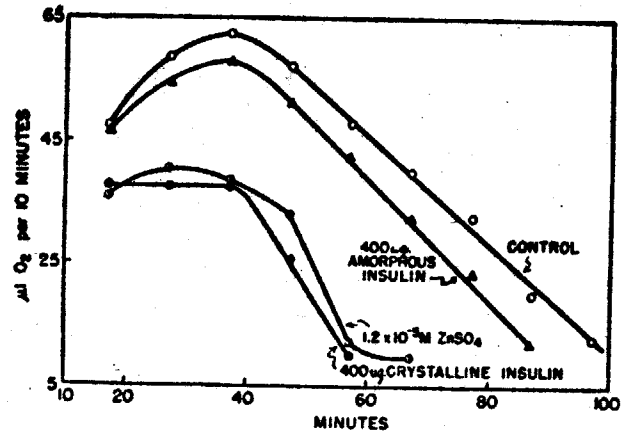

FIG. 5. The effect of amorphous insulin, crystalline zinc insulin, and zinc ions on the oxidation of citrate by a rat kidney homogenate. Each flask contained water to make 3.0 ml., 1.3×10^{-4} *M* cytochrome c, 10×10^{-3} *M* K adenylate, 0.017 *M* K phosphate (pH 7.4), 0.003 *M* $MgCl_2$, 0.01 *M* K citrate, 0.8 ml. isotonic KCl homogenate of rat kidney, and KCl to give a final concentration of 0.06 *M*.

shortening of duration of activity by insulin with minced skeletal muscle of the cat, dog, and rabbit, and with minced dog cardiac muscle when citrate was added as the substrate. This inhibition cannot be accounted for by the zinc content of the amorphous insulin (0.005% zinc).

Relative Inhibition of Rat Kidney and Liver Homogenates

The relative effects of varying amounts of zinc ions on the different respiratory systems from rat kidney and from rat liver with citrate, α-ketoglutarate, and malate and pyruvate as substrates is given in Table I. In each case, with comparable amounts of tissue, zinc caused a greater decrease in the maximum oxygen uptake of the kidney system than with the liver homogenate. This is in contrast to the results with the succinoxidase system where the kidney was less affected by zinc ions. These data probably reflect the greater lability of certain respiratory enzymes of rat kidney as compared with those of rat liver, as well as the apparently greater content in rat liver of protective substances which have a greater affinity for zinc ions than the respiratory enzymes have.

Discussion

When succinoxidase is the only Krebs cycle oxidative system that is operative, such as in the assay system for this enzyme, the inhibition by zinc and copper (10) is in effect irreversible. Barron and Kalnitsky (15) have presented data to indicate that this inhibition involves a linkage between zinc and the —SH groups of the protein molecule. From their data, it is also evident that the succinic dehydrogenase-zinc complex has a dissociation constant, although a very small one, for glutathione was shown to reverse the inhibition caused by zinc.

In the "phosphorylating" system, as reported in this communication, the zinc inhibition is reversed by providing conditions which would allow many other enzymes to remain active. Webb *et al.* (16) have recently reported that malonate inhibition of rat heart slice respiration occurs initially, but that the respiration subsequently increases to a peak which is greater than the control respiration. The mechanism may be similar to that of the reversal of zinc inhibition. The reversibility of the zinc inhibition of the succinoxidase system appears to be unique in the oxidative events of the Krebs cycle. No other system tested showed this phenomenon although essentially the same conditions were used.

The effect of small concentrations of zinc on the oxidation of α-ketoglutarate and citrate may be that of promoting the breakdown of the system by accelerating the loss of labile components and the deterioration of the mitochrondria. Pardee and Potter (17) have concluded from their studies that these labile components are destroyed enzymatically under conditions unfavorable to phosphorylative oxidation. The insulin inhibition of citrate oxidation may also be a quickened loss of labile components.

It has been shown that high-energy phosphate is required for stability of such a system (17), and insulin may accelerate the depletion of the high-energy phosphate in some way. At any rate, the affinity of zinc for insulin appears to be less than for the enzymes studied here. Insulin offers no protection against zinc toxicity in these systems. Thus it appears likely that the biological activity of insulin does not depend upon the presence of zinc.

Acknowledgments

The author's thanks are due to Professor James B. Sumner for his valuable help and criticism in this investigation, and to the Rockefeller Foundation for financial assistance.

Summary

Zinc ions in small concentrations (2×10^{-5} *M*) inhibit rat liver and kidney succinoxidase systems by forming an inactive complex with the enzyme. Under conditions which favor oxidative phosphorylation (isotonic KCl homogenates, Mg ion, and muscle adenylate or adenosine triphosphate), inhibition by zinc occurs, but this inhibition is transitory. The rate of oxidation subsequently rises to a level even higher than shown by the control data. A later fall to a lower level of activity can be attributed to the zinc inhibition of other Krebs cycle intermediates, particularly the oxidation of α-ketoglutarate and citrate. The addition of a protein such as insulin has no protective action against zinc inhibition, and crystalline zinc insulin causes the same inhibition as does zinc sulfate. With the exception of succinoxidase, the oxidizing enzymes of rat kidney are more readily inhibited by zinc ions than are those of rat liver.

Amorphous insulin, in itself, inhibits citrate oxidation by rat kidney homogenates.

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Zinc Poisoning

SIR,—In 1954 we came to live in a bungalow which had been built for us on a site some 120 ft. (37 m.) from the road and water main: this had entailed the laying of a very considerable length of galvanized iron piping. After a few months my wife and I had to admit to one another that we were not feeling well but had tried to put it down to the change we had made in our lives and situation, but the following symptoms developed to such an extent that we really began to feel quite ill:

Mental.—Irritability; difficulty in concentration; great drowsiness; "cloudiness" in the head and great mental fatigue; headaches, to which neither of us is subject in the ordinary way; and, in my wife's case, "buzzing" in the head.

Pain.—Stiffness and pain in the muscles of the neck and back; constant pain in the arms; and, finally, such pain in the one or other leg that sleep was becoming impossible.

Digestive Symptoms.—Loss of appetite; heartburn; some nausea. We both lost about 7 lb. (3.2 kg.) in a few months. My wife's weight is pretty constant and I have not varied more than about a pound either way for the last 10 or 15 years.

Eyes and Nose.—Some agglutination of lids and nasal coryza.

General Symptoms.—Increasing and most disturbing general lassitude and fatigue.

Very belatedly I sent a sample of the water to the Public Analyst's laboratory in Reading (in May, 1955), and was informed that the water we had been drinking for many months contained 40 parts per million of zinc. Understanding that a reasonably allowed amount is 5 p.p.m., I thought that this was of sufficient importance to report to the Ministry of Health. I had a most courteous reply from the Ministry from which I gathered that if we lived long enough all the zinc would be washed out of the pipe, but the essence of the reply lay in this paragraph: "I should point out that there have been a number of investigations into the effect on people of water containing a high proportion of zinc. In one case a hamlet had a supply for many years of which the zinc content varied between 9.8 and 22.8 p.p.m. and no ill effects were discovered after personal interviews with every one of the inhabitants, while the practitioner in the village thought that intestinal irritation and constipation were no more common there than anywhere else."

There is, of course, a difference between a maximum of 22.8 and 40 p.p.m., but the implication here is that there is no such thing as zinc poisoning and that the symptoms we complained of could hardly be due to zinc. We brought in a fresh supply of water direct from the main by a polythene pipe, and we got gradually better, though it took some few months before we felt anything like right.

Now in the *Monthly Bulletin of the Ministry of Health* for December last[1] eight outbreaks of acute poisoning from food which had been contaminated by zinc are reported as having occurred during the years 1942–54. I find it a little difficult to reconcile the two statements. It is reported that a few odd meals contaminated by zinc can cause acute poisoning, but according to their reply to me the Ministry will not allow that food cooked for several months in water containing 40 p.p.m., and that such water used regularly for drinking purposes, are at all likely to do any harm.

There is, apparently, no ordinary clinical or laboratory test for zinc in the system.—I am, etc.,

Wargrave, Berks. GEO. LAURENCE.

REFERENCE

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Studies on Growth, Copper Metabolism and Iron Metabolism of Rats Fed High Levels of Zinc[1,2]

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High levels of dietary zinc (0.5 to 1.0%) fed to rats cause a depression in growth, the development of anemia, a decrease in heart and liver cytochrome oxidase activities, and a decrease in the copper content of the liver (Sutton and Nelson, '37; Smith and Larson, '46; Gray and Ellis, '50; Van Reen, '53; Duncan et al., '53; Grant-Frost and Underwood, '58). Smith and Larson ('46) found that a supplement of a liver extract partially alleviated the depression in growth. The nature of the active factor(s) in liver extract has not been determined. Duncan et al. ('53) reported some beneficial effect on the subnormal growth by adding folic acid to the high-zinc diets of rats. Supplements of copper to diets containing 0.75 to 1.0% of zinc partially overcome the zinc-induced anemia (Smith and Larson, '46; Duncan et al., '53; Grant-Frost and Underwood, '58). Additional copper also prevents the depression in cytochrome oxidase activity (Van Reen, '53; Duncan et al., '53). Thus, there is ample evidence to support the hypothesis that zinc interferes with copper metabolism in some way that has, as yet, not been determined.

In view of the lack of information concerning certain aspects of the zinc toxicity problem in rats, the present study was conducted to investigate the nature of the active factor(s) in liver, capable of alleviating subnormal growth in zinc-fed rats, to examine the nature of the interference of zinc with copper metabolism, and to determine the possibility of iron involvement in the zinc-induced syndrome.

MATERIALS AND METHODS

Male and female rats (Wistar and Sprague-Dawley strains) from 4 to 6 weeks old were used for the various phases of this study. The animals were maintained on experiment for 5 weeks, housed in individual wire-bottom cages, and given free access to feed and water. Rats in each experiment were randomized into replications according to initial body weights. The test treatments were assigned at random to individual cages within a replication.

The basal diet, the control, used in this study consisted of the following (in per cent): casein 19;[3] corn starch 63;[4] vegetable fat 10;[5] mineral mix 4;[6] vitamin mix 2;[7] cellulose 2;[8] and oleum percomorphum.[9] Materials tested were added to the basal diet at the expense of equal amounts of starch. Since previous workers have fed

Received for publication May 13, 1960.

[1] Published with the approval of the Director of the North Carolina Agricultural Experiment Station as paper no. 1171 of the Journal Series. The data were taken from a thesis submitted by the senior author in partial fulfillment of the requirement for the Doctor of Philosophy degree.

[2] Supported in part with a grant from the Herman Frasch Foundation and presented in part at the 136th American Chemical Society national meetings, September, 1959.

[3] Vitamin-Test Casein, Nutritional Biochemicals Corporation, Cleveland.

[4] Corn Products Sales Co., Baltimore.

[5] Crisco, Procter and Gamble Company, Cincinnati.

[6] Salt Mixture W, Nutritional Biochemicals Corporation, Cleveland.

[7] Each 100 gm of the vitamin mix contained (in milligrams) biotin 1; folic acid 5; 0.1% vitamin B_{12} (with mannitol) 0.1; thiamine·HCl 25; pyridoxine·HCl 25; 2-methyl-naphthoquinone 50; riboflavin 50; nicotinic acid 50; Ca pantothenate 150; and (in grams) p-aminobenzoic acid 5; inositol 5; choline 7.5; DL-methionine 30; and corn starch 52. Folic acid was furnished by American Cyanamid Company, Pearl River, New York; all other vitamins were contributed by Merck and Company, Rahway, New Jersey.

[8] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[9] Each 1000 gm of diet contained 24 drops of oleum percomorphum, Mead Johnson and Company, Evansville, Indiana.

zinc in the forms of carbonate, chloride or oxide, the first experiment was designed to compare the effects of these three zinc compounds on growth, hemoglobin formation, liver copper and iron and heart cytochrome oxidase activity. In all other experiments zinc was fed as the carbonate. Supplements of copper and iron were in the forms of sulfate.

Oxyhemoglobin determinations, by the method of Shenk et al. ('34), were made on blood samples taken from the tails of the rats, at the termination of each experiment.

At the end of an experiment, rats from several replications were sacrificed, and the hearts and the livers were removed. The hearts were homogenized immediately in a Potter-Elvehjem homogenizer with 5 ml of cold 0.03 M phosphate buffer (pH 7.4). Cytochrome oxidase activities of appropriate dilutions of the crude homogenates were assayed spectrophotometrically at 550 mμ with a Beckman DU spectrophotometer (Cooperstein and Lazarow, '51). A stock solution of cytochrome C was prepared by dissolving cytochrome C[10] in cold 0.03 M phosphate buffer (pH 7.4) so that there were 12 mg of cytochrome C per ml. This solution was reduced with sodium hydrosulfite. A standard assay mixture consisted of 40 μ moles of phosphate buffer (0.1 M) at pH 7.4, 0.021 μ moles of reduced cytochrome C, 0.02 to 0.04 ml of diluted enzyme (volume depended on activity), and sufficient water to bring the total volume to 1 ml. The enzyme was added to the assay mixture, and the decrease in optical density between 15 and 75 seconds was recorded. Enzyme activity was expressed as the optical density change per minute per milligram of protein rather than as the first-order velocity constant (Cooperstein and Lazarow, '51) because repeated tests showed that under the conditions of these assays the oxidation rates remained linear for at least three minutes. This is in agreement with the findings of others (Fitch and Beevers, '55; Miller and Evans, '55). Protein contents of the diluted homogenates were determined by the method of Lowry et al. ('51).

The copper and the iron content of the livers was determined by the [illegible] Parks et al. ('47) and [illegible] respectively, as modified by Matrone et al. ('47). The zinc content of the livers was determined by a method outlined by Sandell ('59).

A radioisotope experiment with Cu^{64} was conducted to determine if zinc affected the absorption or the utilization and the excretion of copper in the rat. Four control rats and 4 zinc-fed rats were taken from one of the growth trials after it was completed and placed in individual metabolism cages with access to water and the respective experimental rations. Each rat received 100 μc of radiocopper[11] by stomach tube. Forty-eight hours after the administration of the radiocopper, the rats were sacrificed; the hearts, spleens, livers, kidneys, and gastrointestinal tracts were removed and the radioactivity of each was determined by means of a scintillation counter. Feces and urine samples from each rat were also collected, and the radioactivities of these were measured. Using the same procedures an experiment was conducted with Fe^{59} to observe the effect of zinc on absorption, utilization, and excretion of iron. Each rat received 5 μc of radioiron[11] by stomach tube. The radioactivities of the hearts, spleens, livers, kidneys, right and left femurs, gastrointestinal tracts, feces and urine samples were also estimated by means of a scintillation counter.

In the investigation of the nature of the active factor(s) in liver extract,[13] an ash of the liver was obtained by the wet-ashing method. Solvent fractionation of the liver extract was carried out with either methanol or acetone. The liver extract, in 50-gm amounts, was extracted for approximately 24 to 36 hours with either solvent. A Soxhlet extractor was used for this extraction step. When acetone was used as the solvent, a precipitate settled out in the boiling flask as the acetone cooled. This precipitate, designated as "compound X" in this study, was collected on filter paper by means of a suction filter, allowed to dry at room temperature, and saved for use in an assay. The acetone fraction was concentrated to appr[illegible]

[illegible] Sigma Chemical Company, St. Louis, Missouri.

[illegible] Oak Ridge Nuclear Laboratories, Oak Ridge, Tennessee.

[illegible] Fraction L, Nutritional Biochemicals Corporation, Cleveland.

mately 100 ml at reduced pressure. The methanol fraction, obtained when methanol was used as the extracting solvent, was also concentrated to approximately 100 ml at reduced pressure.

A randomized block design was used in the animal experiments and all data were subjected to an analysis of variance. Statements of significance are based on odds of at least 19 to 1 ($P \leq 0.05$).

RESULTS AND DISCUSSION

Experiment 1. As shown in table 1, the addition of zinc to the diet of rats caused marked decreases in growth, liver copper, and liver iron. Although the hemoglobin levels of the zinc-fed rats were lower than the controls, the effect of zinc on hemoglobin was not as great as has been reported in the literature. Zinc in the diet was associated with a slight decrease in heart cytochrome oxidase activity and a large increase in the concentration of zinc in the liver. In general, the results from feeding the three zinc compounds were similar.

Experiment 2. Since the work of Smith and Larson ('46) with liver extract was carried out prior to the discovery of vitamin B_{12}, it was reasoned that this compound could be a part of the active factor(s) in liver. This vitamin, in combination with folic acid and ascorbic acid, was added to the zinc diets in a second experiment. The results shown in table 2 indicated that the rats receiving additional vitamins gained more and had a higher concentration of iron in the livers than the zinc-fed rats. However, the difference was not statistically significant. The added vitamins apparently had no effect on the hemoglobin levels, the copper contents of the livers or the cytochrome oxidase activities of the hearts of the zinc-fed rats.

Experiment 3. In this experiment the effectiveness of liver extract for alleviating subnormal growth was compared with that of the ash of liver extract since it has not been established whether or not the active principle was organic or inorganic. The addition of 10% of liver extract to the zinc diet caused a highly significant increase ($P \leq 0.01$) in growth, hemoglobin level, and liver iron (table 3). There was also a significant increase ($P \leq 0.05$) in the liver

TABLE 1

Effect on the rat of high dietary zinc from various sources[1,2]

Source	Zinc in diet	Weight gain at 5 weeks	Hemoglobin	Liver constituents[3]			Heart cytochrome oxidase[4]
				Cu	Fe	Zn	
	%	gm	gm/100 ml blood	μg/gm dry weight			ΔOD/min/mg protein
None	—	145 ± 8[5]	13.58 ± 0.46[5]	15.69 ± 0.57[5]	425.72 ± 43.01[5]	37.21 ± 3.26[5]	10.50 ± 1.42[7]
$ZnCO_3$	0.75	191 ± 9[6]	10.03 ± 0.51[7]	6.47 ± 0.69	230.53 ± 29.39	378.39 ± 40.62	6.74 ± 0.73
	1.00	95 ± 9[6]	11.11 ± 0.75[5]	6.16 ± 0.51	240.56 ± 11.91	497.70 ± 40.57	5.91 ± 0.46
$ZnCl_2$	0.75	108 ± 12[6]	11.92 ± 0.10[6]	5.30 ± 0.52	232.54 ± 56.25	398.73 ± 63.92	7.79 ± 1.86
	1.00	62 ± 6[6]	10.97 ± 0.68[10]	3.96 ± 0.07	161.88 ± 10.59	452.25 ± 49.86	6.74 ± 1.13
ZnO	0.75	106 ± 9[6]	12.40 ± 0.37[7]	5.42 ± 0.90	173.26 ± 42.84	367.01 ± 47.98	7.63 ± 2.74
	1.00	76 ± 7[6]	10.39 ± 1.18[6]	4.11 ± 0.23	321.08 ± 91.95	526.66 ± 55.84	5.46 ± 1.74

[1] Wistar rats averaging 58 gm in weight initially.
[2] Figures represent mean ± standard error.
[3] Each figure is the mean of 4 animals unless otherwise indicated.
[4] Optical density change at 550 mμ.
[5] Mean of 16 animals.
[6] Mean of 15 animals.
[7] Mean of 8 animals.
[8] Mean of 6 animals.
[9] Mean of 7 animals.
[10] Mean of three animals.

TABLE 2

Effects of extra vitamins on zinc-fed rats[1,2]

Diet	Weight gain at 5 weeks[3]	Hemoglobin[3]	Liver constituents			Heart cytochrome oxidase[6]
			Cu[4]	Fe[4]	Zn[5]	
	gm	gm/100 ml blood	μg/gm dry weight			AOD/min/mg protein
Control	145±8	12.58±0.46	15.60±0.57	405.70±43.91	37.21±3.26	10.[illegible]±3.43
Control+extra vitamins[7]	146±7	13.13±0.28	16.61±0.70	470.05±75.62	37.89±3.73	11.42±1.28
Control+0.75% Zn	114±6	10.62±0.69[8]	5.05±0.56	321.89±26.37	322.[illegible]±20.10	7.10±1.02
Control+0.75% Zn+extra vitamins	125±8	11.26±0.35	6.41±0.59	281.59±28.75	325.29±20.30	5.48±0.47
Control+3.0% Zn	91±6[9]	12.52±0.63	5.14±0.47	290.82±45.84	510.10±80.41	5.60±0.64
Control+3.0% Zn+extra vitamins	103±5	10.93±0.[illegible]	5.02±0.20	320.68±26.16	453.04±46.40	5.70±0.74

[1] Sprague Dawley rats averaging 60 gm in weight initially.
[2] Figures represent mean ± standard error.
[3] Each figure is the mean of 10 animals unless otherwise indicated.
[4] Each figure is the mean of 13 animals in the control treatment. Mean of 8 animals in all other treatments.
[5] Mean of 10 animals in the control treatment. Mean of 8 animals in all other treatments.
[6] Optical density change at 550 mμ.
[7] Each figure is the mean of 8 animals.
[8] Including extra vitamin B_{12} + 500 μgm folic acid + 0.15% ascorbic acid.
[9] Mean of 10 animals.
[10] Mean of 33 animals.
[11] Mean of 35 animals.

TABLE 3

Response of zinc-fed rats[1] to supplements of liver extract and liver extract ash[2]

Diet	Weight gain at 5 weeks[3]	Hemoglobin[3]	Liver constituents[4]			Heart cytochrome oxidase[5,6]
			Cu	Fe	Zn	
	gm	gm/100 ml blood	μg/gm dry weight			AOD/min/mg protein
Control	196±7	13.28±0.17	12.91±0.44	332.87±37.00	36.13±3.27	6.55±0.49
Control+0.75% Zn	128±10[7]	7.36±0.36[7]	4.93±0.48	118.46±7.22	334.26±23.47	4.43±0.42
Control+0.75% Zn+10% liver extract	163±9	11.14±0.32	6.52±0.41	209.32±12.40	294.86±17.59	3.77±0.29
Control+0.75% Zn+liver extract ash[8]	87±6[9]	8.64±0.25[9]	5.45±0.42[10]	110.84±5.56[10]	402.93±35.75[10]	4.16±0.71[11]

[1] Sprague Dawley rats averaging 58 gm in weight initially.
[2] Figures represent mean ± standard error.
[3] Each figure is the mean of 16 animals unless otherwise indicated.
[4] Each figure is the mean of 13 animals unless otherwise indicated.
[5] Optical density change at 550 mμ.
[6] Each figure is the mean of 9 animals unless otherwise indicated.
[7] Mean of 14 animals.
[8] Equivalent to 10% liver extract.
[9] Mean of 12 animals.
[10] Mean of 10 animals.
[11] Mean of 6 animals.

copper and a further reduction in cytochrome oxidase activity.

The ash of the liver extract caused an additional reduction in growth when added to the zinc diet (table 3). There was a significant effect from the ash on hemoglobin level, but no significant effect on either liver copper, or liver iron or on the heart cytochrome oxidase activity. The failure of the ash to significantly increase the concentration of copper and iron in the livers was unexpected in view of the increases obtained with equivalent amounts of unashed liver extract which contained 77 µg and 681 µg per gm. of dry weight of copper and iron respectively. Presumably, the oxides of these metals, the form probably prevailing in the ash, were poorly utilized. This possibility seems reasonable in view of the fact that iron oxide has been reported to be poorly utilized by the chick (Livchjern and Hart '39), the rat (Mitchell and Schmidt, '26), and the pig (Hart et al., '30).

The results of this experiment suggest that the factor(s) in liver extract capable of alleviating substantial growth is primarily organic in nature.

Experiment 4. In the observed as previously shown, there was a marked decrease in the iron content of the livers of the rats fed zinc as compared with that of the controls. Thus there is an indication that zinc was also affecting iron metabolism in some manner. The possibilities are that (1) zinc has a direct effect on iron metabolism, and (2) the adverse effect on iron metabolism is a result of the effect of zinc toxicity on copper metabolism. An experiment was designed to test these two hypotheses by studying the effects of adding copper and iron supplements to the zinc diet. As shown by the data in table 4, a level of 0.05% of copper only partially [illegible] the hemoglobin level of zinc-fed rats. A level of 0.04% of iron effected the same [illegible] increase. When both minerals were added to the diet of zinc-fed rats the hemoglobin levels equaled those of control rats. The [illegible] of copper were [illegible] the liver copper to [illegible] normals, whereas [illegible] completely [illegible]

TABLE 4

Response of zinc-fed rats to supplements of copper and iron[a]

Diet	Weight gain	Hemoglobin	Liver concentration Cu	Liver concentration Fe	Liver concentration Zn	Heart cytochrome oxidase
	gm	gm/100 ml blood	µg/gm dry weight			[illegible]
Control	104±9	13.09±0.18	12.80±0.58	344.54±47.26	38.72±[illegible]	6.0[illegible]
Control+0.75% Zn	110±04	6.97±0.39[b]	4.20±0.51	121.76±8.18	341.11±20.07	4.8[illegible]
Control+0.75% Zn+0.05% Cu	110±9	10.31±0.37	18.35±0.90	108.00±0.00	349.68±21.03	8.10±0.07
Control+0.75% Zn+0.04% Fe	116±8	10.31±0.29	4.84±0.33	107.82±16.08	350.04±27.64	4.89±0.49
Control+0.75% Zn+0.05% Cu+0.04% Fe	97±10	12.98±0.38	20.20±2.50	179.00±7.75	408.62±24.63	7.36±0.72

[a] Sprague-Dawley rats averaging 53 gm in weight initially.
[b] Figures represent mean ± standard error.
[c] Each figure is the mean of 12 animals unless otherwise indicated.
[d] Each figure is the mean of 10 animals.
[e] Optical density change at 550 mµ.
[f] Each figure is the mean of 6 animals.

liver iron. Supplemental copper did not increase the concentration of iron in the liver, but actually was associated with a further reduction. Iron had no effect on copper accumulation in the liver. These results would tend to support the hypothesis that zinc is directly interfering with iron metabolism. Grant-Frost and Underwood ('58) have suggested that a deficiency of some dietary essential, in addition to copper, affecting hematopoiesis is occurring as a consequence of the high zinc intakes. The results of this experiment indicate that the additional dietary essential involved may be iron.

The effect of added copper on the cytochrome oxidase activity in the zinc-fed rat is similar to that previously reported by Duncan et al. ('53) and Van Reen ('53). Iron would not be expected to have any effect on cytochrome oxidase activity in view of the results of several investigators (Cohen and Elvehjem, '34, Schultze, '39; Gubler et al., '57) who have shown that cytochrome oxidase activity is not influenced by iron deficiency but is markedly reduced in copper deficiency.

Experiment 5. Since supplemental vitamins were not as effective in preventing subnormal growth as the liver extract, it is possible that the adverse effect of zinc on growth is being mediated through the inactivation of some other essential factor(s). Potter and Dubois ('48) showed that zinc was one of the compounds which was capable of inhibiting succinic dehydrogenase by its ability to react with the SH groups of the enzyme. Barron and Kalnitsky ('47) showed that a linkage between zinc and the -SH groups of succinoxidase resulted in an inactivation of the enzyme. Kunkel ('51) confirmed the inhibition of succinoxidase by zinc and also found that zinc inhibited the oxidation of α-ketoglutarate and citrate. Thus, it may be reasoned that the inactivation of metabolically important sulfhydryl groups by zinc is involved and that the sulfhydryl compounds in liver extract might be responsible, in part, for the alleviating effect observed.

With this possibility in mind an experiment was designed testing two sources of protein, casein and egg albumen, in combination with [illegible] levels of copper, [illegible] of iron, and various levels of vitamin B[illegible], folic acid and ascorbic acid. Two additional test diets were included. One contained 1% of cysteine; the other contained 5% of liver extract. The results of this experiment (table 5) indicate that there was apparently no difference between the treatments across the casein diets and the corresponding treatments across the egg albumen diets. The addition of 1% of cysteine was also without effect. The failure of egg albumen and cysteine to alleviate the subnormal growth of zinc-fed rats would suggest that the sulfhydryl groups *per se* present in the liver extract were not the active factor. The results on growth suggest that the amounts of copper and the iron supplements used in this experiment may have approached toxic levels. It is also apparent that the addition of the extra vitamins, in combination with the copper and the iron supplements, was not beneficial in alleviating the subnormal growth. However, a level of 5% of liver extract seems to be sufficient to alleviate the subnormal growth of the zinc-fed rats since the mean weight gain of the rats receiving this treatment was not significantly different from the mean weight gain of the control animals.

The improvement in the hemoglobin levels of the zinc-fed rats supplemented with the other ingredients was probably due to the presence of copper and iron.

The data indicated that increasing levels of vitamins added to the diets containing casein were associated with slight increases in the levels of copper in the livers of the rats. However, when the level of the vitamin supplement was increased, there appeared to be a corresponding decrease in the level of iron in the liver.

The rats fed all supplemented diets, except those containing zinc only, had cytochrome oxidase activities equal to or greater than the controls. The ability of 5% of supplemental liver extract to overcome the depression in cytochrome oxidase activity was unexpected in view of the results of experiment 3 (table 3) which showed that 10% of liver extract had no beneficial effect on the enzyme activity. The reason for this difference is not apparent.

[illegible] National Biochemicals Corporation, Cleveland.

TABLE 5

Response of zinc-fed rats[1] fed various supplements[2]

Diet	Weight gain at 5 weeks[3]	Hemoglobin[3]	Liver constituents[4]			Heart cytochrome oxidase[5,6]
			Cu	Fe	Zn	
	gm	*gm/100 ml blood*	*μg/gm dry weight*			*ΔOD/min/mg protein*
Basal, casein	220±5	13.14±0.53	11.47±0.41	339.54±43.44	26.14± 1.42	14.05±1.46
Basal, casein+0.75% Zn	150±7	6.73±0.34	3.64±0.22	137.76±14.77	364.74±32.43	8.05±0.81
Basal, casein+0.75% Zn+0.02% Cu+0.03% Fe+10× vitamins[7]	120±8	12.42±0.31	15.42±0.99	303.33±52.35	464.04±35.24	23.20±2.44
Basal, casein+0.75% Zn+0.02% Cu+0.03% Fe+100× vitamins[8]	130±8	12.00±0.35	18.72±2.13	244.95±26.79	434.14±46.82	22.66±4.02
Basal, casein+0.75% Zn+0.02% Cu+0.03% Fe+500× vitamins[9]	140±8[10]	11.84±0.43[10]	18.69±1.25	208.98±15.28	419.70±16.30	16.40±1.59
Basal, casein+0.75% Zn+0.02% Cu+0.03% Fe+500× vitamins+1% cystine	122±11	11.82±0.36	16.08±0.42	289.88±10.62	359.55±37.28	15.99±2.73
Basal, casein+0.75% Zn+1% lime	198±6[10]	10.35±0.40[10]	6.80±0.53	146.39±11.43	299.27±21.07	12.44±1.12
Basal, egg albumen	210±7	12.96±0.20	12.38±1.30	261.55±16.37	31.44± 1.43	14.48±3.69
Basal, egg albumen+0.75% Zn	135±6	6.24±0.45	3.70±0.82	143.60±32.12	338.05±16.85	10.43±1.48
Basal, egg albumen+0.75% Zn+0.02% Cu+0.03% Fe+500× vitamins	157±9	12.42±0.27	14.52±1.49	142.58±17.97	273.20±34.83	10.02±4.78

[1] Sprague Dawley rats averaging 80 gm in weight initially.
[2] Figures represent mean ± standard error.
[3] Each figure is the mean of 8 animals unless otherwise indicated.
[4] Each figure is the mean of 4 animals.
[5] Optical density change at 550 mμ.
[6] Each figure is the mean of three animals.
[7] Included 0.2 ppm vitamin B_{12}+10 ppm folic acid+0.1% ascorbic acid.
[8] Included 2 ppm vitamin B_{12}+100 ppm folic acid+0.1% ascorbic acid.
[9] Included 10 ppm vitamin B_{12}+500 ppm folic acid+0.1% ascorbic acid.
[10] Mean of 7 animals.

Rat assay experiment. In a further attempt to isolate the active factor(s) in liver extract, the materials obtained from the solvent fractionation procedure were incorporated into a rat assay experiment. The data, table 6, indicate that the active factor(s) in liver cannot be extracted with acetone since the mean weight gain of the rats consuming the zinc diet supplemented with the acetone fraction was not significantly different from that of the rats consuming the zinc diet. Also, the mean weight gain of the rats consuming the zinc diet containing 5% of acetone-extracted liver was not significantly different from that of the rats fed the zinc diet supplemented with 5% of liver. The mean weight gain of the rats fed both of these diets was significantly different ($P \leq 0.01$) than that of the rats fed only zinc. The data also suggest that the active principle is soluble in methanol since the rats consuming the zinc diet supplemented with the methanol fraction had a mean weight gain significantly greater ($P \leq 0.01$) than that of the rats consuming only zinc.

Radiocopper experiment. In the experiment with radiocopper, approximately 96 and 98% of the activity recovered was present in the gastrointestinal tracts and the feces of the control and the zinc-fed rats, respectively. The difference between these two values was not significant. Thus, it appears that zinc had little effect on the absorption of copper. Table 7 shows the effect of zinc on the distribution of the absorbed radiocopper in some tissues and urine. There was a significant increase ($P \leq 0.05$) in the amount of activity in the urine of the zinc-fed rats as compared with the activity of the urine of the controls. There was approximately a 20% decrease in the activities of the livers of the zinc-fed rats which was a significant change ($P \leq 0.05$) when compared with the controls. The addition of zinc to the diet caused no significant changes in the uptake of radiocopper by the heart, kidney and spleen. Thus, it appears that high intakes of zinc interfere with the utilization of copper and cause it to be excreted via the urine.

Radioiron experiment. In the experiment with radioiron, approximately 97 and 98% of the recovered activity was present in the gastrointestinal tracts and the feces of the control and the zinc rats, respectively. These two values were not signifi-

TABLE 6

Response of zinc-fed rats to test materials obtained by extraction procedures[1]

Diet treatment	Mean weight gain at 5 weeks
	gm
Control	225 ± 5
Control + 0.75% Zn	106 ± 11
Control + 0.75% Zn + 5% liver extract	170 ± 8
Control + 0.75% Zn + 5% acetone-extracted liver	168 ± 7
Control + 0.75% Zn + acetone fraction (≈ 5% liver)	121 ± 10
Control + 0.75% Zn + 0.1% "compound R"	111 ± 5
Control + 0.75% Zn + methanol fraction (≈ 5% liver)	151 ± 6

[1] Each figure is the mean and the standard error of the mean of 10 rats.

TABLE 7

Effect of zinc on the distribution of absorbed radioactive Cu^{64}

Tissue or urine	Distribution of radioactivity[1]	
	Control rats	Zinc-fed rats
	%	%
Heart	1.0 ± 0.2	1.1 ± 0.2
Spleen	[illegible]	2.2 ± 0.4
Kidneys	17.0 ± [illegible]	12.4 ± 1.8
Liver	[illegible] ± [illegible]	37.0 ± [illegible]
Urine	[illegible]	[illegible]

[1] Each figure [illegible] the mean calculated from 4 rats.

TABLE 8

Effect of zinc on the distribution of absorbed radioactive Fe^{59}

Tissue or urine	Distribution of radioactivity	
	Control rats[1]	Zinc-fed rats[2]
	%	%
Heart	3.8±0.1	2.1±0.5
Spleen	14.0±0.0	10.3±1.7
Kidneys	5.4±0.4	3.5±0.3
Liver	65.0±0.9	76.5±2.8
Femurs	10.0±0.7	6.1±0.7
Urine	1.2±0.3	0.9±0.1

[1] Each figure represents the mean and the standard error of the mean calculated from three rats. One rat died while the radioactive iron was being administered.

[2] Each figure represents the mean and the standard error of the mean calculated from 4 rats.

cantly different from each other. This would suggest that zinc had little effect on the absorption of iron. The summary of the results of this experiment (table 8) reveals that zinc caused significant decreases ($P \leq 0.05$) in the uptake of radioiron by the heart and the femurs. There was a significant increase ($P \leq 0.05$) in the radioactivities of the livers of the zinc-fed rats. While these results give some indication that zinc may be interfering with the utilization of iron, it is evident that a complete picture has not been attained. There is the possibility that 48 hours was not enough time for equilibrium conditions to become established among the various iron compartments of the body. Unfortunately, radioactivity data on the blood was not taken.

SUMMARY

Young rats were fed purified diets containing high levels of zinc (0.75 and 1.0%) in the presence and absence of various combinations of copper, iron, vitamin and liver extract supplements. Under these conditions effects of zinc toxicity on growth, hemoglobin formation, liver copper and iron levels, and heart cytochrome oxidase activity were observed in several experiments.

The factor(s) of liver extract that alleviates the subnormal growth of zinc-fed rats appears to reside primarily in the organic portion of the extract. There is an indication that certain vitamins, such as vitamin B_{12} and folic acid, may be involved. The data suggest that the active principle can be extracted from the liver with methanol.

In addition to supporting the accepted hypothesis that zinc interferes with copper metabolism, results of this study indicate that zinc directly interferes with iron metabolism.

Supplements of iron had no apparent effect on the heart cytochrome oxidase activities of rats on a high zinc diet indicating that copper is the important factor connected with restoration of the enzyme activity.

Results of an isotope experiment suggest that zinc interferes with copper metabolism by decreasing the utilization and increasing the excretion of copper in the rat, but apparently has little effect on the absorption of copper.

Results of another isotope experiment indicate that zinc does not interfere with the absorption of iron, but interferes, in some manner, with the utilization of iron.

Throughout this entire study, the addition of zinc to the diet resulted in a marked increase in liver zinc. None of the dietary components tested counteracted this tremendous increase in the accumulation of zinc in the liver.

ACKNOWLEDGMENTS

The authors wish to acknowledge the technical assistance of Mrs. C. R. Bunn, Mrs. V. W. Smart, and E. L. Thomason.

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JOUR. NUTRITION 74 (MAY): 51-57.

Effect of Source and Level of Dietary Protein on the Toxicity of Zinc to the Rat[1,2]

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The interactions of zinc with other dietary constituents are especially important in a study of zinc toxicity. It appears that no particular symptom may be specifically attributed to the zinc alone, but that the toxicity syndrome is observed as deficiency symptoms of several other nutrients.

Early studies with zinc by Sutton and Nelson ('38) and Smith and Larson ('46) indicated that the hypochromic microcytic anemia caused by feeding rats a relatively high concentration of zinc was actually the result of impaired utilization of iron for hemoglobin formation and this condition was corrected by the addition of copper salts to the diet. Later studies by Van Reen ('53) and Duncan et al. ('53) have extended this zinc-copper-iron interrelationship to include the iron-containing enzymes, catalase and cytochrome oxidase. These enzymes decrease in activity with high levels of dietary zinc and return to normal or even higher-than-normal values when copper salts are added above the required level.

Excessive dietary zinc causes a decrease in the concentration of copper in the liver of the rat and a recent study reported by Cox and Harris ('60) indicates that there is also a decrease in the concentration of iron in the livers of rats fed zinc at a high level. From these results it was suggested that it was this decrease in iron, and not copper, which is responsible for the anemia and reduction in activity of the iron containing enzymes. Grant-Frost and Underwood ('58) have stated that a high zinc intake may produce a deficiency of dietary components other than copper and iron necessary for normal hematopoiesis. Further support is added to this suggestion by the ability of animals to consume much larger concentrations of zinc than of many other metallic elements.

Still another dietary interaction involving zinc and a source of protein was reported by O'Dell and Savage ('60) and Kratzer and others ('59). They observed that chickens and turkeys fed isolated soybean protein as a source of protein showed a higher zinc requirement than those fed protein from casein or other sources. In the former work evidence was presented to show that the phytic acid in the soybean oil meal was responsible for this effect. In the work by Kratzer and others the zinc requirement was reduced by autoclaving the soybean protein or by the addition of EDTA (ethylenediaminetetraacetic acid) to the ration. This effect was explained on the basis that the zinc bound to the soybean protein and unavailable to the poults became available when the protein was denatured or when the zinc-EDTA chelate was formed.

The study reported here was undertaken to measure the effect of dietary source of protein, casein or soybean oil meal, and dietary level of protein, 20 or 30%, on rats fed zinc at a high level. Factors considered were final weights, hemoglobin values, liver accumulation of total copper, iron and zinc and liver deposition of copper[64] and zinc[65] from a single oral dose containing these radioactive elements.

Received for publication December 19, 1960.

[1] This investigation was supported in part by Public Health Grant H-1318.

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TABLE 1

Weight gains and hemoglobin values for rats fed normal and high levels of zinc with casein and soybean oil meal diets

Added Zn, ppm	Protein source and level															
	20% protein								30% protein							
	Casein						Soybean oil meal				Casein			Soybean oil meal		
	0	2500	5000	7000	5000[1]	5000[2]	0	2500	5000	0	2500	5000	0	2500	5000
Av. weight, gm																
Males	289	282	180	148[3]	272	216	310	298	263[3]	320	313	213	281	290	283[3]
Females	198	200	161	114[4]	179	139	202	194	198[5]	250	204	185	203	186	198[5]
Av. Hb., gm/100 ml																
Males	13.4	11.5	7.3	5.7[3]	11.9	13.9	14.2	13.6	12.4[6]	14.5	13.6	6.0	14.2	14.1	13.3[6]
Females	13.6	12.3	7.7	5.7[6]	13.5	13.5	13.9	13.9	12.8[6]	14.4	13.7	10.0	14.0	13.9	14.1[6]

[1] 500 ppm Cu added as $Cu_3(PO_4)_2$.
[2] 500 ppm Cu + 1000 ppm Fe added as $Cu_3(PO_4)_2$ and $Fe_2(SO_4)_3$.
[3] Two died before experiment terminated.
[4] One died before experiment terminated.
[5] Significantly heavier than corresponding groups receiving casein, ($P < 0.01$).
[6] Significantly higher than corresponding groups receiving casein, ($P < 0.01$).

EXPERIMENTAL

The experimental design is shown in table 1. The basal diets were composed of casein or soybean oil meal (50% protein); sucrose; 4% of mineral mix (U.S.P. XIV, '55); 2.2% of vitamin mix;[4] and 4% of cottonseed oil.[5] The casein or soybean oil meal and the sucrose in each ration were varied to give the 20 or 30% protein level required. Three per cent of purified cellulose was added to the casein rations. Zinc was added as zinc carbonate, copper as cupric phosphate and iron as ferrous sulphate.

The radioactive copper was received from Oak Ridge as cupric nitrate. This was converted and administered to the animals as cupric chloride. The radioactive zinc received from Oak Ridge[6] as zinc chloride was used in this form.

One-hundred-eighty piebald rats of the Long Evans strain, raised in our laboratory, weighing about 50 gm each, were divided into 15 groups each containing 6 males and 6 females. These animals were fed the rations ad libitum during the time of the trial. After all the animals had received the diets for 8 weeks, two males and two females from each group were given an oral dose of about 1 ml containing 250 μc of copper[64] and 2 μc of zinc[65]. Twenty-four hours later the animals were weighed, killed, hemoglobin values measured, and the livers were taken for analysis of the radioactive and total copper and zinc and total iron. The remaining 120 rats (60 males and 60 females) were killed 5 days after the first group, body weights and hemoglobin measured and the livers taken for analysis of total zinc, iron and copper.

For the copper[64] and zinc[65] assay the livers were dried under infrared lamps, pre-ashed in beakers over Bunsen burners

[4] Vitamin mix included: (in mg/100 gm of ration) α-tocopherol, 11; ascorbic acid, 100; inositol, 11; choline chloride, 165; menadione, 5; p-aminobenzoic acid, 11; niacin, 10; riboflavin, 2.2; pyridoxine·HCl, 2.2; thiamine·HCl, 2.2; Ca pantothenate, 6.6; biotin, 0.044; folic acid, 0.2; vitamin B_{12}, 0.003; also (in I.U.) vitamin A, 1980 and vitamin D, 220.

[5] Wesson Oil, The Wesson Oil Company, New Orleans, Louisiana.

[6] Oak Ridge National Laboratory, Oak Ridge, Tennessee.

and then ashed for 4 hours in a muffle furnace at 500°C. After ashing, the livers were broken up in the beakers with glass stirring rods, transferred to 5-ml glass beakers and the radioactivity measured with a thin window Geiger-Mueller tube. The activity of both the copper^{64} and the zinc^{65} was measured within 12 hours after sacrifice, and the activity of the zinc^{65} alone was measured again a week later when all the copper^{64} had decayed to a point that there were no detectable counts in the standard. The activity of the copper^{64} was determined by difference. Total copper and zinc was determined by the method of McCall et al. ('58) and total iron by the phenanthroline method as described by Sandell ('50). Hemoglobin was determined by the acid hematin method. The analytical data were tested by analysis of variance as described by Snedecor ('59).

RESULTS AND DISCUSSION

The first visible symptom of zinc toxicity in the rat was a graying or fading of the dark areas of the haircoat. This was not a general overall fading but occurred in a definite, individual pattern for each animal and was more pronounced over the head and across the nose. At one stage there was a similarity in appearance between the faces of the affected rats and the familiar masks of the raccoon. As the severity of the toxicity continued the dark haircoats became silvery-brown in appearance and finally changed to the dull, yellowish-brown color so common in anemia. In advanced stages of zinc toxicity, alopecia was observed.

During the third week of treatment the first signs of a haircoat change occurred in the animals receiving 20% of casein with 5000 and 7000 ppm of added zinc. A similar fading of haircoat began to appear in those animals receiving 30% of casein with 5000 ppm of added zinc and those receiving the 20% casein diets with 2500 ppm of added zinc during the 4th and 5th weeks of the experiment. The first signs of fading occurred in the animals receiving 30% of casein with 2500 ppm of added zinc during the 6th week of the experiment. The last indication of a change in haircoat occurred during the 8th week of the experiment with those animals receiving 20% of soybean oil meal with 5000 ppm of added zinc. No change in haircoat was observed in animals receiving the 20% casein diet with 5000 ppm of added zinc plus the copper, or the added zinc plus copper and iron; and there was no sign in those animals receiving the 20% soybean diet with the zero or 2500 ppm of added zinc, or any of the animals receiving the 30% soybean diet.

The addition of 5000 ppm of zinc with or without the added copper and iron and the addition of 7000 ppm of zinc to the 20% casein diets restricted the rate of gain of the animals from the beginning of the experiment, although the animals that received added copper and added copper plus iron appeared normal in all other respects. Those animals receiving 30% of casein with 5000 ppm of added zinc grew normally for the first month and then lost weight until the end of the experiment. The average weights at time of sacrifice are shown in table 1.

The average hemoglobin values for the animals used in this study (table 1) followed the same general pattern as the weights for each treatment except for those groups receiving the added copper and iron. No definite numerical values were assigned to the appearance of the animals but in each instance appearance paralleled the hemoglobin values. Measurement of hemoglobin was made only at the time of sacrifice; however, the animals receiving 30% casein diets with 2500 ppm of added zinc and 20% of soybean with 5000 ppm of added zinc showed definite signs of achromotrichia with a normal hemoglobin range. This would indicate that the drop in hemoglobin value occurs sometime after the visible signs of zinc toxicity.

The average values for the percentage dose of copper^{64} and zinc^{65} observed in the livers of those rats receiving the radioactivity are shown in table 2. Little decrease occurred in the absorption of either copper^{64} or zinc^{65} under the conditions of this experiment. Interpretation of these data was difficult owing to the variability of the individual results and the small number of animals used for the radioactive study. It was not possible to use

TABLE 2

Liver deposition of an oral dose of Cu^{64} and Zn^{65} in rats fed normal and high levels of zinc with casein and soybean oil meal diets

	Protein source and level														
	20% Protein									30% Protein					
	Casein						Soybean oil meal			Casein			Soybean oil meal		
Added Zn, ppm	0	2500	5000	7000	5000[1]	5000[2]	0	2500	5000	0	2500	5000	0	2500	5000
% Dose CuC_4															
Males	2.6	1.1	3.1	7.0	0.4	0.1	1.3	0.2	0.4	1.4	5.9	0.1	3.9	1.2	1.2
Females	2.1	0.9	0.2	1.1	1.4	4.3	3.8	1.1	0.7	2.6	2.6	1.1	4.3	0.8	5.1
% Dose Zn^{65}															
Males	1.8	0.5	0.4	0.6	0.5	1.7	3.4	0.4	3.2	1.4	2.5	1.6	3.2	0.2	0.3
Females	3.4	1.1	0.4	2.9	4.4	5.1	1.6	0.4	2.2	1.4	0.7	2.3	2.1	0.7	0.4

[1] 500 ppm Cu, as $Cu_3(PO_4)_2$, added.
[2] 500 ppm Cu, as $Cu_3(PO_4)_2$, + 1000 ppm Fe, as $Fe_2(SO_4)_3$, added.

TABLE 3

Accumulation of total copper, zinc and iron in livers of rats fed normal or high levels of zinc with casein and soybean oil meal diets

	Protein source and level														
	20% Protein									30% Protein					
	Casein						Soybean oil meal			Casein			Soybean oil meal		
Added Zn, ppm	0	2500	5000	7000	5000[1]	5000[2]	0	2500	5000	0	2500	5000	0	2500	5000
Av. Cu[5]															
Males	33.0	14.0	17.0	12.5	16.0	24.0	27.0	24.5	16.0	22.5	22.5	15.5	28.5	22.5	17.5
Females	39.0	34.0	22.0	14.5	35.5	54.0	44.5	41.0	29.5	47.5	27.5	21.5	44.5	37.0	35.0
Av. zinc[5]															
Males	29.0	49.5	114.0	204	63.0	82.0	26.5	33.0[3]	75.0[3]	23.5	70.0	62.0	31.0	33.5[3]	57.0[3]
Females	22.0	69.0	164.0	255	165.0	196.0	29.5	31.5[3]	133.0[3]	27.0	87.5	129.0	37.0	34.0[3]	89.0[3]
Av. iron[5]															
Males	166	78[4]	82[4]	68[4]	47[4]	137[4]	112	106[4]	109[4]	126	75.0[4]	140[4]	115	105[4]	69[4]
Females	288	200[4]	134[4]	83[4]	87[4]	182[4]	237	161[4]	157[4]	245	121[4]	117[4]	219	190[4]	109[4]

[1] 500 ppm Cu, as $Cu_3(PO_4)_2$, added.
[2] 500 ppm Cu, as $Cu_3(PO_4)_2$, added, plus 1000 ppm Fe, as $Fe_2(SO_4)_3$, added.
[3] Significantly less than corresponding groups receiving casein, ($P < 0.05$).
[4] Fe in treated groups. Significantly less than control groups, ($P < 0.05$).
[5] Micrograms/gram of fresh weight of tissue.

large numbers because of the small amount of Cu^{64} available as a result of its short half-life, 13 hours.

Values showing the concentration of total copper, zinc and iron in the livers of the rats are given in table 3. Although a slight decrease was observed in the concentration of copper in the livers of the rats receiving the high-zinc diets this difference was not significant and probably did not produce the anemia which occurred in the animals. There is a decrease in liver copper concentration in all of the animals receiving high-zinc diets, even in those that received the soybean oil meal diets and showed no signs of zinc toxicity.

The concentration of zinc in the livers of both the males and the females increased as the dietary level of zinc increased, although significantly less zinc accumulated in the livers of those animals receiving the soybean oil meal rations. Both males and females receiving the 30% casein diet with 5000 ppm of added zinc had less accumulation of zinc in the liver than those receiving the 20% of casein with the 5000 ppm of zinc with added copper and iron. These results are unusual in that those animals receiving the 30% casein diets showed severe symptoms of zinc toxicity with low hemogloblin values, whereas those fed 20% of casein with the added copper and iron had normal hemoglobin levels and exhibited no particular signs of zinc toxicity.

A decrease was observed in the iron concentration in the livers of both the males and females for all groups corresponding to the increase in zinc ingested, including a slight decrease in that group receiving the added iron in the diet. The decrease in iron concentration in the animals receiving the soybean oil meal diets without any sign of zinc toxicity was equal to the decrease of iron in the animals receiving the casein diets, which had low hemoglobin values and other signs of severe zinc toxicity.

The results of zinc toxicity in the rat, including the hypochromic microcytic anemia, poor growth, reduction in liver catalase, and cytochrome oxidase, as well as the fading in haircoat color, have been explained previously on the basis that the increased level of zinc blocks the absorption or the utilization of copper, or of iron. In the reports, all of the symptoms of zinc toxicity were eliminated by the addition of copper to the diets, and in later work, Cox and Harris ('60) showed that the addition of iron to the diet had the same effect. In the present study the addition of either copper or copper and iron to the diets of the animals receiving the toxic level of zinc, increased the hemoglobin to the normal range and eliminated all other signs of zinc toxicity except the poor growth. It does not seem possible, however, that the small differences in the total copper, zinc, or iron content of the livers of these animals are enough to account for these changes.

The data obtained from this study suggest that the conditions arising from ingestion of a toxic level of zinc result from interactions with zinc and protein and which also involved other mineral elements. Zinc, copper and iron form stable chelates with amino acids and protein fragments (Chaberek and Martell, '59) and it is possible that the excess zinc could tie up enough of a required protein compound to impair the utilization of copper and iron. This would agree with the findings of Pal and Christensen ('59) that there is a strong possibility that metal chelates act as carrier or parts of a carrier for amino acid transport.

The limiting action of zinc under these conditions would have to be due to the combination of zinc with a specific compound within the protein supplement (soybean oil meal) and not just any chelate. The work of Kratzer and others ('59) showed that soybean protein increased the requirement of the chick for zinc and the addition of EDTA to this ration apparently made the zinc more available to the chick. Zinc and EDTA, in solutions at a physiological pH, form a very stable chelate. Perry and Perry ('59) have shown that the injection of EDTA changes the normal pathway of excretion of zinc increasing the urinary excretion of zinc by 10 times.

If the protein source makes this much difference on the availability of zinc, copper and iron, then it might be expected that the difference was effected by the

amino acid concentrations in the different proteins. A comparison of the amino acid content of soybean meal (Kuiken et al., '49) and that of casein (Almquist, '54) shows that, with the exception of arginine, these two protein sources have approximately the same amount of the essential amino acids. Gurd and Goodman ('52) have stated that zinc is tightly bound to the imidazole group of histidine which suggests the possibility that a histidine-zinc chelate may account for the differences. In soybean meal and casein, however, the histidine content is essentially the same. Van Etten and co-workers ('59) separated soybean protein into the soluble heat-coagulated and acid-precipitated fractions and found that the amino acid composition of these different fractions was essentially the same.

These considerations suggest the possibility that a structural feature in the protein is responsible for this difference in metabolic handling of metallic elements. The stability of a mineral-amino acid chelate depends to a large extent upon the relative position of the amino groups. From the data presented here it is proposed that specific protein compounds in soybean oil meal form stable chelates with zinc which decrease the accumulation of zinc in the livers when otherwise toxic levels are ingested and eliminate the adverse interaction with copper and iron. Utilization of copper and iron and possibly protein transportation within the body appear to be affected by this combination.

SUMMARY

Evidence was presented to show that the amount of zinc required to produce toxic symptoms in the rat and the severity of the symptoms depend upon the source and level of the dietary protein.

Rats fed excessive zinc in 20 or 30% soybean protein diets were significantly heavier, and showed higher hemoglobin levels and less total zinc accumulation in the livers than those animals receiving the same level of protein from casein.

The source of protein had no effect on the percentage of dose of radioactive copper or zinc or the total copper accumulation in the livers.

The addition of 500 ppm of copper and 500 ppm of copper plus 1000 ppm of iron to the diets of rats receiving 5000 ppm of zinc in a 20% casein protein diet increased the hemoglobin level and decreased the total accumulation of zinc in the liver but did not increase growth rate.

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POULTRY SCI. 35:956-958. (1956).

A Comparison of the Growth of Chicks Fed Diets Containing Different Quantities of Zinc

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(Received for publication March 31, 1956)

THE essentiality of zinc in the nutrition of both plants and animals appears to be well established. Zinc is widely distributed and, supposedly, is needed in such small quantity that there is little likelihood of animals suffering from a deficiency. Moreover, there have been a few reports indicating that zinc may have a poisonous or toxic effect when added to the diet of animals.

Insko, Lyons and Martin (1938) reported that, in their experiments with chicks, supplementing a diet containing 0.61% percent of calcium, 0.49 percent of phosphorus, and only 4.5 p.p.m. of manganese with 30 p.p.m., each, of zinc and aluminum caused a marked increase in lameness or perosis. Lyons, Insko and Martin (1938) injected a zinc sulphate solution intraperitoneally into chicks four times weekly, starting when the chicks were five days old and continuing until they were forty-five days old. The total quantities of zinc injected per chick were 6.45 mg., 12.90 mg., and 19.35 mg. They reported that these injections appeared to retard growth. Sampson, Graham and Hester (1942) reported on the feeding of 1,000 p.p.m. of zinc, as zinc lactate, to weanling pigs and to pigs weighing 75 to 100 pounds. The larger pigs showed no ill effects from this high level of zinc, but the weanling pigs developed unthriftiness, lameness, and arthritis.

Other recent experimental work, however, has indicated that additions of zinc to the diets of pigs has been beneficial under certain circumstances.

Tucker and Salmon (1955), found that comparatively high levels of zinc (about 50 and 100 p.p.m.), supplied as zinc carbonate in amounts of 0.01 percent and 0.02 percent of the diets, were beneficial in the diets of pigs as an aid in combating parakeratosis, a skin disease which appeared to develop most readily when high-calcium diets were fed in dry lot. As a result of their findings, which currently are being substantiated in practical feeding operations, there is a demand from feed manufacturers for mineral mixtures containing enough zinc to provide these higher levels in the pig feeds they make. The question has arisen, can such mineral mixtures be used safely in feeds for other classes of livestock? The experiment reported in this paper was set up to ascertain whether such high levels of zinc, and even higher levels, would affect the growth and efficiency of feed utilization of young growing chickens.

EXPERIMENTAL

Two hundred day-old New Hampshire chicks were distributed at random into ten groups in such a way that the live weights of the chicks in any group were as nearly as possible like those in all the other groups. The chicks were kept in all-metal battery brooders for four weeks. The room temperature was held at 75°F. and the relative humidity was maintained at 50 percent. When the chicks were four weeks old, they were transferred to metal developing batteries in another room where the temperature was held at 70°F. and the

TABLE 1.—*Formula of the basal diet*

Ingredient	Percent
Ground yellow corn	61.0
Soybean oil meal (44% protein)	30.8
Fish meal	3.0
Dicalcium phosphate	1.9
Calcite flour	1.68
Riboflavin supplement (B·Y-500[1])	.75
Salt	.45
25% dry mixture choline chloride	.12
Trace-mineral pre-mix (Delamix[2])	.10
Antibiotic and vitamin B_{12} supplement (Pro-Pen "2:3"[3]	.10
Vitamin A supplement (Nopcay "10"[4])	.04
Vitamin E supplement (Myvamix[5])	.0315
Vitamin D_2 supplement (Delsterol[6])	.025
Niacin	.003
Calcium pantothenate	.0005
Total	100.0000[7]

[1] Contains 227 milligrams riboflavin per pound.
[2] Contains 6% manganese, 0.12% iodine, 2% iron, 0.2% copper, and 0.02% cobalt.
[3] Contains 2 grams procaine penicillin and 3 milligrams vitamin B_{12} per pound.
[4] Contains 10,000 I.U. vitamin A per gram.
[5] Contains 20,000 I.U. vitamin E per pound.
[6] Contains 1,500 I.U. vitamin D_2 per gram.
[7] Vitamin K supplement (Klotogen F) added at the rate of 5 grams per ton of feed.

relative humidity was kept at 50 percent. Both battery rooms were artificially lighted for fourteen consecutive hours each day. Distilled water was supplied in galvanized drinking pans throughout an experimental period of nine weeks. A practical-type, corn-soybean meal, all-mash diet was used as the basal diet. The formula of the diet is given in Table 1.

Spectrographic analysis of the basal diet showed that it contained 36 p.p.m. of zinc. This diet was fed to one group of chicks. The diets of the other nine groups were supplemented with zinc oxide. Enough of the zinc oxide was added to the diet of each group to provide a series of diets in which the quantities of total zinc were in a geometrical progression having a ratio of 1.414.

Samples of water were taken from drinking pans like those used for the chicks. The water was allowed to remain in these pans for the average length of time that water remained in the pans used for the chicks, before it was sampled. Analyses indicated that enough zinc dissolved from the surface of the pans to add 3 p.p.m. to the drinking water. If it be assumed that the weight of water consumed is two to three times that of the feed eaten, an additional 6 to 9 p.p.m. of zinc should be added to the levels provided in the feed to get the total quantity of zinc consumed by the chicks. The total consumption of zinc by the chicks that received the maximum level of 814 p.p.m. of zinc in their diet was therefore approximately 820-823 p.p.m.

RESULTS AND DISCUSSION

A summary of the results obtained is presented in Table 2. It is apparent that none of the levels of zinc had an effect on the average live weight of the chicks, or the feed efficiency, to the age of nine weeks. The groups receiving the highest levels of zinc grew as well, and used their feed as efficiently, as did those receiving the lowest levels.

In the work of Insko, Lyons and Martin, already cited, the addition of zinc and aluminum to a diet very low in manga-

TABLE 2.—*Average live weights and feed efficiency to nine weeks of age of chicks receiving graduated levels of zinc in their diet*

Zinc added to the feed	Total zinc in the feed	Total zinc in the diet[1]	Average live weights at nine weeks[2]	Feed efficiency to nine weeks of age[3]
p.p.m.	p.p.m.	p.p.m.	grams	
0	36	42– 45	1,132.4	.382
15	51	57– 60	1,214.8	.382
36	72	78– 81	1,138.8	.383
66	102	108–111	1,115.0	.378
108	144	150–153	1,150.2	.382
168	204	210–213	1,154.9	.384
252	288	294–297	1,225.6	.388
371	407	413–416	1,211.3	.375
540	576	582–585	1,163.5	.383
778	814	820–823	1,195.3	.383

[1] Total zinc in the feed plus 6 to 9 p.p.m. zinc obtained in the drinking water.
[2] Average of the average for the males and the average for the females.
[3] Unit gain in live weight per unit of feed consumed.

nese (4.5 p.p.m.) appeared to cause a high incidence of perosis. The addition of zinc to the diet of the chicks in this experiment, however, did not produce perosis, probably because the manganese content of the diet was much higher (78 p.p.m.) than that of the diet used by Insko and associates.

The results of this experiment indicate that mineral mixtures containing sufficient zinc oxide to provide 100 to 200 p.p.m. added zinc in a manufactured feed for pigs may be used without harm in chick starting-and-growing feeds. They also show that growing chicks can tolerate, without apparent harm, as much as 800 p.p.m. of zinc in their diet.

SUMMARY

An experiment was conducted with young growing New Hampshire chicks to study the effect, on their growth and efficiency of feed utilization, of adding to their diet graduated levels of zinc in the form of zinc oxide. A practical-type, corn-soybean meal, all-mash diet, containing 36 p.p.m. of zinc, was used as the basal diet. The quantity of zinc added to the basal diet ranged from 15 to 778 p.p.m., and the total quantity of zinc consumed in the feed and water ranged from about 42 to about 820 parts per million parts of diet. The growth and efficiency of feed utilization were equally good on all diets, and were not affected by the added zinc.

ACKNOWLEDGMENT

The authors are indebted to A. J. Sutherland of the Lime Crest Research Laboratory, Limestone Products Corporation of America, for the spectrographic analyses.

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THE EFFECT OF SUPPLEMENTARY QUANTITIES OF ZINC ON THE INCREASE IN GROWTH AND THE FEED CONVERSION IN SWINE

S. Milosavljevic, S. Pavlovic, S. Antic

The parakeratosis in swine is not interesting as a skin disease since from the age of four months approximately the changes in the skin generally disappear spontaneously. From the point of view of economics much more interesting are the changes in the growth and in the conversion (utilization) of food. Since before the manifestation of the changes on the skin there occur a reduced growth and weaker feed conversion these are also important for the timely diagnosis of these sicknesses. From the economic point of view the reduced growth and the inadequate conversion of food have a considerable effect on economic factors and often can result in in a situation in which there is doubt as to the profitability of the fodder. For this reason in testing the effect of the various factors of parakeratosis we took as the main criterion for the evaluation of their effect the level of growth and conversion of food.

Our previously published results , as well as data obtained from other authors, showed that under conditions of feeding which lead to disturbance in the metabolism the examined group of swine which received a supplement of zinc in their feed had greater growth and better food conversion. These results led some authors to the assumption that zinc is a specific element of growth. In order to verify this assumption we tested the effect of supplementary amounts of zinc in the food on the increase in growth and on the improvement in food conversion under conditions of feeding which did not lead to the occurrence of parakeratosis in the control groups. We give the results of that study in this paper.

Materials and modus operandi

At the end of 1959 and at the beginning of 1960 two examinations were carried out at the Station for Practical Education and Research of the Veterinary School in Belgrade-Krnjaca. The swine were given the same food with which all of the swines in that station were fed (Table 1). In the first experiment the swines were fed dry food, whereas the swines of the second group got an addition of 10 g and those of the 3rd group an addition of 30 g of $ZnSo_4$ per 100 kilos of feed. The swine of the second experiment got the same basic meal in the wet state, and the swine of the fifth group got 10 g and the swine of the sixth group got 20 g of $ZnSO_4$ per 100 kg. In each group there were 8 swine of the English great white race of swine of the same average age of 2.5 months at the beginning of the experiment. In the forming of the

we proceeded as described in our previous work (10) so that the group would be as similar as possible with respect to age, weight, sex and inheritance succession. All of the swine were examined at the same time. The swine in the first group were given dry food at will. The swine of the second group were fed wet food twice a day and the amount of food was determined according to the appetite shown , i.e. according to the amount of food consumed during 30 minutes. The experiment lasted 3 1/2 months . All of the swine were measured individually once a week in consideration of the total amount of food expended and the expenditure of food per kilo of growth.

Results and discussion

The results of the growth, expenditure and conversion of food are given in Table 2. The figures on the average daily expenditure of food show that in the first experiment the swine of the second and third groups, which received $ZnSO_4$, consumed a daily average of food through the mouth which was less than the swine of the first group which did not get zinc. In the second experiment , in the case of feeding with wet food, likewise the swine which were given zinc consumed a smaller daily amount of food on the average than the swine in the fourth control group. These results show that the addition of zinc reduced the appetite in swine.

The figures on growth (Table 2) show that in the first experiment the greatest average daily growth occurred in the swine of the control group and the swine in the examined groups which received additional amounts of zinc had lesser growth.

The swine of the third group, which received 20 g of zinc sulphate per 100 kilos of feed had less growth than the swine of the second group which received a lower supplementary amount of zinc in their food. In the second experiment the swine of the fifth group had a somewhat greater and those of the sixth group a somewhat smaller growth than the swine of the first control group.

The average amount of food expended per kilogram of growth in the first experiment is almost the same in the first and second group, whereas it is greater in the swine of third group (Table 2). In the second experiment the fifth group had a somewhat lesser and the sixth group a somewhat greater expenditure of food per kilogram of growth than the fourth group. These figures show that the swine of the second and fifth experimental groups with 10 g of zinc added had an insignificantly greater conversion and the swine of the third and sixth experimental groups, with 20 g of zinc added, had a lower food conversion than the swine of the fifth and fourth control groups.

Table 1

1 = composition of the food

2= Experiment

3 First

4 Second

5 Group

6 Barley

7 Corn

8 bran

9 Super protein

10 Whiting

11 So

12 Zinc sulphate

13 Food

14 Dry

15 Wet

Table No. 2 Results of growth, expenditure and conversion of food

1 = Experiment

2 = first

3 = Second

4 = Group

5 = Zinc sulphate

6 = food dry wet

7 = no. of swine in group

8 = Average initial weight

9 = Average final weight

10 = Average daily growth

11 = Average daily expenditure of food

12 = average expenditure of food per kilo of growth

Tabela br. 1. Sastav hrane

Ogled	Prvi			Drugi		
Grupa	I	II	III	IV	VI	V
Prekrupa ječam	40	40	40	40	40	40
Prekrupa kukuruza	33,5	33,5	33,5	33,5	33,5	33,5
Mekinje	15	15	15	15	15	15
Superbelko	10	10	10	10	10	10
Kreda	1	1	1	1	1	1
So	0,5	0,5	0,5	0,5	0,5	0,5
Cink sulfat	—	0,010	0,020	—	0,010	0,020
Hrana	suva			nakvašena		

Tabela br. 2. Rezultati prirasta, utroška i iskorišćavanja hrane

Ogled	Prvi			Drugi		
Grupa	I	II	III	IV	VI	V
Cink sulfat	—	0,010	0,020	—	0,010	0,020
Hrana	suva			nakvašena		
Broj prasadi u grupi	8	8	8	8	8	8
Pros. početna težina	15,120	15,150	15,160	15,190	15,110	15,170
Pros. završna težina	74,060	70,560	70,190	64,940	66,140	57,800
Pros. dnevni prirast	0,556	0,523	0,519	0,469	0,479	0,402
Pros. dnevni utrošak hrane	2,030	1,907	1,942	1,692	1,594	1,474
Pros. utr. hrane za kg prirasta	3,651	3,649	3,740	3,604	3,494	3,665

The results obtained show that the swine of the group which was given additional amounts of zinc sulfate had an appetite which was lower on the average and lesser growth than the swine in the control groups. With regard to the food conversion the groups which received 10 g of zinc sulphate had a somewhat better food conversion and the groups with 20 g of zinc sulphate had a poorer food conversion than the swine of the control group fed the same feed but without the addition of zinc sulphate. The swine of the third and sixth group which feceived 20 g of zinc sulphate had poorer growth and poorer food conversion than not only the swine of the corresponding control groups but also the swine of the second and fourth group which received 10 g of zinc sulphate. This shows that the supplementary addition of zinc sulphate in the food not only does not result in an increase in growth and food conversion but even has a negative effect on them. According to these results and other results which we published earlier, zinc sulphate has a favorable effect on growth and food conversion only under the conditions in which they have been reduced as the result of the appearance of parakeratosis . However, when no conditions are present for the appearance of parakeratosis, the giving of supplementary amounts of zinc sulfate for the purpose of stimulating the fattening process is unwarranted and can even lead to negative results. It should be pointed out that during the course of the entire period of experiments there were no changes in the swine of both... (ends abruptly here..translator's note)

THE EFFECT OF SUPPLEMENTARY QUANTITIES OF ZINC ON THE GROWTH INCREASE AND FEED CONVERSION IN SWINE

S. Milosavljević, S. Pavlović, S. Antić

On the ground of the obtained results can be drawn the following conclusions.

The most groups ot pigs that were given supplementary quantities of zinc-sulphate in their diets, both dry and wet, had reduced appetite and growth unlike those pigs from their corresponding control groups.

The pigs of the experimental group that were given 10 g. zinc-sulphate per 100 kg feed had somewhat better feed conversion while the pigs from the group that were given 20 g. of zinc-sulphate had worse feed conversion than those pigs from their corresponding control groups.

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Arch. Biochem. Biophys. 32:414-23 (1951)

Cation Activation of Desoxyribonuclease

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Received March 20, 1951

Introduction

The activation of desoxyribonuclease by magnesium ions was first reported by Fischer *et al.* in 1941 (1), and since then has been amply confirmed by others (2-6). Manganese was found by McCarty to be equally effective, zinc very much less so, while calcium and ferrous ions were ineffective (4). Previous studies from this laboratory indicated that a wide variety of salts was capable of activating desoxyribonuclease (5). This broad range of activators for the enzyme was questioned by Kunitz in a very interesting paper (6). Kunitz reported that his crystalline desoxyribonuclease in the absence of added Mg^{++} was appreciably activated when quartz vessels were used, and much more activated when Pyrex glass containers were used; in the presence of added magnesium, however, the degree of activation was the same whether quartz or Pyrex glass vessels were employed. Kunitz suggested that both Pyrex glass and quartz might furnish Mg^{++} or Mn^{++} or both as impurities during the enzymatic digestion.

These observations of Kunitz impelled us to re-examine and critically repeat and extend our previous experiments on the cation activation of desoxyribonuclease. In so doing, we have worked with all-Pyrex and with all-quartz vessels, with quartz-distilled water of measured low conductivity, and with spectroscopically—and chemically—analyzed salts. At no time have we ever noted a measurable activation of desoxyribonuclease in either Pyrex or quartz containers in the absence of added salts. We have, moreover, not only confirmed our earlier observations on the activation by calcium, strontium, barium, and zinc, but have made the new observations that cobalt is as potent an activator

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as magnesium or manganese, ferrous ion only a little less so, and nickel and cadmium relatively weaker but appreciable. The conditions under which the various cations are maximally effective may vary from one to the other, and these differences help to explain some of the divergent results in the literature.

Experimental

The sodium salt of desoxyribonucleic acid was prepared from calf thymus by a modified Hammersten procedure. It was further purified by the Chloroform-gel method. The N content was 14.5%, P 8.9%.

Quartz vessels of various types and sizes were purchased from the Hanovia Chemical and Mfg. Co. Spectroscopic analysis of a sample of these containers by the National Bureau of Standards revealed less than 1 part/10,000 of alkali earth metals. Pipets were prepared and calibrated from quartz tubing by Mr. F. Highhouse of this Institute. Ordinary distilled water was distilled twice in a quartz apparatus and stored in quartz containers; the conductivity of this water varied from 1.03 to 1.49×10^{-6} ohm^{-1} $cm.^{-1}$. No attempt was made to keep it CO_2-free. The nucleate was dissolved at 1.0% concentration in this water and dialyzed for 18 hr. in a cellophane bag against frequent changes of this water. The substrate solution was made up fresh every day and kept in a quartz container.

The enzyme was a once-crystallized desoxyribonuclease supplied by the Worthington Biochemical Laboratory.

The salts employed were mostly the chlorides of Mg, Mn, Co, Fe, Ni, Ba, Ca, Sr, Zn, Cd, Pb, Hg, and Cu. All of these salts except Mg, had less than 1 part/10,000 of Mg as measured spectroscopically and by chemical analysis.

The enzyme activity was measured by following the appearance of nonacid-precipitable products of the nucleate [cf. (6)]. The reaction mixture generally consisted of 1 ml. of a 0.1% solution of nucleate, 1 ml. of enzyme solution containing 5 μg. protein, 1 ml. of activator, 1 ml. of either water or buffer solution, plus water to make the total volume 6 ml. During the digestion period 1-ml. aliquots were withdrawn and mixed with 4 ml. of cold 0.25 *N* HCl, centrifuged at 10,500 r.p.m. for 10 min. in a refrigerated centrifuge, and the supernatant was analyzed at 260 mμ in a Beckman spectrophotometer.

Comparative Studies in Quartz and in Pyrex Vessels

Parallel studies were made entirely in quartz vessels and entirely in Pyrex vessels. Digests were first prepared of 1 ml. nucleate, 1 ml. of enzyme solution, 3 ml. water, a few drops of dilute acetic acid to bring the final pH to 5.2, plus sufficient water to bring the final volume to 6 ml. Aliquots were removed and analyzed spectrophotometrically every 10 min. for 50 min. The pH remained nearly constant during this period of time. The temperature was 37.5°. Some twelve experiments were performed in each type of container. In no case was there any

evidence of enzymatic activity. When 1 ml. of 0.02 *M* $MgSO_4$ was substituted for 1 ml. of the water in the digest, the rate of activation of the enzyme in Pyrex and in quartz vessels was identical.

A sample of the quartz-distilled water possessed a conductivity when freshly prepared of 1.42×10^{-6} mho./cm. After standing for 1 hr. in either a Pyrex or quartz container this value did not change, nor did it change after standing further for 24 hr. If any material dissolved from the vessels into the water, it made no contribution to the conductivity. Digests of 5 ml. of 0.1% nucleate, 5 ml. of enzyme solution, and 20 ml. water possessed an initial conductivity in either Pyrex or quartz vessels of 2.61×10^{-5} mho/cm.; after 1 hr. of incubation at 37.5°, the conductivity in the former vessel was 3.33×10^{-5} mho/cm. and in the latter 3.32×10^{-5} mho/cm. It is interesting to note that some slight reaction is shown by the increase in conductivity, although it cannot be apparently measured by the acid-precipitability test employed. Nevertheless, the extent of even this very slight reaction is identical in Pyrex and in quartz vessels.

A fine Pyrex powder was prepared by grinding in a ball mill, and carefully washed with dilute HCl, water, alcohol, and ether. Amounts of this powder from 20 mg. to 1 g. were added to digests of the enzyme with nucleate in quartz vessels. The surface area of this powder was not estimated but must have been considerable. When 1 g. of this powder was suspended in 10 ml. water with conductivity of 1.42×10^{-6} mho/cm., the conductivity of the mixture rose after 1 hr. of standing to 1.04×10^{-4} mho/cm. When 150 mg. of the powder was added to a digest of nucleate and enzyme in either Pyrex or quartz vessels, there was no evidence of any activation of the enzyme (Fig. 1.). When the digest contained 1 ml. of 0.5 *M* sodium acetate–acetic acid buffer at pH 5.0 in place of 1 ml. water (final pH 5.2), there was a marked activation of the enzyme, which was the same in Pyrex or in quartz vessels and the same whether 150 mg. of Pyrex powder was added or not (Fig. 1). However, in the presence of 1 ml. of 0.02 *M* $MgSO_4$ the added powder depressed the activation of the enzyme (Fig. 1). With more powder, the depression of activation was still greater. With 20 mg. or less of the powder, little or no interference with the activation by Mg^{++} was evident. Each component of the digest, namely nucleate, enzyme, buffer, and Mg^{++} solution was separately incubated with 300 mg. of Pyrex powder for 1 hr. at 37.5° before mixing with the other components. In every case, the results were nearly identical with each other and with a digest in which all the components plus the powder were incubated together.

These experiments with added Pyrex powder are rather heroic, for the exposure of an enzymatic reaction to so much surface area may be unreasonable and extreme. However, there was no interference by a large amount of the powder with the buffer activation of the enzyme, and relatively little with the Mg^{++} activation. It is possible that the silicate which dissolved from the powder, and which increased the electrical conductivity of the mixture, also combined with some of the Mg^{++} and reduced its effective concentration. With less powder present (20 mg. or less), this effect is inappreciable, even though such small amounts of powder still possess considerable surface area. No activation effects were noted when badly scratched Pyrex vessels were used.

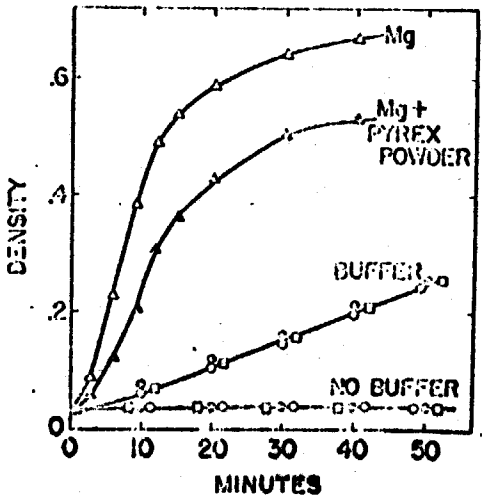

FIG. 1. Desoxyribonuclease activity in Pyrex and in quartz containers; pH 5.2 in all runs. ○ Pyrex, O quartz, □ quartz plus 150 mg. Pyrex powder; Δ quartz, digest containing Mg^{++}; ▲ quartz, digest containing Mg^{++} plus 150 mg. Pyrex powder.

The activation of the enzyme by the acetate buffer (Fig. 1) confirms our earlier observations on the activation by univalent ions (5). Considerable care was taken in the preparation of several samples of the buffer, and no detectable amounts of Mg^{++} were present. All samples

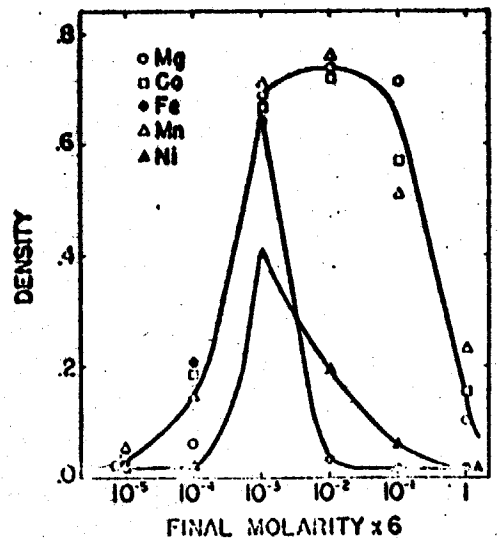

FIG. 2. Effect of concentration of added salts on enzymatic activity. Period of incubation at 37.5° was 30 min.; pH 5.2.

gave identical activating results.[2] Identical digests of 5 ml. of 0.1% nucleate solution, 5 ml. buffer, 5 ml. of enzyme solution, and 15 ml. water were incubated for 1 hr. in quartz and in Pyrex vessels. The initial conductivity in both vessels was 4.72×10^{-4} mho/cm. After incubation, the conductivity was 5.77×10^{-3} mho/cm. in quartz, and 5.83×10^{-3} mho/cm. in Pyrex. The acid-soluble fraction in each experiment was identical within the experimental error.

Activation by Bivalent Ions

Digests were prepared of nucleate, enzyme, and varying amounts of added salts. The pH was set at 5.2 at each concentration of salt. No buffer was present, but the pH did not drift more than 0.2 in any case over the 30-min. digestion period employed. Figures 2 and 3 show the results obtained. Mg^{++}, Co^{++}, and Mn^{++} were effective to nearly the same degree in the activation of desoxyribonuclease. Under the conditions used, namely 5 μg. enzyme, and 1 mg. nucleate (3 μmoles of nucleic acid phosphorus), the maximum activation for all three ions is achieved with 10 μmoles. Ferrous and nickelous ions exert optimal activation at a concentration one-tenth that of Mg^{++}, Co^{++}, or Mn^{++}. Indeed, at the maximum effective concentration for the latter three ions, ferrous ion is practically ineffective (Fig. 2). The maximum activation for Fe^{++} and Ni^{++} is thus achieved with about 1 μmole of salt.

The maximum activation for Ba^{++} is obtained with about 5 μmoles, for Ca^{++} and Sr^{++} with 10, and with Zn^{++} and Cd^{++} with about 0.5–1 μmole salt (Fig. 3).

Per micromole of nucleate phosphorus, the optimal effective amount of each ion in micromoles is: Mg^{++}, Mn^{++}, Co^{++}, Ca^{++}, and Sr^{++}, 3; Ba^{++} 1.7; Fe^{++}, Ni^{++}, Zn^{++}, and Cd^{++}, 0.3.

The marked influence of the concentration of salt, as illustrated by Co^{++} and Fe^{++} in Fig. 2, is further emphasized by the time course of the activation shown in Fig. 4. At final concentrations of Co^{++} of 1.7×10^{-3} *M* and 1.7×10^{-4} *M*, the rate of digestion is slightly greater at the higher concentration. At the same concentrations of Fe^{++}, the rate of digestion is considerably different for each concentration, the rate being lower at the higher concentration.

The time course of the activation with Ca^{++}, Sr^{++}, and Ba^{++} shows a considerable lag during the early part of the digestion, as illustrated

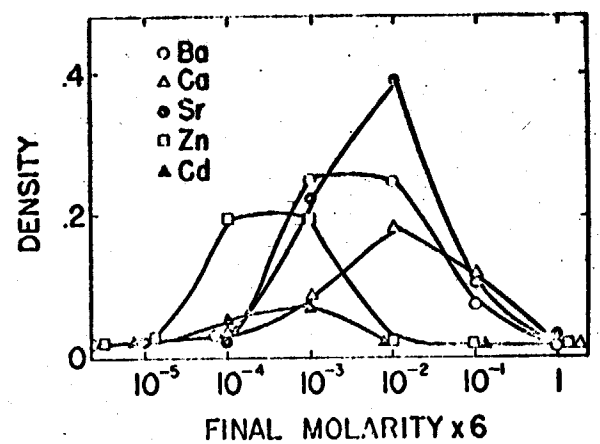

FIG. 3. Effect of concentration of added salts on enzymatic activity. Period of incubation at 37.5° was 30 min.; pH 5.2.

[2] An earlier report (5) described the activation of desoxyribonuclease by added arginine monohydrochloride. We could confirm this finding with a commercial sample of arginine. When, however, this sample was purified through the benzilidine procedure (7), it no longer activated the enzyme.

Finally, these ions found ineffective at any concentration in activating the enzyme were Pb^{++}, Hg^{++}, Cu^{++}, Fe^{+++}, Al^{+++}, and Cr^{+++}.

Effect of pH

Digests were prepared consisting of 1 ml. of 0.1% nucleate, 1 ml. of enzyme solution containing 0.5 μg. protein, 1 ml. of salt solution to give a final concentration of 5×10^{-4} *M*, sufficient dilute HCl or NaOH to obtain the desired pH, plus water to bring the final volume to 6 ml. The pH was determined before and after incubation.

As shown by Kunitz, the optimal pH for the digestion of sodium deoxyribonucleate by nuclease in the presence of Mg^{++} is about 6.5

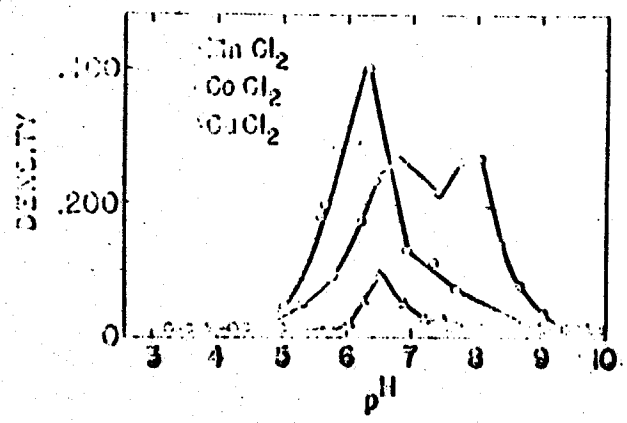

FIG. 7. Effect of pH on activity of enzyme in presence of added salts. Period of incubation at 37.5° was 30 min.

(6). We have confirmed this finding (Fig. 6). With Fe^{++}, however, the optimal pH for the digestion is at 5.7 (Fig. 6). The optimum pH with Co^{++} is close to that of Mg^{++} and to Ca^{++}. With Mn^{++} two peaks are noted, one at 6.8 and the other at about 8.0 (Fig. 7). These values have been repeatedly obtained.

SUMMARY

The activation of deoxyribonuclease on deoxyribonucleate, known to occur with Mg^{++} and Mn^{++}, has been shown to occur equally well with Co^{++}, to nearly the same extent with Fe^{++}, and to a lesser extent with Ca^{++}, Ba^{++}, Sr^{++}, Ni^{++}, Cd^{++}, and Zn^{++}. The conditions

under which the optimal activation is revealed vary among these ions. Thus, Mg^{++}, Mn^{++}, and Co^{++} may show marked activation under conditions in which Fe^{++} is nearly ineffective. Since too high a concentration of an ion may be as ineffective as too little, concentration-activation curves were determined for each ion. Per micromole of nucleic acid phosphorus, the optimal effective amount of each ion in micromoles is as follows: Mg^{++} 3, Mn^{++} 3, Co^{++} 3, Fe^{++} 0.3, Ni^{++} 0.3, Ba^{++} 1.7, Ca^{++} 3, Sr^{++} 3, Zn^{++} 0.3, and Cd^{++} 0.3.

The optimum pH for the activation with Mg^{++}, Co^{++}, and Ca^{++} is about 6.5, that with Fe^{++} is at 5.7, while Mn^{++} shows two optima at pH 6.8 and 8.0.

Experiments conducted in Pyrex and in quartz vessels showed the same results, and indicated that there was no activation of deoxyribonuclease in the absence of added salts.

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Biochem. J. 111(3): 22P (1969)

Influence of Metal Ions on the Orthophosphatase and Inorganic Pyrophosphatase Activities of Human Alkaline Phosphatase

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It is now recognized that mammalian alkaline phosphatases (EC 3.1.3.1) are able to hydrolyse both pyrophosphate and orthophosphate substrates (Cox & Griffin, 1965; Moss, Eaton, Smith & Whitby, 1967; Fernley & Walker, 1967), and it seems probable that a single type of active centre is responsible for activity towards both classes of substrate (Eaton & Moss, 1967*a*). However, Mg^{2+} ions have a differential effect on the two activities of alkaline phosphatase, activating the hydrolysis of orthophosphates but inhibiting that of pyrophosphates (Fernley & Walker, 1967; Eaton & Moss, 1967*a*,*b*). The influence of Zn^{2+} and Mg^{2+} ions on the activities of purified human intestinal alkaline phosphatase (Moss *et al.* 1967) has now been examined in more detail, and the results indicate that the differential effects of these ions are consistent with the suggestion that both catalytic activities are properties of a single type of active centre.

Both the inorganic pyrophosphatase and orthophosphatase (substrate, *p*-nitrophenyl phosphate) activities of the enzyme were measured at pH 9·0 in tris–HCl buffer; at this pH both substrates are hydrolysed at measurable rates over a wide range of concentration. Addition of Zn^{2+} ions to the pyrophosphatase assay system almost completely inhibits release of inorganic phosphate when the concentration of Zn^{2+} is greater than that of the substrate, inorganic pyrophosphate. As the concentration of pyrophosphate is increased above that of Zn^{2+}, competitive inhibition of the pyrophosphatase activity is seen. These observations can be explained by assuming that, when Zn^{2+} ions are in excess, all the substrate is in the form of a Zn^{2+}–pyrophosphate complex which is not broken down by the enzyme, or is only slowly attacked. As the substrate concentration is increased free pyrophosphate appears and is hydrolysed, but the Zn^{2+}–pyrophosphate complex is a competitive inhibitor of this hydrolysis. The Zn^{2+}–pyrophosphate complex is also a competitive inhibitor of the orthophosphatase activity when Zn^{2+} and pyrophosphate are added to this assay system in proportions calculated to produce the complex.

A similar explanation seems to be valid for the effect of Mg^{2+} ions on the pyrophosphatase activity of alkaline phosphatase. However, the inhibition of pyrophosphate hydrolysis by the Mg^{2+}–pyrophosphate complex is less marked than that by the Zn^{2+} complex, and the stoicheiometry of the interactions of the two metal ions with pyrophosphate appears to be different.

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Considering low zinc requirement of laboratory animals fed casein as source of protein it is surprising that, in recent years, deficiency symptoms have appeared in animals maintained under practical conditions(1). This has probably come about because of more common use of soybean meal in rations inasmuch as several investigators have found zinc in soy protein less available than that in animal proteins(2,3,4). Evidence has been presented that heat treatment increases availability of zinc in soy protein(5,6) although this observation has not been confirmed under our laboratory conditions. Phytin accounts for about 70% of phosphorus in soybean meal, and during extraction of soy protein, phytic acid forms a complex with protein(7). That isolated soy protein has a high affinity for zinc led us to test whether or not a casein-phytic acid complex would decrease availability of zinc to the growing chick.

Methods and material. To prepare casein-phytic acid complex, casein was suspended in distilled water to make a slurry, and phytic acid was added equivalent to 5% of dry weight. After standing overnight the mixture was dried in oven at 60°C. A similar complex was prepared with soy protein* using 4% of phytic acid. Phytic acid analyses were performed by the method of Pringle and Moran(8) except that samples were extracted twice for 18 hours. The original soy protein contained 0.5% of phytic acid phosphorus and the casein-phytic acid complex, 0.8%. According to analysis about one-fourth of phosphorus in commercial phytic acid was inorganic, and total phosphorus contents of proteins were: soy protein 0.9%; casein 0.7%; casein-phytic complex 1.8%. Basal diet, type of chicks and experimental conditions were as previously described(9) except that the battery was plastic coated. Other diets fed were modifications of the soy protein basal. Twenty-six % of soy protein-phytic acid complex, 18% of casein and 5% of gelatin, or 19% of casein-phytic acid complex and 5% of gelatin were substituted for soy protein in the respective diets and glucose hydrate was adjusted accordingly. Other additions were made at the expense of glucose.

Results of 2 trials are summarized in Table 1. Rate of growth on casein-gelatin basal diet was not improved by zinc supplementation, and it may be concluded that approximately 9 p.p.m. of zinc as it occurs in casein-gelatin diet is adequate for growth of the chick. Growth on soy protein basal diet, which by analysis contained the same amount of zinc, was markedly improved by zinc supplementation. In agreement with earlier observations (9) addition of more than 15 p.p.m. of zinc was required to give maximum response on this type of diet.

TABLE I. Availability of Zinc for Chick Growth.

Phytic acid-P in diet, %	Supplements	Wt at 4 wk (g) Trial 1	Trial 2
	A. Casein-gelatin basal		
.0	None	460 (10)†	417 (10)
.0	Zn, 55 p.p.m.‡	469 (10)	446 (10)
.15	Casein-phytic acid complex	296 (10)	153 (7)
"	*Idem*, Zn, 55 p.p.m.	473 (10)	395 (9)
.29	1.2% Ca phytate§		438 (10)
"	*Idem*, Zn, 55 p.p.m.		476 (9)
	B. Soy-protein basal*		
.12	None	162 (9)	122 (9)
"	Zn, 15 p.p.m.	382 (9)	391 (9)
"	" 55 "	473 (10)	410 (9)
.26	Soy protein-phytic acid complex	97 (6)	94 (2)
"	*Idem*, Zn, 15 p.p.m.	227 (10)	201 (9)

* Zinc content of basal diets was 9 ± 0.5 p.p.m.
† Ten male crossbred chicks were started in each group. No. of survivors is shown in parentheses.
‡ Zinc added as $ZnCO_3$.
§ Nutritional Biochemicals Corp.

When the casein-phytic acid complex and gelatin served as source of protein, growth rate was about the same as that on basal soy protein diet. These 2 diets contained approximately the same amounts of phytic acid phosphorus and zinc. Supplementation of casein-phytic acid diet with zinc supported near maximum rate of gain. In contrast to phytic acid in combination with casein, calcium phytate added to casein-gelatin basal diet had little or no effect on growth rate.

Chicks that received artificially prepared soy protein-phytic acid complex grew slowly and exhibited severe symptoms of zinc deficiency. When this diet, which contained 0.26% of phytic acid phosphorus as compared to 0.12% in basal soy diet, was supplemented with 15 p.p.m. of zinc, rate of growth was superior to that on basal diet. Thus 15 p.p.m. of zinc more than overcame the depressing effect of added phytic acid protein complex.

Although these results do not prove that the natural inhibitor in soy protein is phytic acid, they strongly suggest that phytic acid is involved in making zinc unavailable. Commercial soy protein used contains about 0.12% of phytic acid phosphorus, and a similar quantity of phytic acid complexed with casein makes zinc less available. The results suggest that phytic acid must be in combination with protein to make zinc unavailable, because addition of calcium phytate to the diet had little or no effect. It is known that phytic acid forms a complex with soy protein(7) and that proteins complexed with phytic acid are resistant to digestion by proteolytic enzymes such as pepsin(10). It is also possible that failure of calcium phytate to make zinc unavailable is due entirely to its insolubility.

Summary. Zinc in isolated soy protein is less available than that in casein. Zinc in a casein-phytic acid complex, which contains an amount of phytic acid comparable to that found in isolated soy protein, is also less available than that in untreated casein. Addition of calcium phytate to casein-gelatin type diet had little or no effect on zinc availability.

Authors gratefully acknowledge assistance of Dr. F. E. Pickett, Dept. of Agri. Chem., who performed zinc analyses by polarographic method, and A. E. Staley Manufacturing Co., Decatur, Ill., which supplied phytic acid.

* Contribution from Missouri Agri. Exp. Station, Journal Series No. 2066. Approved by Director.

†C-1 Assay Protein, Archer-Daniels-Midland Co., Cincinnati, O.

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Received October 27, 1959. P.S.E.B.M., 1960, v103.

Proc. Soc. Exptl. Biol. Med. 103: 304-306 (1960)

Poultry Sci. 43: 415-419 (1964)

Zinc Availability in the Chick as Affected by Phytate, Calcium and Ethylenediaminetetraacetate*

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(Received for publication August 21, 1963)

IN THE original description of zinc deficiency in the chick, O'Dell and Savage (1957) reported that the zinc in soy protein was less available than that in casein and gelatin. This difference between plant and animal proteins for the chick has been confirmed in several laboratories (Morrison and Sarett, 1958; Moeller and Scott, 1958; Ziegler *et al.*, 1958) and similar observations have been made with swine (Smith *et al.*, 1962).

The observation that the addition of phytic acid to animal protein diets decreased the availability of zinc both to the chick (O'Dell and Savage, 1960) and to the pig (Oberleas *et al.*, 1962), has led to the hypothesis that the difference in availability between animal and plant protein is due to the phytate content of the latter. Several groups of investigators have shown that autoclaving either the total diet or the plant protein source makes the zinc more available (Supplee *et al.*, 1958; Kratzer *et al.*, 1959; Lease *et al.*, 1960; and Smith *et al.*, 1960). Since phytic acid is rather easily hydrolyzed by autoclaving it seems likely that the beneficial effect of heat treatment resulted from the destruction of phytate.

It is well established that excess dietary calcium aggravates the symptoms of parakeratosis in swine fed corn-soybean diets, apparently decreasing the availability of zinc (Tucker and Salmon, 1955; Luecke *et al.*, 1957; Conrad and Beeson, 1957; and Lewis *et al.*, 1957) but this effect was not observed in pigs fed casein as the source of protein (Oberleas *et al.*, 1962). Pensack *et al.* (1958) observed that the calcium level was without effect on zinc availability when chicks were fed a casein-gelatin type diet whereas O'Dell *et al.* (1958) noted a calcium effect when chicks consumed a diet based on soy protein. Thus, there appears to be an interrelationship among calcium, zinc and the protein source used in the diet. If phytate is responsible for the decreased availability of zinc in plant protein, then one might expect an interaction among calcium, zinc and phytate.

Phytate may well exert its effect on zinc availability by binding zinc as a chelate or metal complex. In this regard it is significant that the addition of ethylenediaminetetraacetate (EDTA) to plant protein diets has increased the availability of zinc to various species: the turkey poult (Kratzer *et al.*, 1959), the chick (Lease *et al.*, 1960), the rat (Forbes, 1961) and the pig (Smith *et al.*, 1962). The mechanism by which EDTA makes the zinc more available is not yet clear.

The objectives of this study were to determine whether or not there is an interrelationship among zinc, calcium and phytate, in the chick, to investigate the relationship of phytate and EDTA as regards zinc availability and to evaluate the relative physiologic binding of zinc by different forms of phytate.

* Contribution from the Missouri Agricultural Experiment Station, Journal Series No. 2642. Approved by the Director.

TABLE 1. *Composition of basal diet*

	%
Casein	25.0
Gelatin	10.0
Arginine HCl	0.36
Glycine	1.0
DL-Methionine	0.5
Glucose Hydrate[1]	47.94
Cellulose[2]	3.0
Soybean Oil	5.0
Salts[3]	5.0
$CaCO_3$	1.0
Choline Cl	0.2
Vitamin Premix[4]	1.0

[1] Cerelose, Corn Products Co., Argo, Ill.
[2] Solka Floc, Brown Co., Berlin, N. H.
[3] The salts mixture supplied the following in grams per 100 grams of diet: $CaHPO_4$, 1.66; KH_2PO_4, 0.75; $CaCO_3$, 1.07; NaCl, 0.50; $FePO_4$ (Soluble), 0.35; $MgSO_4 \cdot 7H_2O$, 0.12; $MgCO_3$, 0.10; $MnSO_4 \cdot H_2O$, 0.10; KCl, 0.08; $CuSO_4 \cdot 5H_2O$, 0.0062; NaF, 0.0056; KI, 0.0024; $KAl(SO_4)_2 \cdot 12H_2O$, 0.00094; $CoCl_2 \cdot 6H_2O$, 0.00024.
[4] The vitamin premix in glucose supplied the water soluble vitamins and a soybean oil premix the fat-soluble vitamins at the levels previously described (O'Dell *et al.*, 1958).

EXPERIMENTAL

Day-old male crossbred Vantress ×White Rock chicks were placed in electrically heated batteries which had been painted with an epoxy resin. Feed and distilled water were supplied *ad libitum*, the latter in glass water fountains. The chicks were individually weighed and examined weekly during a four-week period.

The composition of the basal diet is shown in Table 1. In the first experiment acid-washed casein was used but in the latter experiments a commercial grade casein was employed. The basal diet contained 1.2% calcium and 0.8% phosphorus and the zinc content, which varied from 12 to 17 p.p.m., is indicated in the tables for each experiment. All additions were made at the expense of glucose. Zinc was added as $ZnCO_3$, calcium as $CaCO_3$, EDTA as the disodium salt and phytate in various forms. The usual method of phytate addition was as a dry premix (5.5% phytic acid) with casein (O'Dell and Savage, 1960) but for comparison phytic acid was premixed with glucose immediately before compounding the diet. The phytic acid, as well as the mixed diets were analyzed for phytate phosphorus by a modification of the method of Early (1944) in which phosphorus was determined colorimetrically.

RESULTS AND DISCUSSION

The results of the first experiment designed to test the possible interaction of calcium, zinc and phytate are presented in Table 2. In confirmation of earlier results (O'Dell and Savage, 1960) the addition of 0.5% of phytic acid to the casein-gelatin diet depressed the growth rate when zinc was suboptimal but had no deleterious effect in the presence of adequate zinc. Phytic acid at the 1% level was even more damaging in the absence of added zinc. Doubling the calcium content of the diet in the absence of phytic acid had no detrimental effect on the growth

TABLE 2.—*Interaction of calcium, zinc and phytate. Experiment 1: Groups of 10 chicks per trial*

Diet No.	Dietary supplements			Chick at 4 weeks	
	Total Ca	Phytic acid	Zn	No.	Avg. weight
	%	%	p.p.m.		g.
A[1]	1.2	0	0	40	497 ± 13[2]
B	1.2	0	55	40	548 ± 11
C[3]	1.2	0.5	0	40	340 ± 10
D	1.2	0.5	55	39	528 ± 9
E	1.2	1.0	0	20	212 ± 13
F	1.2	1.0	55	17	528 ± 24
G	2.4	0	0	39	498 ± 8
H	2.4	0	55	39	532 ± 12
I	2.4	0.5	0	38	291 ± 11
J	2.4	0.5	55	37	568 ± 13
K	2.4	1.0	0	18	216 ± 17
L	2.4	1.0	55	18	523 ± 15

[1] By analysis Diet A contained 11, Diet C, 13, Diets E and G, 12, and Diet I, 14 p.p.m. of zinc.
[2] Standard error of mean.
[3] Statistical analysis by Student's "t" test showed the average weights of following diets to be significantly different, $P<0.01$; A>C, C>E, G>I, I>K, B>A, D>C, J>I, L>K and C>I.

rate as shown by comparison of diets A and G and B and H. However, in the presence of 0.5% phytic acid and with no added zinc, excess calcium depressed growth significantly (C *vs.* I). When a zinc supplement was added to these diets the excess calcium was innocuous (D *vs.* J). Thus, excess calcium, in presence of phytic acid, significantly increased the response to zinc supplementation, but in absence of phytate it had no effect.

The results described above strongly suggest an interaction of calcium, zinc and phytate. An analysis of variance of the data relating to calcium and zinc levels and with and without 0.5% of added phytic acid revealed no significant calcium-phytic acid interaction, a small effect due to calcium-zinc interaction ($P<.05$), a highly significant zinc-phytic acid interaction ($P<.01$) and a highly significant three-way interaction among calcium, zinc and phytic acid ($P<.01$). It seems clear that calcium exerts a depressing effect on the biological availability of zinc only in the presence of phytate and that the effect of phytate is enhanced by excess calcium. Similar results have been observed in swine (Oberleas *et al.*, 1962).

Since EDTA appears to enhance the availability of zinc when added to soy protein diets and the phytate content of the protein is probably responsible for the zinc binding, the direct effect of EDTA in the presence of added phytic acid was tested in the second experiment. The results, tabulated in Table 3, show that a near maximum growth rate occurred when the chicks received 17 p.p.m. of zinc in a casein-gelatin diet (M). The addition of 0.5% of phytic acid (O) caused a marked growth depression and other symptoms, typical of zinc deficiency including shortening of the long bones, poor feathering and a stilted gait. Significantly, the further addition of 0.1% EDTA supported a growth rate approaching that of the control (Q *vs.* N) and eliminated all gross symptoms of the deficiency. This level of EDTA did not completely alleviate the detrimental effect of 0.5% phytic acid because supplementary zinc produced a small but significant ($P<0.025$) increase in growth. (Compare Q and R.) Although the mechanism by which EDTA increases the availability of zinc in the presence of phytate is not known, it seems probable that EDTA and phytate bind zinc competitively. Zinc phytate is a highly insoluble compound at the pH of the small intestine and would no doubt be poorly absorbed because of its insolubility. The zinc-EDTA chelate is soluble under the same conditions and, if not absorbed as such, would tend to solubilize zinc in the presence of phytate and possibly release it to the intestinal mucosa for absorption.

TABLE 3.—*Effect of EDTA and phytate on zinc availability. Experiment 2: Three trials with 10 chicks per group*

Diet No.	Dietary supplements			Avg. weight 4 weeks
	EDTA	Phytate	Zn	
	%	%	p.p.m.	g.
M[1]	0	0	0	570 ± 13[2]
N	0	0	55	587 ± 11
O	0	0.5	0	395 ± 15
P	0	0.5	55	558 ± 10
Q	0.1	0.5	0	529 ± 9
R	0.1	0.5	55	567 ± 11

[1] Commercial casein used in all diets. Zinc analysis for Diet M and O, 17 and 18 p.p.m., respectively.
[2] Standard error of mean.
[3] Statistical analysis by Student's "t" test: Q>O, $P<0.01$; R>Q, $P<0.025$.

It is known that phytic acid readily complexes with soy protein (Smith and Rackis, 1957) as well as many other proteins (Barré and Labat, 1962). In their first experiments O'Dell and Savage (1960) mixed phytic acid with casein with the intent of forming a protein-phytate

TABLE 4. –*Effect of chemical form of phytate and its mode of addition*[1]

Experiment 3: Three trials with 10 chicks per group

Diet No.	Amount and form of added phytate	Zinc added	Avg. weight at 4 weeks
		p.p.m.	g.
M[2]	None	0	570 ± 13[3]
N	None	55	587 ± 11
O	0.5% Phytic Acid-Casein Complex	0	395 ± 15
P	0.5% Phytic Acid-Casein Complex	55	558 ± 10
S	0.5% Phytic Acid-Glucose Premix	0	364 ± 14
T	0.5% Phytic Acid-Glucose Premix	55	568 ± 11
U	0.8% Calcium Phytate	0	560 ± 9
V	0.8% Calcium Phytate	55	544 ± 12
W	1.6% Calcium Phytate	0	516 ± 14
X	1.6% Calcium Phytate	55	572 ± 11

[1] Commercial casein used in all diets. Data for diets M–P are the same as those in Table 3.
[2] Standard error of mean.
[3] Statistical analysis by Student's "t" test: $P<0.01$; M>0, and S and W.

complex, although the formation of a complex was not verified. The question then arose whether or not it was necessary to premix the casein and phytic acid in order to make the zinc biologically unavailable. The results of experiment 3, designed to answer this question, are shown in Table 4. The effect of an insoluble form of phytate, the calcium salt, was also investigated. Clearly, phytic acid premixed with cerelose was as effective in biologically binding zinc as that which was complexed with casein. Of course, this does not preclude the possibility that phytate was complexed with protein while in the digestive tract. Calcium phytate, added at a level equivalent to 0.5% phytic acid, had no effect on zinc availability. This is in agreement with an earlier observation (O'Dell and Savage, 1960) that 1.2% calcium phytate had no detrimental action. Although the effect was small, calcium phytate at the 1.6% level reduced growth rate by a statistically significant amount. From these results it appears that phytate must be in the diet in a soluble form in order to exert the maximum binding effect. It seems probable that the calcium-phytate interaction effective in decreasing zinc availability is mediated by a mechanism other than simple adsorption of the zinc ion on an insoluble calcium phytate aggregate.

The results of this study show that there is a three-way interaction among the calcium, zinc and phytate ions. Presumably these three ions react to form a more insoluble or at least a less available complex than is formed by any two ions alone. In fact, there is no evidence that calcium exerts a detrimental effect on zinc availability in the absence of phytate ion. It is possible that a protein-phytate interaction is also involved, but, if so, the complex must be formed in the digestive tract because phytic acid premixed with glucose was as effective as that complexed with casein before mixing in the diet. EDTA increases zinc availability in the presence of phytate, presumably preventing the formation of the unavailable calcium-zinc-phytate complex and maintaining a soluble zinc-EDTA complex which can be either absorbed as such or can release zinc ion to the intestinal mucosa for absorption.

SUMMARY

Male chicks were maintained for 4 weeks on casein-gelatin type diets supplemented with various combinations of calcium, zinc, phytate and ethylenediaminetetraacetate. Addition of phytic acid markedly decreased the biological availability of zinc whether complexed with the casein or simply premixed with glucose. Preformed calcium phytate had relatively little effect on zinc availability.

Excess calcium in the absence of phytate or, in the presence of adequate zinc, had no deleterious effect on growth, but in the presence of phytate it aggravated the zinc deficiency symptoms. There was clearly a calcium-zinc-phytate interaction which decreased biological availability. Ethylenediaminetetraacetate added along with phytic acid largely counteracted the detrimental effect of the phytic acid.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr. E. E. Pickett of the Station Spectrographic Laboratory for the zinc analyses which he did by atomic absorption spectrometry. We are also grateful to Merck and Co., Rahway, N. J., Hoffman-LaRoche Inc., Nutley, N. J., and Distillation Products Industries, Rochester, N. Y., for their generous contribution of the vitamins used in this investigation.

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ZINC TOXICITY IN RUMINANTS. I. EFFECT OF HIGH LEVELS OF DIETARY ZINC ON GAINS, FEED CONSUMPTION AND FEED EFFICIENCY OF LAMBS[1]

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Zinc has been shown to be a dietary essential for lambs (Ott *et al.*, 1964), and work has suggested that their requirement is similar to levels which occur in natural feeds (Ott *et al.*, 1965). On the basis of this work, zinc supplementation of lamb rations is practiced as insurance against zinc deficiency. In supplementing the animal's diet with any mineral, it is not only desirable to know the requirement but also the tolerance and toxicity levels as well. Since zinc has been shown to be toxic to nonruminant animals (Scott and Fisher, 1938; Sutton and Nelson, 1937; Sampson *et al.*, 1942), a series of experiments was designed to determine the toxicity threshold of dietary zinc for lambs and to characterize the toxicity symptoms.

Material and Methods

Four experiments were conducted to determine the maximum level of zinc which could be fed to lambs without causing detrimental effects on performance. The first two experiments were feeding trials designed to establish the tolerance and toxicity levels, while the second two experiments were included to elicit the mechanisms of some of the responses.

Experiment I. Thirty wether lambs weighing between 22 and 29 kg. were allotted according to weight to one of five groups. Each lamb was assigned to an individual 1.22-m. x 2.44-m. pen with concrete floors equipped with slotted platforms to eliminate contamination from bedding. After a 2-week preliminary period on the basal diet (table 1), the previously selected groups were assigned at random to the following levels of supplemental zinc: 0, 0.5, 1.0, 2.0 and 4.0 gm. per kg. of diet. The zinc was added to the basal diet as ZnO (U.S.P. grade) prior to pelleting.

Gains and feed consumption were recorded weekly. At the end of 6 weeks two lambs from each treatment group were slaughtered for tissue samples. The remaining lambs were fed for an additional 4 weeks.

Experiment II. The second experiment was designed to confirm the results of the first experiment and to measure the tolerance levels within a narrower range and in smaller increments. Eighty western lambs weighing 28.3 to 38.6 kg. were allotted according to weight to eight lots of 10 animals each. After a 10-day preliminary period on the basal diet (table 1), the lots were assigned at random to the following levels of supplemental zinc: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 gm. per kg. of diet. Zinc oxide was again used as the source of zinc, and the data collected were similar to those in the first experiment.

Experiment III. In order to assess the effect of extremely high levels of zinc intake on lambs, an experiment was designed to increase the zinc intake above the voluntary level. Eight wether lambs averaging 37.6 kg. were divided into four comparable groups. After 10 days on the basal + 1.0 gm. of zinc per kg. of diet, the lambs were dosed daily with 0, 2.0, 4.0 or 6.0 gm. of zinc as zinc sulfate. The zinc was dissolved in 35 ml. of water and administered directly into the rumen via a stomach tube. The contents of the stomach tube were washed into the rumen with an additional 50 ml. of water. The treatment was repeated daily. Daily gains, feed consumption and water consumption were recorded.

Experiment IV. Since palatability has been shown to be a factor in zinc toxicity in other species (Scott and Fisher, 1938), an experiment was designed to determine the acceptability of diets containing various levels of zinc by lambs. Eight lambs were confined to a single pen and given access to diets containing 0, 0.5, 1.0 and 4.0 gm. of supplemental zinc per kg. of diet. Two feeders were provided for each diet, and their location within the pen was changed at random each day. The experiment was conducted in three phases of 10, 12 and 16 days. Between each phase the lambs were provided only the basal diet in the same feeders for periods equal to or longer than the test periods. Only daily feed consumption was recorded.

Data collected on experiment I were analyzed by multiple regression, while data collected on other experiments were analyzed by orthogonal polynomials (Snedecor, 1956).

Results and Discussion

Experiment I. The first indication of zinc toxicity was a reduction in feed consumption (table 2). Although the effect was quite evident the first few days of the experiment, consumption returned toward normal as the experiment progressed. Only the lambs which received the 2.0 and 4.0 gm. of zinc per kg. of diet showed any noticeable depression in feed intake at the end of 6 or 10 weeks. The lambs which received 0.5 and 1.0 gm. of zinc per kg. of diet consumed more feed, gained faster and had better feed efficiency than the controls. Feed consumption and gain decreased in the groups consuming high levels of zinc. Lambs receiving 4.0 gm. of zinc per kg. of diet lost weight during the experiment. The only external symptoms of toxicity were emaciation and weakness. Although one lamb died in each of the treatment groups, except in the group receiving 4.0 gm. of zinc per kg. of diet, there appeared to be no common cause of death, nor could the deaths be attributed to the zinc intake.

Experiment II. The high levels of zinc caused a reduction in feed consumption during the first few days of the experiment. Thereafter, a partial recovery of appetite occurred, but the overall consumption was reduced in the groups receiving 1.5 gm. of zinc per kg. of diet or more (table 3). A significant ($P<.01$) linear decrease in rate of gain accompanied the reduced feed consumption. The effect on gains was even greater than on feed consumption and occurred at a lower level of intake (1.0 gm. of zinc per kg. of diet). Increasing levels of dietary zinc also resulted in decreased feed efficiency and increased mineral intake.

Five lambs died or became moribund during the last 2 weeks of the experiment (three from the group receiving 3.0 gm. of zinc and two from the group receiving 3.5 gm. of zinc per kg. of diet). Symptoms generally observed included extension of the limbs, convulsions and opisthotonos. The symptoms appeared as an acute attack, and death occurred within 6 to 8 hr. Examination by personnel at the Animal Disease Diagnostic Laboratory failed to reveal any gross or histological lesions characteristic of the affected animals. Endocardial hemorrhages, lung congestion, pleuritis and enteritis were observed in some of the lambs, but there was no consistency in their occurrence. Some brain damage was also observed, but this was not evident in all animals. Lack of consistency of the observations made it difficult to determine the exact cause of death or even to attribute the

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[3] These data are from a thesis submitted by the senior author to the Graduate School, Purdue University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

TABLE 1. BASAL DIET FOR LAMBS[a,b]

Ingredient	Percent
Dehydrated alfalfa meal (17% protein)	20.0
Ground corn cobs	39.5
Ground yellow corn	32.0
Soybean meal (44% protein)	7.0
Iodized salt+cobalt[c]	0.5
Steamed bonemeal	1.0
Vitamin A and D concentrate[d]	+
Total	100.0

[a] Complete ration was pelleted after mixing.
[b] Basal diet contained 35 mg. of zinc per kg., 0.65% calcium and 0.35% phosphorus.
[c] Mixture contained 0.62 gm. $CoSO_4 \cdot 7H_2O$ per kg. of salt.
[d] Vitamin A and D mix provided 4400 I.U. of vitamin A and 880 I.U. of vitamin D per kg. of ration.

TABLE 2. INFLUENCE OF HIGH LEVELS OF ZINC ON GAIN, FEED CONSUMPTION AND FEED EFFICIENCY BY LAMBS

Zinc added to diet	Gain		Feed consumption		Feed to gain ratio	
	6 weeks[a]	10 weeks[b]	6 weeks	10 weeks[c]	6 weeks	10 weeks[c]
gm. kg.	kg. day					
0	0.11	0.15	1.26	1.31	11.57	8.94
0.5	0.17	0.18	1.42	1.49	8.4[illegible]	8.[illegible]
1.0	0.19	0.19	1.28	1.26	6.7[illegible]	6.7[illegible]
2.0	0.08	0.10	1.10	1.12	13.1[illegible]	12.05
4.0	—0.15	—0.13	0.71	0.62	.....	.....

[a] Significant treatment ($P<.01$), linear ($P<.01$), quadratic ($P<.01$) and cubic ($P<.05$) effects.
[b] Significant ($P<.01$) treatment, linear and quadratic effects.
[c] Significant ($P<.01$) treatment effect.

TABLE 3. INFLUENCE OF HIGH LEVELS OF ZINC ON GAINS, FEED AND MINERAL CONSUMPTION AND FEED EFFICIENCY BY LAMBS

Zinc added to diet	Average daily gain[a]	Average daily feed	Feed to gain ratio	Average daily mineral consumption[b]
gm./kg.	kg.	kg.		gm.
0	0.19	1.75	9.28	20
0.5	0.23	1.80	7.90	36
1.0	0.16	1.75	10.94	38
1.5	0.13	1.52	12.82	46
2.0	0.09	1.44	16.42	61
3.5	0.00	1.35		61
4.0	0.05	1.34	22.95	67
6.0	0.00	1.12		63

[a] Significant treatment ($P<.01$), linear ($P<.01$) and cubic ($P<.05$) effects.
[b] Mineral mixture was composed of two parts dicalcium phosphate, one part salt and was fed *ad libitum*.

effect directly to zinc. The loss of nine additional lambs within a month after all lambs had been changed to a low zinc diet served only to confuse the issue. However, all of these lambs had previously received at least 1.5 gm. of zinc per kg. of diet.

Experiment III. Since reduced gains in the first experiments could be attributed at least in part to the decreased feed consumption, the third experiment offered an opportunity to determine the mechanism involved. During the preliminary period the lambs readily consumed the diet containing 1.0 gm. of zinc per kg. However, both feed and water consumption decreased in the groups receiving 4.0 or 6.0 gm. of zinc the first day they were dosed (figures 1 and 2). By the end of the sixth day, lambs on the 6.0-gm. level were eating no feed, and water consumption was about 25% of that consumed by the controls.

The experiment was terminated on the 11th day, after the death of one of the lambs which had been receiving 6.0 gm. of zinc daily. At this time average daily gains for the four treatments were: no supplemental zinc, 0.20; 2.0 gm. of zinc per day, 0.0; 4.0 gm. of zinc per day, —0.76; and 6.0 gm. of zinc per day, —1.09 kg. per day. Differences in gains were significant ($P<.05$). Since administration of high levels of zinc to lambs caused marked depression in feed and water consumption, the resulting emaciation and dehydration may account for a large portion of the decrease in weight.

Experiment IV. Results of the palatability studies are shown in table 4. The lambs consumed the rations containing 0.5 and 1.0 gm. of supplemental zinc per kg. of diet with equal preference. The diet containing 4.0 gm. of zinc per kg. was almost completely refused. Thus, high levels of dietary zinc can be detected by lambs and will be refused if a choice is given.

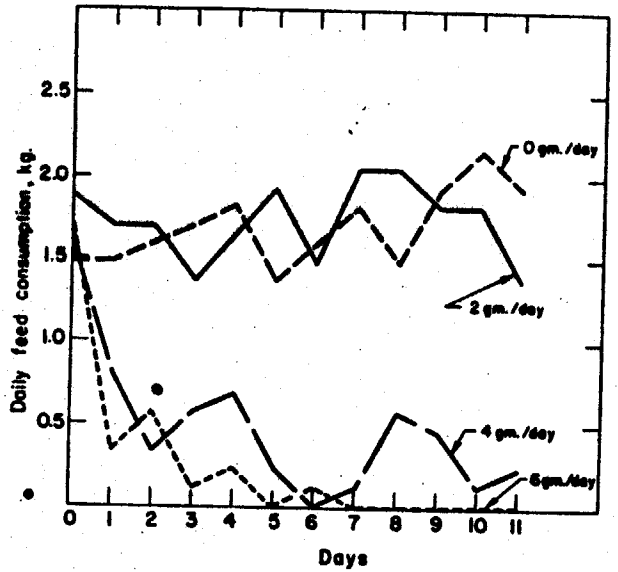

Figure 1. Feed consumption by lambs after being dosed with high levels of zinc.

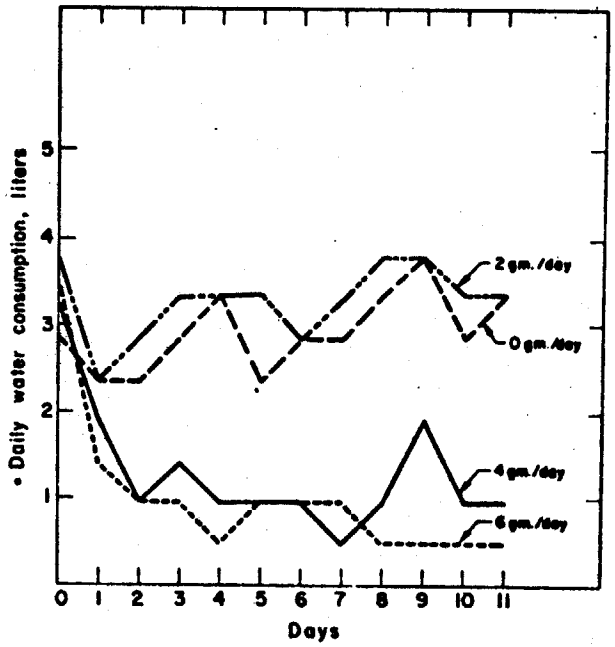

Figure 2. Water consumption by lambs after being dosed with high levels of zinc.

TABLE 4. CONSUMPTION OF DIETS CONTAINING VARIOUS LEVELS OF ZINC BY LAMBS

Zinc added to diet	Phase I (7 days)	Phase II (13 days)	Phase III (10 days)	Average
gm./kg.	kg./day			
0	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible] gm. of zinc per kg. [illegible] study.

Discussion

Reduced gains, feed consumption and feed efficiency have been described in connection with zinc toxicity in other species, including the cat (Scott and Fisher, 1938), rat (Grant-Frost and Underwood, 1958; Sadasivan, 1951) and pig (Sampson *et al.*, 1942). Responses to high levels of dietary zinc appear to be similar in lambs and nonruminant animals. The toxicity threshold in lambs is lower than that for most nonruminants but similar to that reported by Grimmett *et al.* (1937) as being toxic to young pigs fed milk.

Lowered feed consumption due to high-zinc intake was also described by Thompson *et al.* (1950) when lambs were fed 1.[illegible] zinc sulfate. This would be a zinc level similar to the levels showing marked appetite depression in this study. Work by Hemingway *et al.* (1964) has recently shown, however, that 2[illegible] gm. of zinc per head per day causes no detrimental effect on reproduction in ewes.

High levels of dietary zinc have been implicated in the death of pigs (Grimmett and McIntosh, 1956) and verified experimentally

by Brink *et al.* (1959). Lack of consistency in the exact cause of death from high-zinc levels which occurred in this study was found to some extent in the pigs. Some of the tissue changes which occurred were common to both species.

Summary

Four experiments were conducted with lambs to determine the levels of dietary zinc which could be tolerated without affecting performance. Zinc consumption above 1.5 gm. per kg. of diet caused depressed feed consumption, while 1.0 gm. of zinc per kg. of diet caused reduced gains, decreased feed efficiency and increased mineral consumption. Decreased feed consumption was shown to be in part a palatability effect. Water consumption was also suppressed by force feeding 4.0 to 6.0 gm. of zinc daily. No other external symptoms of the toxicity were observed, but prolonged consumption of high levels of zinc caused death.

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83. ZINC TOLERANCE AND TOXICITY STUDIES WITH CALVES. E. A. Ott*, W. H. Smith, H. E. Parker, R. B. Harrington and W. M. Beeson, *Purdue University, Lafayette, Indiana.*

Two experiments were conducted to determine the tolerance and toxicity of zinc in the diets of fattening calves. In the first experiment 16 calves were divided into four comparable groups and fed a complete corn, corncob basal diet supplemented with 0, 1000, 2000 or 3000 mg. Zn/kg. of diet as ZnO. Average daily gain, feed consumption and feed per 100 lb. gain for the above treatments were, respectively, as follows in pounds: 2.69, 19.95, 741; 2.25, 23.94, 1064; 0.48, 18.48, 3804; and —0.13, 15.10, 0.0. Increased salt and mineral consumption was noted. Blood and hair zinc changes were followed. In the second experiment, 60 calves were allotted at random within sex and breed to six treatment groups using steers and heifers in replicating lots. The diet was a complete pelleted feed incorporating 100, 500, 900, 1300, 1700 and 2100 mg. of Zn/kg. of diet. At 71 days average daily gain, feed consumption and feed efficiency for the above treatments were, respectively, as follows in pounds for the steers: 2.11, 21.70, 1027; 2.24, 21.01, 934; 1.82, 18.59, 1020; 1.58, 20.45, 1298; 0.33, 17.59, 5400; and 0.55, 18.91, 3410; and for the heifers: 1.77, 17.95, 1014; 1.85, 17.22, 933; 1.60, 17.26, 1076; 1.30, 18.44, 1422; 0.71, 17.79, 2499; and 0.42, 16.52, 3964. Blood tissue and hair samples were collected for zinc analysis.

ANN. SURG. 165(3):432-436. (1967).

Acceleration of Healing with Zinc Sulfate

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ZINC is a potent catalyst of wound healing and zinc deficiency may be a common cause of delayed tissue repair. It is curious that this element has been ignored for so long in studies of human nutrition. Physiologically, zinc is similar to iron in many respects and is present in nearly the same concentrations. It is more plentiful in the human body than other metals such as cobalt, iodine and fluorine and is intimately involved with such vital processes as protein synthesis and cellular respiration. To a great extent, knowledge has been limited by difficulties in analysis for only recently with the advent of sophisticated chemical technics, could careful studies of zinc metabolism be made. As these investigations have progressed in the last few years, zinc has been identified as an important factor in repair of tissue.

The use of zinc to promote healing is not new. The ancient Egyptians used it topically in the form of calamine and since then zinc oxide, zinc sulfate and zinc stearates in the form of powders, salves and ointments have continued to be used. It is likely that some of the element is absorbed through the skin, particularly through injured and granulating tissues.

Our interest in the systemic use of zinc began with a laboratory accident. During a series of wound healing studies in rats, a remarkable acceleration of healing was noted in a group fed a contaminated diet. After considerable searching, zinc was finally identified as the beneficial impurity and further experiments in rats confirmed that zinc was a potent factor in healing.[5] Subsequent observations in patients suggested that zinc also played a role in human healing. The purpose of this report is to present the results of a carefully controlled study which suggests that dietary zinc supplements can accelerate the rate of healing in man.

Materials and Methods

Extension of zinc therapy from animal studies[5] was a problem until it was found that the wound formed by excising pilonidal sinuses in young airmen was very satisfactory for healing studies. These wounds should reflect the behavior of normal granulating tissues as the excisions were carried into normal tissues on all sides and as the lesions were left open to heal by second intention. The excision sites were dressed daily with Balsam of Peru and the volumes were measured by taking impressions of

Submitted for publication October 21, 1966.

The contents of the paper reflect the authors' personal views and are not to be construed as a statement of official Air Force policy.

This research was supported in part by a grant from the Aerospace Medical Division, under approval of the Surgeon General, U. S. Air Force; in part by grant HE 10213, National Institutes of Health, and grant RH 00042, Division of Radiological Health, Bureau of State Services, U. S. Public Health Service; and in part by the Horatio H. Butt Fund of the University of Rochester.

each cavity at approximately 5-day intervals with a rapidly setting, innocuous alginate hydrocolloid, Jeltrate, as previously described,[4] and as shown in Figure 1. After 25 days, many volumes were less than 1 ml. and accurate measurement of volume by this method was no longer possible.

The second method of quantifying the rate of wound healing was the determination of the number of days required for complete healing. Healing was considered complete when the entire surface was covered with epithelium and no further scab formation took place which proved to be a remarkably clear end point.

Twenty patients were studied. These airmen were young and healthy and continued their usual diets during the study. They were randomly divided into treatment groups on the basis of serial numbers, with odd numbers assigned to control and even numbers to zinc therapy. Ten patients became controls and were maintained on their usual diets *ad lib* without medication during the period of healing. The remaining ten formed the therapy group and were given daily dosages of zinc sulfate in addition to their usual diets *ad lib*. The medicated patients were supplemented with capsules containing 220 mg. of zinc sulfate USP ($ZnSO_4 \cdot 7H_2O$) at a dosage of one capsule three times a day in order to supply the equivalent of an additional 150 mg. of elemental zinc per day.* This dosage was well tolerated by all patients. The capsules were taken either with milk or after a meal to avoid possible gastro-intestinal irritation by zinc sulfate: the salt has an emetic dose of 2 Gm. All patients were maintained on regular house diets until three days after operation, when they were returned to full duty and resumed their usual dietary habits. All patients healed without difficulty and, to date, there have been no recurrences.

* Supplied by Smith, Kline & French Laboratories, Philadelphia, Pa.

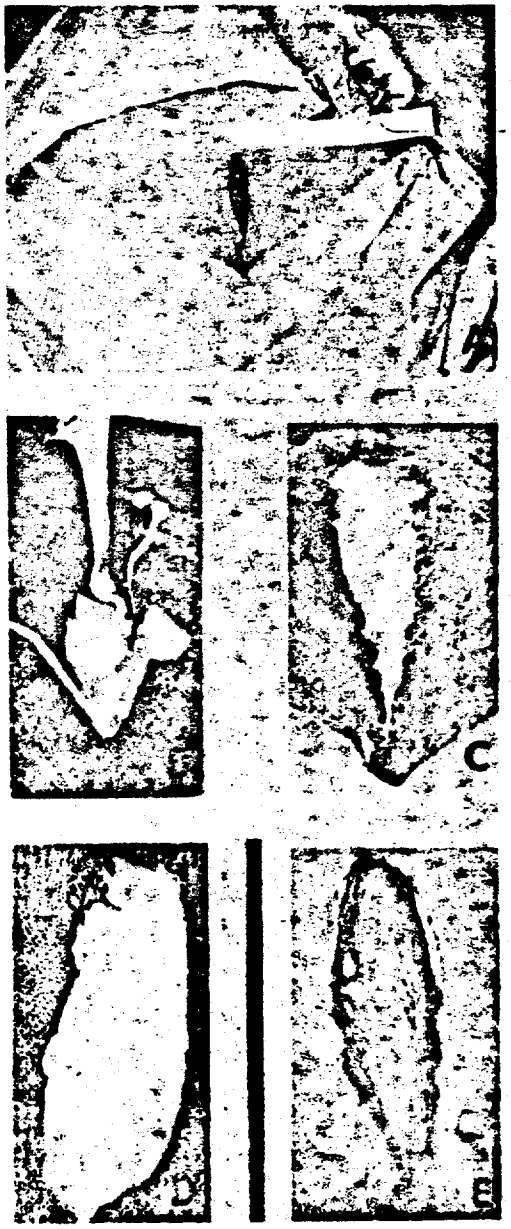

FIG. 1. The measurement of human healing. The granulating wound (A) is filled with Jeltrate (B). The alginate sets rapidly (C) and can be removed easily as an impression (D). The volume of the wound can be measured by immersing the impression in a graduated cylinder of water and recording the volume displaced. The impression can also be used to make a permanent cast of the wound (E) for later comparisons.

TABLE 1. *Wound Healing in Controls and Airmen Medicated with Zinc Sulfate USP*

Patient	Age	Wound Volume (ml.)	Days for Complete Healing	Healing Rate (ml./day)
		Controls		
1	27	10	40	0.25
2	21	2.5	46	0.05
3	40	40	48	0.83
4	23	47	52	0.89
5	23	28	63	0.44
6	24	52	71	0.73
7	23	42	85	0.50
8	24	7.5	95	0.08
9	23	45	121	0.37
10	22	49	180	0.27
MEAN ± SE	25.0	32.3	80.1 ± 13.7	0.44 ± 0.09
		Zinc Sulfate USP, 220 mg. t.i.d.		
1	19	100	33	3.03
2	24	28	34	0.82
3	22	30	43	0.70
4	18	44	44	1.00
5	28	35	46	0.76
6	40	140	46	3.04
7	28	30	48	0.63
8	23	50	51	0.98
9	22	49	52	0.94
10	22	39	61	0.64
MEAN ± SE	24.6	54.5	45.8 ± 2.6	1.25 ± 0.30
Significance			$p < 0.02$	$p < 0.01$

Results

The significant acceleration of wound healing produced by zinc therapy is emphasized by the data in mean healing values summarized in Table 1. In the control group, wounds healed in 80.1 + SE 13.7 days.* In the zinc-medicated patients, wounds healed in 45.8 + SE 2.6 days ($p < 0.02$). Thus wounds of patients receiving 150 mg. of metallic zinc daily were healed 34.3 days earlier than the controls even though their initial wounds were almost twice as large (54.5 ml. vs. 32.3 ml.). Acceleration is also shown in the rate of wound closure. The control wounds closed at the rate of 0.44 ml./day, whereas those of patients medicated with 150 mg. of zinc closed at the rate of 1.25 ml./day, or almost three times faster ($p < 0.01$).

* Standard error.

There was a considerable difference in the appearance of the wounds between the two groups. The wounds of the airmen on zinc sulfate therapy demonstrated cleaner, pinker and *healthier* granulations with considerably less purulent exudate.

Although the medicated group healed more rapidly throughout the course of the experiment, the differences were small during the first 15 days. As shown in Figure 2, the effect of zinc sulfate therapy is greatest during the second half of the healing period in the epithelization phase. Acceleration of epithelization in the terminal stages of healing is similar to the pattern observed in our earlier animal studies.[5]

Discussion

It is not surprising that zinc should play an important role in human wound healing. Considerable experimental evidence has

been available that this element is essential for wound repair. Zinc-deficient cattle, poultry, sheep and swine spontaneously develop ulcerating lesions, especially of their legs, which heal only if adequate dietary zinc is supplied. Miller *et al.*,[5] noted the dependency of healing on adequate zinc stores, and suggested that poor healing in cattle can be used as a sign of zinc deficiency.

Zinc probably acts primarily at the wound site where it is incorporated into enzyme systems. Savlov *et al.*[6] demonstrated that radiozinc is preferentially concentrated in healing tissues with a peak of activity during the first 7 days after injury, followed by a gradual decrease. The migration of zinc into the wound appears to be temporary since at the time of complete scarring, 100 days after injury, these investigators found no evidence of zinc-65 in the scar although the radioisotope was still detectable in most tissues.

Zinc is the metal moiety in a number of essential enzyme systems.[7] Metalloenzymes known to contain zinc include alkaline phosphatase, carbonic anhydrase, carboxypeptidase and the following dehydrogenases: alcohol, glutamic acid and lactic acid. In addition, zinc acts as a cofactor to a number of enzymes including arginase, carnosinase, dehydropeptidase, glycyglycine dipeptidase, histidine deaminase and tripeptidase, as well as oxaloacetic carboxylase and some of the lecithinases and enolases. Evidence accumulated from studies in microorganisms also supports the thesis that the action of zinc is in the area of protein and nucleic acid synthesis. Zinc deficiency in *Mycobacterium smegmatis*, for example, interferes with the synthesis of RNA and thus secondarily inhibits the synthesis of DNA and other proteins. Whether similar pathways are affected in humans remains to be shown.

It is desirable to offer readily available zinc to the organism during periods of wound healing as zinc appears to be essential to protein synthesis. Studies on rats first demonstrated that such an approach was sound. The addition of extra zinc to a commercial rat feed accelerated the healing rate of wounds and burns in these animals by 30 per cent.[7] The present studies on young airmen on normal diets suggest that zinc salts are also beneficial during tissue repair in humans. The healing of standard granulating wounds was accelerated by 34.3 days, or 43 per cent, over that in controls (80.1 + SE 13.7 days vs. 45.8 + SE 2.6 days). Most of this acceleration occurred during the stage of epithelization. Perhaps this is related to the fact that 20 per cent of the body's zinc stores is in the skin, concentrated in the epithelium and epithelial structures.[8]

It is becoming apparent that zinc deficiency may be a common problem in our

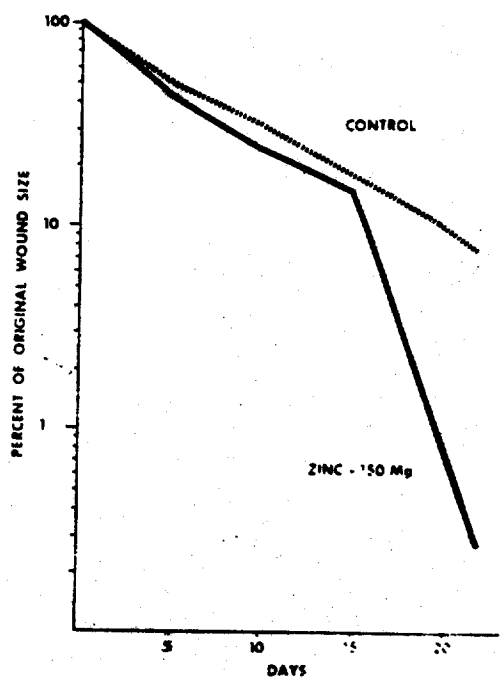

FIG. 2. The effect of zinc on wound healing in man. Almost all of the acceleration occurs after the second week of healing, during the stage of epithelization. Almost 20% of the body's zinc stores are in the epithelium.

patient population. Studies in 47 patients with severe burns have shown a marked zinc deficit beginning shortly after the burn and lasting as long as 2 to 3 months after injury.[3] More recent observations in postoperative patients have shown a marked zincuria with a fall in zinc stores during the early postoperative period.[1]

Zinc is obtained only in a limited number of foods and in these the zinc content is often variable; the element is primarily absorbed and excreted by the small intestine. In the chronically malnourished patient such as the aged, the obese, the faddist or those with serious debilitating illnesses severe zinc deficits due to the negative zinc balance can and probably do easily develop. This may account for wound dehiscences, poor healing and frequent hypoproteinemia so often seen in this group. Since zinc medication was so beneficial in young, healthy airmen with relatively minor wounds, it is likely to be even more so in seriously ill patients with severe nutritional problems. Further studies of these groups of patients are now underway.

Fortunately, zinc sulfate is an extremely safe compound for oral use and toxicity levels are about 30 times the dosages used in these studies. It is well tolerated and offers a safe and effective method of promoting healing, especially in the marginally nourished patient with deficient zinc stores.

Summary

Zinc is intimately involved in healing. Zinc-deficient animals heal poorly, zinc-65 localizes preferentially in healing tissues and the addition of extra readily available zinc salts accelerates healing in rats.

Zinc also appears to play an important role in human healing. Patients with severe burns develop significant zinc deficits and it is likely that patients with other healing problems also have zinc stores inadequate to meet the demands of healing tissues.

Oral medication with 220 mg. of zinc sulfate USP three times a day, resulted in the acceleration of healing by 43 per cent in a well controlled study of young men with granulating wounds. Zinc therapy deserves a trial as an adjunct in the treatment of patients with healing problems.

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J. Gen. Physiol. 26: 391-404 (1943)

STUDIES ON THE MECHANISM OF HYDROGEN TRANSPORT IN ANIMAL TISSUES

VI. Inhibitor Studies with Succinic Dehydrogenase*

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(Received for publication, November 12, 1942)

In 1938, Hopkins and coworkers (1, 2) showed that succinic dehydrogenase could be inactivated by oxidized glutathione (GSSG) and could be reactivated by reduced glutathione (GSH). They interpreted these results to mean that the active enzyme requires intact —SH groups and that when these are converted to the —S—S— form of the enzyme, the dehydrogenase is inactivated. Any assumption that the functioning of the enzyme involved an oscillation between the SH and the —S—S— form of the enzyme seemed to be definitely eliminated, however, by the fact that the —S—S— form could not be reduced by succinate. Thus the function of the SH group in succinic dehydrogenase has remained an unsolved problem.

Although many proteins contain SH groups, very little is known about the structural relationship of the SH group to the rest of the molecule. Even in the case of egg albumin, in which the SH groups have received the most careful study, the mechanism by which the SH groups of native egg albumin are shielded from some sulfhydryl reagents and not from others remains obscure (3). In the case of succinic dehydrogenase, the presumptive SH group (1, 2) is associated with function, and the reaction of the protein with sulfhydryl reagents should be demonstrable on the basis of determinations of the amount of active enzyme remaining. Furthermore, since it is an oxidative enzyme, the measurement of oxygen uptake makes possible a continuous appraisal of the amount of active enzyme at any given moment. We have previously established the test conditions for the measurement of the activity of this enzyme (4, 5). The rate of oxygen uptake is a valid measure of succinic dehydrogenase activity in this system since cytochrome *c* and cytochrome oxidase, which are needed to complete the reaction with oxygen, are present in excess. The activity of the enzyme is so great under the proper conditions that the extraneous matter present in the enzyme preparation does not interfere with the study of the reaction.

At present, inhibitor studies appear to constitute the only available means of establishing the presence of SH groups in succinic dehydrogenase and of determining their rôle in the function of the enzyme.

* This work was supported by the Jonathan Bowman Fund for Cancer Research.

In the work to follow, the nature of the SH groups in succinic dehydrogenase has been studied by testing the inhibitory action of some 38 different chemical compounds over a wide range of concentrations and in the presence and absence of the substrate. In addition, malonate, which is itself an inhibitor, was shown to decrease the action of other inhibitors. On the basis of the inhibitor experiments, it has been possible to approximate the structure of the succinate-activating center of the dehydrogenase and to provide an explanation for the earlier results of Hopkins, thus reopening the possibility that the SH group may be involved in hydrogen transport in this enzyme.

EXPERIMENTAL

Enzyme System.—Studies in this laboratory (4, 5) have led to the development of an enzyme preparation in which the components of the succinoxidase system are believed to approximate their native functional activity more closely than in systems hitherto studied. Rat liver homogenates were freshly prepared and kept in ice water until pipetted. The reaction mixtures contained 0.8 ml. of 0.1 M sodium phosphate pH 7.4, 0.4 ml. of 10^{-4} M cytochrome *c*, 0.3 ml. of 4×10^{-3} M $CaCl_2$, 0.3 ml. of 4×10^{-3} M $AlCl_3$, 0.3 ml. of 0.5 M recrystallized sodium succinate pH 7.4, 0.1 ml. of 10 per cent liver homogenate in 0.033 M sodium phosphate pH 7.4, plus water and inhibitor solution to give a final volume of 3.0 ml. About once a week the calcium, aluminum, and cytochrome were made up into one solution of which 1 ml. was pipetted into each flask. All solutions were stored in the cold and kept in ice water during the pipetting. The rate of oxygen uptake was measured in a conventional Warburg apparatus at 38°C. The need for the various components has been previously demonstrated (4, 5). The enzyme system as set up will not oxidize succinate beyond the fumarate stage.

Inhibitor Experiments.—During the course of this work, a wide variety of compounds were tested for toxicity to the succinoxidase system. The results are summarized as briefly as possible in Table I. Although the concentration of inhibitor was varied over a wide range, only one concentration is reported. In most cases, the presence of succinate decreased the action of the inhibitors; therefore, the enzyme was incubated in the absence of its substrate, and the activity was measured by delaying the succinate addition. In these cases, less inhibitor was required for a given amount of inhibition than in the case of experiments in which succinate was present at the beginning. In the delayed succinate experiments, the action of the inhibitor was usually complete by the time the substrate was added, while in those cases where succinate was present from the beginning, the per cent inhibition usually increased with time. In the latter experiments, it is thus necessary to specify the time at which the per cent inhibition was computed, and in Table I the percent inhibition is stated for the second and fourth 10 minute intervals. Although most of the inhibitors would give 100 per cent inhibition if added in sufficient quantity, an attempt was made to report those concentrations which gave 50 to 80 per cent

TABLE I.

Inhibition of Succinoxidase

Test system described in text. Inhibition calculated from Q_{O_2} values, with controls in each run.

No.	Compound	Substrate added at 20 min.		Substrate added at the beginning		
		Inhibitor concentration	Inhibition	Inhibitor concentration	Inhibition 10-20 min.	Inhibition 20-40 min.
	Quinones and related compounds					
			per cent		*per cent*	*per cent*
1	Quinone	M/100,000	65	M/30,000	50	68
2	1,4 Naphthoquinone	M/100,000	43	M/30,000	21	30
3	2-Methyl naphthoquinone	M/10,000	53	M/1,000	44	45
4	9:10 Anthraquinone	Saturated	0	Saturated	0	0
5	Hydroquinone	M/100,000	65	M/30,000	11	35
6	Catechol	M/100,000	35	M/30,000	3	5
7	Resorcinol	M/10,000	0	M/1,000	0	0
	Azo compounds and diamines					
8	*p*-Phenylenediamine	M/100,000	65	M/30,000	26	54
9	*p*-Aminophenol	M/30,000	43	M/100,000	17	46
10	*o*-Phenylenediamine	M/30,000	37	M/1,000	7	7
11	N-Methyl-*p*-phenylenediamine	M/100,000	65	M/30,000	43	72
12	*p*-Aminodimethylaniline	M/100,000	71	M/30,000	45	77
13	2-Methyl N^4, N^4 dimethyl 1,4 phenylene-diamine (meta)*	M/100,000	72	M/30,000	15	26
14	2-Methyl N^1, N^1-dimethyl 1,4 phenylenediamine (ortho)*	M/100,000	68	M/30,000	0	0
15	*p*-Aminoacetanilid	M/1,000	4	M/100	50	53
16	Sulfanilamide	M/100	0	M/100	0	0
17	*p*-Nitrosodimethylaniline	M/2,000	4	M/2,000	22	18
18	*p*-Aminoazobenzene	M/2,000	43	M/2,000	36	35
19	*p*-Dimethylaminoazobenzene‡	M/200	55	M/200	25	24
20	Methyl orange	M/1,000	33	M/200	42	48
21	Methylene blue	M/3,000	63	M/3,000	20	20
	Sulfhydryl reagents					
22	Iodine	M/3,000	52	M/3,000	4	4
23	Iodoacetate	M/1,000	52	M/100	17	31
24	Iodoacetamide	M/1,000	90	M/100	68	83
25	Maleic acid	M/25	17			
26	*p*-Chloro-mercuro-benzoic acid	M/100,000	30	M/30,000	63	70
27	Ferricyanide	M/1,000	89	M/1,000	32	58
	Metal cations					
28	$Copper^{++}$	M/100,000	64	M/30,000	67	83
29	$Zinc^{++}$	M/100,000	30	M/30,000	56	63
30	$Iron^{+++}$	M/3,000	29	M/1,000	32	58
	Toxic anions					
31	$Selenite^{--}$	M/100,000	46	M/30,000	11	20
32	$Arsenite^{---}$	M/10,000	25	M/1,000	16	42

* Obtained through the kindness of Dr. C. J. Kensler, Memorial Hospital, New York.

‡ Dissolved in corn oil and homogenized with the tissue. Corn oil or dye non-toxic alone.

inhibition for the majority of the compounds and to report the inhibition obtained at this same concentration with compounds which were less toxic.

Although it is not possible to discuss each inhibitor at length and to give all the reasons which led to their selection, it may be stated that each compound serves as a control for one or more other compounds in the series, and all of the compounds studied are reported for the sake of completeness. Of the 38 compounds listed, certain key compounds enable one to deduce the nature of the active center in succinic dehydrogenase.

It is desirable at the outset to emphasize the oxidative capacity of the enzyme system. Many of the compounds used are in the reduced form (*p*-phenylenediamine, *p*-aminophenol, hydroquinone, etc.), but in the presence of cytochrome *c* they are rapidly oxidized. It seems certain that the toxicity of these compounds arises from their properties in the oxidized state, as Potter (6) demonstrated in studies with the urease system. This conclusion is supported by the results with the succinoxidase system.

The key compound of the series is *quinone* (*p*-benzoquinone) (No. 1 in Table I), and 21 of the 38 compounds studied are related to this substance. Of the 21 compounds, those which are toxic either contain the quinonoid structure or are converted to a quinonoid structure in this system. It seems likely that any quinonoid compound will inhibit succinic dehydrogenase but that additional factors such as solubility, molecular size, and configuration lower the toxicity as the compound deviates from the properties of quinone. Thus it will be seen that quinone is the most toxic compound in the quinonoid series, although many of the diamines are equally toxic (the difference between 65 per cent and 72 per cent inhibition is not considered significant). Naphthoquinone (No. 2) is nearly as toxic as quinone, but the introduction of a methyl group to give *2-methyl naphthoquinone* (artificial vitamin K) (No. 3) decreases the toxicity almost tenfold.[1] It has been shown (7) that quinone will combine with the SH group, and Fieser (8) has carried out a number of chemical studies demonstrating the same reaction with 2-methyl naphthoquinone. Although the 2-methyl naphthoquinone was also shown to react with certain alcohols and amino acids, it is interesting to note that Fieser concluded, "it is likely that methylnaphthoquinone can combine with proteins most readily by utilization of the sulfhydryl groups rather than the ε-amino groups." In the case of the next compound, *9:10 anthraquinone* (No. 4), no toxicity whatsoever could be observed (Table I), and it is obvious that the reaction postulated for quinone could not occur with this compound.

[1] The toxicity observed clinically with high doses of this compound (8) may be due to interaction with succinic dehydrogenase or a similar enzyme. The tenfold decrease in toxicity resulting from the introduction of one methyl group provides a explanation for the absence of clinical toxicity with *natural* vitamin K since it contains a long side-chain at the 3 position in addition to the 2-methyl group, and reaction with a sulfhydryl group is manifestly impossible.

Turning now to the various quinols, it is seen that *hydroquinone* (quinol) (No. 5) is just as toxic as quinone under strongly oxidizing conditions. When oxidation is less because of succinate, the compound is less toxic than quinone. This result, plus the data on quinone, proves that the action of succinate is twofold: it protects the enzyme against the inhibitors directly, and it slows the conversion of reduced compound to the toxic oxidized form. Catechol (No. 6), which is oxidized to the orthoquinone, further demonstrates this point. Resorcinol (No. 7), which is not autoxidizable and does not reduce cytochrome *c*, is not oxidized in this system. Thus it cannot be converted to the quinonoid structure and is completely non-toxic.

A number of compounds in which the OH groups of the quinols are replaced by amino groups have as the key compound *p*-phenylenediamine (No. 8); this is analogous to hydroquinone. A mixed compound is *p*-aminophenol (No. 9) in which only one of the OH groups is replaced by —NH_2. The ortho structure is represented by *o*-phenylenediamine (No. 10). Methyl groups can be substituted for hydrogen on the ring or in the amino groups (compounds 11 to 14) without loss in toxicity. Compound 14 is not autoxidizable and gives no inhibition when its oxidation by cytochrome is hindered by succinate. All of the *toxic* amino compounds are oxidized to the *quinonoid* structure. Various deviations which decrease the ease of conversion to the quinonoid structure result in great decreases in toxicity. This is most striking in the case of *p*-aminoacetanilide (No. 15) which is essentially non-toxic and is not oxidized to the quinonoid structure in this system. It may be contrasted with *N*-methyl-*p*-phenylenediamine (No. 11) which has a very similar structure but is very toxic and is readily oxidized to the quinonoid diimine. Further illustration of this point is sulfanilamide (No. 16) and *p*-nitrosodimethylaniline (No. 17) which are not oxidized and the azotized compounds *p*-aminoazobenzene (No. 18) and *p*-dimethylaminoazobenzene (No. 19). The latter compound is quite insoluble in water and, when sulfonated, forms methyl orange (No. 20) which is water-soluble and much more toxic than its parent compound though it has less than 1 per cent of the toxicity of quinone. It forms the quinonoid structure but is probably less toxic because of the size of the molecule. The same is probably true for methylene blue (No. 21) which is toxic and possesses the quinonoid structure. Cedrangolo and Adler (9) have shown that methylene blue will oxidize the SH of cysteine and glutathione and that it will inhibit triosephosphate dehydrogenase. They suggested that the mechanism of the inhibition was interaction with the SH of the enzyme. A number of other oxidation-reduction dyes have been shown (10) to be toxic for the succinic system, with toxicity being a function of the potential of the dye. Phenothiazone (11) and pyocyanine (12) also inhibit succinic dehydrogenase. All of the toxic compounds mentioned thus far possess the quinonoid structure. Although they might conceivably react with some group other than the SH group of the enzyme, the evidence seems to point to the SH group.

However, it seems desirable to consider the effect of other compounds which are toxic but which do not possess the quinonoid structure. A number of reagents have been used to titrate the SH groups of proteins such as egg albumin or to inhibit enzymes on the basis of interaction with SH. The literature on most of these compounds has been reviewed by Hellerman (13, 14). The compounds include iodine (No. 22), iodoacetic acid (No. 23), iodoacetamide (No. 24), maleic acid (No. 25), *p*-chloro-mercuro-benzoic acid (No. 26), and ferricyanide (No. 27). Several of these compounds have been shown to react *stoichiometrically* with the SH groups of denatured egg albumin (3) but with varying degrees of completeness in the case of the native egg albumin. It seems reasonable to conclude that they react with the SH groups of succinic dehydrogenase in a similar manner and that the latter are simply less accessible to these compounds than to quinone.

Many metallic ions are known to react with sulfhydryl groups (2, 13, 14). Experiments with copper (No. 28), zinc (No. 29), and iron (No. 30) are included in Table I. The ferric ion is much less toxic than zinc and copper. The latter is stoichiometrically equivalent to quinone, and the rate of inactivation is about the same. The ability of copper to react with thiols has been amply demonstrated by Pirie (15) who prepared a number of crystalline copper derivatives of SH compounds. In addition to the heavy metal cations which react with thiols, certain anions will also combine with the SH grouping. These are the selenite ion (No. 31) and the arsenite ion (No. 32). Bersin (16) reported that thiol compounds will combine with selenite, while Johnson and Voegtlin (17) demonstrated their combination with arsenite. The latter workers prepared a number of crystalline derivatives and postulated that the toxic action of arsenite is due to chemical combination with cellular SH compounds essential to life.

The above 32 compounds represent quinonoid structures, SH reagents, metal cations, and anions. The most toxic compounds are quite similar in their action on the basis of rate of reaction, final inhibition, and effective molarity. There can be little doubt that the common denominator of all these inhibitors is their reaction with the sulfhydryl group of succinic dehydrogenase. The situation is similar to that with egg albumin, concerning which Anson (3) stated, "the SH group, however, is the only protein group known to react with both oxidizing agents and heavy metal compounds."

In the case of the studies with urease (6), it was concluded that the mechanism of reaction between the SH groups and the inhibitors was more likely to be a combination rather than an oxidation of EnSH to En—S—S—En. The same conclusion is reached in the present instance and, since the line of reasoning is the same, it need not be repeated here. The inactivation by combination with SH probably occurs with all the quinonoid compounds, SH reagents, metal cations, and toxic anions and is represented diagrammatically in

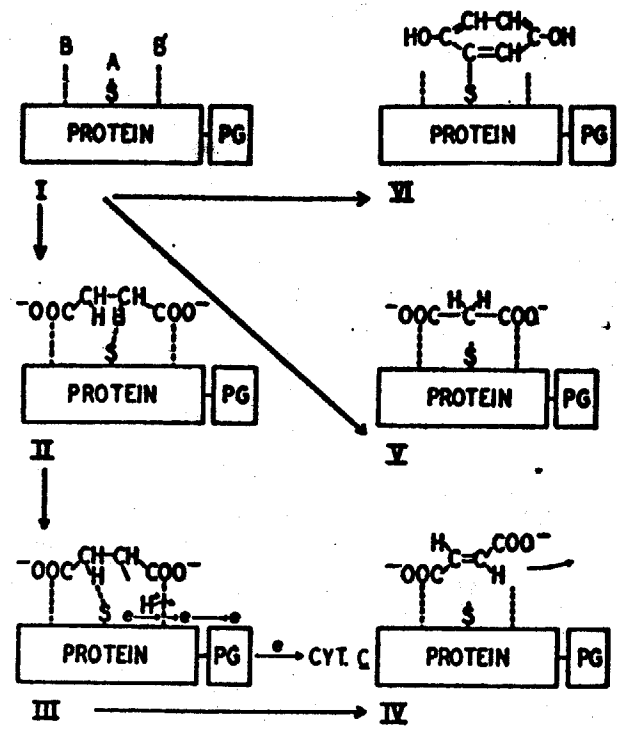

FIG. 1. Schematic representation of the structure of succinic dehydrogenase on the basis of *mutually exclusive* inhibitor reactions. The protein is succinic dehydrogenase, and PG represents its hypothetical prosthetic group.

I = enzyme showing the succinic acid activating center. A is a sulfhydryl amino acid, *e.g.*, cysteine, in a peptide chain; B and B' are the carbonyl affinity points, *e.g.*, —NH— groups of adjacent peptide linkages, capable of forming hydrogen bonds with the carbonyl oxygens.

II = enzyme-succinic acid complex in first stage of succinic acid oxidation.

III = enzyme-succinic acid complex in a possible intermediate stage of succinic acid oxidation.

IV = enzyme-fumaric acid complex resulting from the oxidation of succinic acid.

V = enzyme-malonic acid complex. Only the carbonyl affinity points are involved. Inhibition reversible. Enzyme-sulfhydryl *shielded* by malonate and thereby protected against sulfhydryl reagents.

VI = enzyme-quinol complex, as an example of inhibition by a sulfhydryl reagent (quinone). Analogous complexes formed with other quinonoid compounds, thiol reagents, thiols, heavy metals, arsenite, and selenite.

Fig. 1, stage VI, using quinone as the inhibitor. In the case of urease, it was possible to *prevent* but not to reverse the inactivation due to quinones by adding cysteine to the reaction mixture. This fact gives further support to the

postulated mechanism. However, such experiments are not possible with the present succinic system since, in contrast to the urease system, cysteine is oxidized to a form which inactivates the succinic dehydrogenase as is shown in the next section.

Inhibition by Sulfhydryl Compounds.—In the original experiments by Hopkins *et al.* (1, 2), it was found that R—S—S—R would inactivate succinic dehydrogenase and that RSH would restore the activity. We have tested our succinoxidase system and have found that both cysteine and cystine inactivate the enzyme. Reduced glutathione will cause some inactivation (see Table II). The apparent disagreement with Hopkins *et al.* is only superficial, however, and is easily understood when the respective experimental techniques are considered. Hopkins and his coworkers added large amounts of the sulfhydryl compounds to washed muscle pulp, incubated the mixture, and then washed out the excess of soluble sulfhydryl compound on a Buchner funnel. Thus, the SH compound was never present when the activity of the enzyme was being tested. In our work, we used considerably less enzyme in much greater dispersion so that in fact it could not be washed on a funnel and recovered. Thus, we add much less sulfhydryl and leave it in the final reaction mixture, where it is converted into the oxidized form. Separate experiments in the absence of the enzyme show that RSH is oxidized by cytochrome *c*, and it seems highly probable that the free sulfhydryl radical RS· must be formed as an intermediate in such a reaction even though it dimerizes rapidly to R—S—S—R (14). It seems more likely that the enzyme is inactivated by the formation of En—S—S—R than by oxidation to En—S—S—En (in Hopkins' experiments as well as our own). In either case, the reactivation is undoubtedly due to the splitting of the disulfide linkage by RSH to reconstitute En—SH. Table II also shows

TABLE II

Inhibition of Succinoxidase by Sulfhydryl Compounds

Substrate addition delayed for 30 minutes. Egg albumin "molarity" is the cysteine molarity of the albumin used. Test system as described in text. Activity based on the 10 to 40 minute period after addition of substrate.

Inhibitor molarity	Inhibition				
	Cystine	Cysteine	Reduced glutathione	Egg albumin	
				Native	Denatured
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
M/500		93	18	0	0
M/1,000		69	0	0	0
M/3,000	51*	55*	0	0	0

* Inhibition was zero when succinate was added originally.

that native egg albumin, which will not reduce cytochrome *c*, is non-toxic at concentrations where cysteine is effective. Denatured egg albumin has free SH groups which rapidly reduce cytochrome *c*, but due to steric hindrance these oxidized SH groups cannot react with the SH of native succinic dehydrogenase as effectively as cysteine. However, when the concentration of egg albumin, native or denatured, is raised to higher levels, succinic dehydrogenase can be inhibited. This fact is perhaps analogous to the fact that large quinonoid molecules are much less toxic than quinone but are nevertheless toxic (Table I). The rate of inactivation of the dehydrogenase by the smaller sulfhydryl compounds in the presence of cytochrome system is about the same as the inactivation by copper, quinone, and all of the other inhibitors which are believed to combine with the SH group of the enzyme. It is generally agreed that inactivation of an enzyme by R—S—S—R is due to interaction with the sulfhydryl of the enzyme (1, 2, 13, 14), and such a conclusion is indicated here. Thus, inhibition of the enzyme by the oxidized sulfhydryl compounds appears to proceed by the same mechanism as in the case of the quinonoid compounds, heavy metals, sulfhydryl reagents, arsenite, and selenite.

Inhibition by Malonate.—In contrast to all of the previously mentioned inhibitors which are believed to inactivate the succinic dehydrogenase by combining with the SH group of the enzyme, malonic acid appears to inhibit succinic dehydrogenase by an entirely different mechanism. Malonate is a strong inhibitor of the succinic dehydrogenase because it possesses two —COOH groups and a configuration very similar to that of succinate (18, 19). Previous experiments with minced tissue or slices appear to be complicated by diffusion effects and require up to 0.025 M malonate to block the enzyme completely (20). According to Krebs and Eggleston (20), the inhibition is competitive; *i.e.*, it does not depend upon the absolute concentration of malonate but on the ratio, succinate/malonate. These workers found an inhibition of 50 per cent when the ratio was 9.5/1. The data in Table III confirm their observation that inhibition by malonate is competitive. However, when these data are used to calculate the succinate/fumarate ratio at the points of 50 per cent inhibition, one obtains values of 57.2, 51.8, and 50.6 for the three succinate concentrations employed. These ratios represent a close approximation of the ratios between the dissociation constants for the enzyme-succinate and enzyme-malonate complexes and are about 10 times as high as those reported by Krebs and Eggleston. The difference is probably due to the fact that in our preparation the enzyme is operating at maximum activity. The data support the view that malonate and succinate form similar dissociable complexes with succinic dehydrogenase and that the same affinity points are involved. The nature of these complexes is illustrated diagrammatically in Fig. 1, stages II (succinate) and V (malonate). That the affinity points involve the two carboxylic acid groups is indicated by previous work (2, 18, 19). There is no evidence contrary

to the statement by Hopkins *et al.* (2) that "malonic acid . . . apparently establishes no special relations with thiol groups."

Shielding Action of Malonate.—Hopkins *et al.* reported that malonate and succinate would prevent the inactivation of succinic dehydrogenase by GSSG. We have confirmed the protection by succinate against the SH inhibitors (Table I) and against cysteine and cystine (Table II) and now present evidence demonstrating protection of the enzyme by malonate against the action of quinone. (See Fig. 2.) Similar results were obtained with *p*-phenylenediamine. The data in Fig. 2 also show the difference between the malonate and the quinone inhibition. The results are expressed in terms of the Q_{O_2}, and the observed rate for each 10 minute period is plotted against time. The Q_{O_2} value

TABLE III

Inhibition of Succinoxidase by Malonate. Effect of Substrate Concentration

Test system described in text. Succinate and malonate both added prior to enzyme. Q_{O_2} calculated on basis of 1st 40 minutes.

Substrate concentration	Malonate concentration			
	0	M/300	M/1,000	M/3,000
	Q_{O_2}	Q_{O_2}	Q_{O_2}	Q_{O_2}
M/15	101	30	61	79
M/20	102	24	49	74
M/30	101	13	36	63

thus gives the amount of active enzyme present at any given moment. A detailed examination of Fig. 2 shows the following:

(*a*) Quinone inhibits progressively as shown in curve 2, with almost no inhibition originally and approaching complete inhibition after 60 minutes. When the enzyme is not protected by succinate, the rate of inactivation is at least twice as rapid, as shown by curve 4. These results show that succinate protects the enzyme against quinone and that, when the quinone combines with enzyme, the combination remains inactive. Apparently quinone can react only with enzyme molecules which are not combined with succinate.

(*b*) Malonate exerts its action at once, and the per cent inhibition remains constant (curve 3); the inhibition is no greater when the malonate is incubated with the enzyme in the absence of succinate (curve 5). These results, together with the data in Table II, show that malonate forms a dissociable complex with the enzyme which is comparable to the true enzyme-substrate complex and, furthermore, that the same affinity points are involved for succinate and malonate; that is, malonate does not damage the enzyme but merely competes with succinate for the succinate affinity points.

(*c*) Malonate can prevent quinone from acting on succinic dehydrogenase

whether succinate is added at once (curve 6) or after an incubation period (curve 7). Since the dissociation constant for the enzyme-malonate complex is only about one-fiftieth that of the enzyme-succinate complex (see above), the failure of quinone to inhibit in the presence of malonate is probably due to the fact that there are virtually no uncombined enzyme molecules available

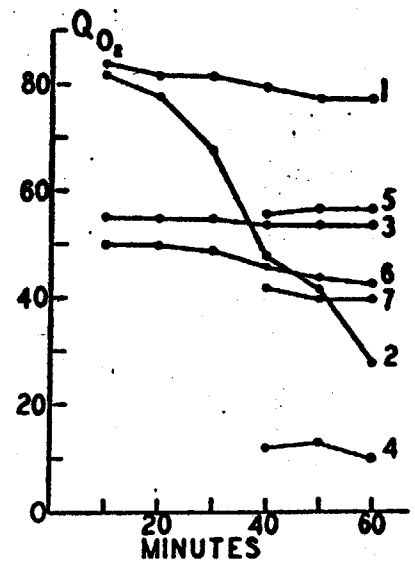

FIG. 2. Shielding of succinic dehydrogenase by malonate against quinone. Rate of inactivation of succinoxidase system (*i.e.*, rate of change in Q_{O_2}) as effected by malonate and quinone, separately and together, with original and delayed succinate additions. See Fig. 1, stages I, II, V, and VI for graphic interpretation of results. Test system as described in text. Final inhibitor concentrations: malonate, M/3,000; quinone, M/50,000. 1, control—neither inhibitor added; succinate originally present. (Loss in enzyme activity is negligible when succinate addition is delayed 35 minutes.) 2, quinone and succinate originally present. 3, malonate and succinate originally present. 4, quinone originally present; succinate added 35 minutes later (rate during next 10 minutes not plotted). 5, malonate originally present; succinate added 35 minutes later (rate during next 10 minutes not plotted). 6, malonate, quinone, and succinate originally present. 7, malonate and quinone originally present; succinate added 35 minutes later (rate during next 10 minutes not plotted).

for the quinone to attack; that is, the malonate shields the enzyme against the quinone. Curves 6 and 7 could also be explained by assuming that malonate reacts with quinone directly and thereby prevents it from exerting its effect. That such is not the case, however, was proved by the following experiment which was suggested by previous experiments with urease (6) showing that this enzyme is very sensitive to quinone: urease was tested with malonate and

quinone since, if the action of malonate in the succinic dehydrogenase system were due to reaction with quinone rather than combination with the dehydrogenase, it should also protect urease against quinone. The results showed that malonate does not inhibit urease and therefore has no affinity for this enzyme, and furthermore, it has no ability to protect urease against the action of quinone.

DISCUSSION

The data presented above deal with the inhibition of succinic dehydrogenase by a group of diverse substances which are believed to act by combining with the SH group of the enzyme, and in contrast to these, a compound, malonic acid, which is believed to act by competing for the carboxylic acid affinity points which form the basis of the succinate-enzyme complex. These fundamentally different mechanisms of combination may be shown schematically as in Fig. 1, in which quinone as an example of the SH inhibitors is shown in combination with the enzyme SH group (stage VI), while succinate and malonate combine with the enzyme on the basis of their —COOH groups (stages II and V).

Experimentation with inhibitors *whose action is mutually exclusive* seems to afford one of the most promising approaches now available for the study of the stereochemistry of enzymes. Though other possibilities most certainly may exist, it is difficult to explain the ability of malonate to protect succinic dehydrogenase against quinone in any terms other than those depicted in Fig. 1, in which the SH group is located *between* the carboxyl affinity points in such a way that, when malonate or succinate is combined with the enzyme, the SH group is *shielded* against attack by the SH reagents.

Hopkins *et al.* demonstrated three facts of paramount interest to this problem: (*a*) malonate will protect active succinic dehydrogenase against inactivation by GSSG; (*b*) enzyme which has been inactivated by treatment with GSSG may be reactivated by treatment with GSH; and (*c*) malonate will *not* prevent GSH from reactivating enzyme which has been inactivated by GSSG. These authors were unable to explain these seemingly paradoxical results and stated only, "That malonic acid protects from GSSG so completely and from GSH not at all suggests structural relations which may prove instructive." We believe that their observations constitute a contribution of fundamental importance which our own work confirms[2] and extends. Furthermore, on the basis of the concept illustrated in Fig. 1, it is easy to see why the results of Hopkins were obtained: if the SH group is located between the carboxyl affinity points, it is obvious that neither succinate nor malonate could approach the carboxyl affinity points (B and B′) when the enzyme is combined with an SH reagent while, on the other hand, if the enzyme is combined with succinate or malonate, the SH reagents cannot approach and react with the SH group.

[2] We do not present data on reactivation of GSSG-inactivated enzyme by GSH and the effect of malonate upon this reaction because of technical differences between our preparation and that of Hopkins.

While it is impossible to provide an answer as to the possible nature of the enzyme groupings which constitute the carboxyl affinity points, it is interesting to attempt a reconstruction of a peptide chain containing cysteine as the SH bearer. When this was done with accurate (Hirschfelder) atomic models, it was found that the —NH— groups adjacent to cysteine were opposite the carbonyl oxygens of the carboxyl groups, while the SH group fell between the —CH_2—groups of succinate. In the case of malonate, the carbonyl oxygens again approximated the —NH— groups, but the —CH_2— group was directed away from the SH, and both hydrogen atoms were on the side away from the SH.

The remainder of Fig. 1 is pure speculation but, since the structural relations shown in stages I, II, V, and VI seem to fit so well with the facts, it seems plausible to suggest that the location of the SH of the enzyme is of functional significance; *i.e.*, that it is involved in the actual mechanism of oxidation of succinate as is indicated in stages II, III, and IV. According to this concept, the enzyme would function by oscillating between the EnSH and EnS· forms, rather than between the thiol and disulfide forms. Succinate would be oxidized to the free radical monodehydrosuccinate in the first step of the oxidation, which would proceed in accordance with the principle of compulsory univalent oxidation described by Michaelis and Smythe (21). The electron might pass to the prosthetic group of the dehydrogenase intramolecularly without the intermediate dissociation of the semioxidized succinate. The idea of intramolecular electron transfer through a protein is not new but has been discussed by Muller (22) and by Szent-Gyorgyi (23) in general terms. The second electron would be disposed of in the same manner as the first, and the two hydrogen atoms would enter the medium as hydrogen ions. Thus the mediation between the two-electron dehydrogenation of the substrates and the one-electron transfer of the cytochrome system could be accomplished.

Whether the above concept is correct or not will remain for future research to ascertain. At any rate, the fact that the enzyme in the disulfide form (whether this be En—S—S—En or En—S—S—R) cannot be reduced by succinate seems, in view of the evidence presented in this paper, no longer to be a sufficient basis for the elimination of the SH groups from the oxidative mechanism of succinic dehydrogenase. The concept that the function of this enzyme involves the alternate oxidation and reduction of EnSH and En—S· may provide the explanation for the rôle of thiol groups in the mechanism of hydrogen transfer, which has been the goal of Hopkins since his discovery of glutathione and for which his papers on succinic dehydrogenase appear to have opened the way.

SUMMARY

1. The mechanism of succinic dehydrogenase action was studied by means of inhibitors.

2. The enzyme is inhibited by a large number of diverse compounds whose only common denominator appears to be their ability to react with SH groups. These compounds include quinonoid structures, sulfhydryl reagents, sulfhydryl compounds, copper, zinc, selenite, and arsenite.

3. In contrast to the above inhibitors, the action of malonate does not appear to involve sulfhydryl groups and is explained on the basis of its affinity for the enzyme groups which react with the carboxyl groups of succinate.

4. The action of malonate and the sulfhydryl reactants is *mutually exclusive*, and this fact suggests the conclusion that the sulfhydryl group of the enzyme is located between the carboxyl affinity points.

5. On the basis of the deduced structure of the succinate-activating center of the enzyme, it is suggested that the enzyme may function by oscillating between the EnSH and EnS· forms, rather than by a thiol-disulfide equilibrium.

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COMPARATIVE METABOLISM OF RADIONUCLIDES IN MAMMALS—I

UPTAKE AND RETENTION OF ORALLY ADMINISTERED Zn^{65} BY FOUR MAMMALIAN SPECIES*

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Received 7 March 1962; in revised form 27 July 1962

Abstract— Uptake and retention of a single oral administration of $Zn^{65}Cl_2$ in mice, rats, dogs, and 4 human subjects were studied by whole-body counting methods. Retention functions for each species were accurately represented by multiple rate equations composed of three exponential terms. The average effective half-time of the longest component (which accounts for approximately 95 per cent of all the disintegrations occurring in the body) of the retention curve for man was 154 days with a range of 149–161 days. A parabolic relationship between species weight and equilibrium level for chronic exposure was derived and used to estimate Zn^{65} MPC_w values for man. The average MPC_w value (168 hr/week exposure) obtained directly from the human data by usual methods was 3.1×10^{-4} μc/ml with a range of 1.7–4.7 $\times 10^{-4}$ μc/ml compared with 2.4×10^{-4} μc/ml when derived from interspecies correlation data.

INTRODUCTION

ZINC is known to be an essential trace element. It functions as a prosthetic group in enzymes concerned with proteolysis and with gaseous and cellular respiration. Zinc may also participate in erythropoiesis and in myelogenous bone marrow function.[1]

Although Zn^{65} is not a product of nuclear fission, it is produced by neutron activation of stable zinc in power reactors and nuclear detonations. Zn^{65} also appears to be produced as a contaminant in cyclotrons by the $Cu^{65}(d, 2n)Zn^{65}$ reaction. Trace amounts of this radionuclide have been detected in people and in foodstuffs from the Hanford area,[2,3] in animals from the vicinity of the Nevada Test Site,[4] in cyclotron workers at this and other laboratories,[5] in reactor workers,[6] and in human beings[7,8] and marine organisms[9,10] from the Marshall Islands. In addition to these geographically isolated cases, Zn^{65} has been detected in plankton and in fish from many areas of the Pacific Ocean[11–13] and in commercial food supplies.[14, 15]

Presence of Zn^{65} in food webs and in people as a contaminant from nuclear weapon tests, reactor effluents, and nuclear research operations motivated the present investigation. This report summarizes comparative metabolic data obtained from four mammalian species and presents an interspecies correlation as a possible basis for deriving, from laboratory animal data, certain parameters of radionuclide metabolism in man.

METHODS AND MATERIALS

Whole-body liquid scintillation counters were used to study uptake and retention of Zn^{65} by mice, rats, dogs and man. Radio-zinc was administered as a single dose of $Zn^{65}Cl_2$. Uptake and retention following oral administration were studied in all species. Additional groups of mice and rats were given Zn^{65} via intravenous injection to assess the validity of the common practice of using rate constants derived from intravenous studies to derive protection guides for radionuclide concentrations in food and in water. The number of animals used, their average ages and weights, routes of administration, and amounts of Zn^{65} administered are given in Table 1. Thirty minutes after

* Work performed under the auspices of the U.S. Atomic Energy Commission.

Zn^{65} administration and at intervals thereafter, all animals were counted in 4π whole body liquid scintillation counters to determine total Zn^{65} activity and its retention as a function of time. Mice and rats were counted in the Los Alamos Small Animal Counter (LASAC I), and dogs and people were counted in the Los Alamos Human Counter (Humco I). Both detectors have been described elsewhere.[26]

Counting standards for mice, rats and dogs were prepared by diluting an amount of Zn^{65} equal to the administered dose with a weight of water equal to the average weight of the respective species. These standards were sealed in polyethylene containers and counted routinely throughout the experiments. Standards for human subjects consisted of aliquots of the administered $Zn^{65}Cl_2$ solution diluted to a volume of 1 gallon.

Whole-body retention data were coded for analysis by an IBM Model 704 computer which was programmed to perform iterative least square calculations.[41] Coding procedures called for retention values (Y) at each time (t) and estimated intercept and rate parameters of the retention function. Each value of Y was weighted statistically by a factor proportional to 1/Y. Computer analysis eliminates subjectiveness inherent to graphic analysis of retention data composed of several exponential functions.

Excreta were collected and measured to check accuracy of retention measurements by determining material balance and to compare measured excretion rate with the derivative of the retention function. Twenty-four hour urinary and fecal samples were collected from all species and diluted with a volume of water equal to the volume of the appropriate standard. Samples and standards were counted under the same geometric conditions. Human fecal samples were sealed in No. 2 tin cans containing formalin, and their measured activities were normalized also to an appropriate standard.

Studies using rats were carried out to demonstrate the time variation of Zn^{65} tissue distribution. Thirty-two adult male animals were given single oral doses of 1.2 μc Zn^{65} as the chloride and counted individually in the whole-body animal counter immediately after administration and just prior to sacrifice. Groups of 4 rats each were sacrificed at 4 hr and at 2, 8, 15, 30, 45, 60 and 112 days after Zn^{65} administration. At sacrifice, γ-activity of liver, gastrointestinal tract, testes, pelt, carcass and remainder was measured in the same geometry used to count the whole animal. The remainder consisted of the accessory sex organs, blood, fat and visceral organs. The carcass (the remains of the animal after visceral organs and pelt had been removed) consisted mainly of skeleton, muscle and some fat. After measurement, the carcass was autoclaved and skeleton and balance of carcass (primarily muscle) were separated and measured individually. Zn^{65} activity of individual tissues and organs at time of sacrifice was expressed as per cent of administered dose.

Table 1. Number of animals, age, weight and dose conditions for the study of Zn^{65} uptake and retention in 4 mammalian species

Species	Route	No.	Age at t_0	Weight (g)	Dose (μ_c)	Last measurement (days)
Mice	Oral	12	53 days	17.0 g	0.5	90
Mice	I.V.	12	46 days	15.5 g	0.5	137
Rats	Oral	6	78 days	266.0 g	1.0	164
Rats	I.V.	6	85 days	289.0 g	1.0	135
Dogs	Oral	3	777 days	10.9 kg	2.0	540
HMZn-1*	Oral		29 years	72.0 kg	1.0	664
HMZn-2	Oral		48 years	56.8 kg	0.6	579
HFZn-3	Oral		31 years	72.7 kg	0.8	431
HMZn-4	Oral		45 years	70.2 kg	0.8	416

* The code for designating human subjects indicates species, sex, nuclide, and case number (e.g. the third subject receiving zinc was a female, whereas subjects 1, 2, and 4 were males).

RESULTS

Data showing retention of orally administered Zn^{65} by mice, rats, dogs and man are presented in Fig. 1. The points represent observed average retention values expressed as per cent of administered dose as a function of time after ingestion. These data were analyzed by a computer programmed to give multiple-rate functions having the general form,

$$R_t = \sum_{i=1}^{n} [a_i e^{-k_i t}] \qquad (1)$$

where R_t is the whole-body retention on any day t, a_i and k_i are intercept and rate constants, respectively, and e is the base of natural logarithms. Units of R_t and a are per cent of administered dose, and k is expressed in reciprocal days. Since retention decreases with time, k is negative.

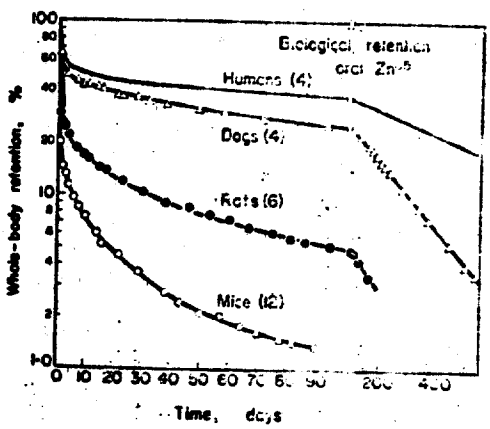

FIG. 1. Retention of orally administered Zn^{65} by 4 mammalian species.

Table 2 gives the computer-derived biological retention functions for Zn^{65} in mice, rats, dogs and 4 human subjects. The equations and measured retention values are plotted in Fig. 1 for mice, rats and dogs. The representative curve for the human subjects, which is shown merely for purposes of comparison with the other species, was obtained from the unweighted averages of the retention parameters given in Table 2. Effective retention functions (Eff. R_t) may be derived form the following expression:

$$\text{Eff. } R_t = \sum_{i=1}^{n} [a_i e^{-(k_i+\lambda)t}] \qquad (2)$$

in which λ is the physical decay constant for Zn^{65}, and other terms are the same as given in equation (1). Also, the area under each effective retention function from t_0 to t_∞ is given by the sum of the integrals $\int_0^\infty a_i e^{-(k_i+\lambda)t} dt$ and is as follows:

$$\text{total area} = \sum_{i=1}^{n} \left[\frac{a_i}{(k_i + \lambda)}\right]. \qquad (3)$$

Table 3 gives parameters of the effective retention functions for each species and the effective area under each component.

Observed and calculated retention of Zn^{65} by rats following oral administration is tabulated in Table 4. Column 1 shows the time of measurement in days. For these times, column 2 shows the average observed retention and column 3 shows the average retention derived from the computer analysis. Column 4 shows the average retention derived from an equation in which the smaller rate constants (k_2 and k_3) were taken from the computer-derived retention

Table 2. Biological retention functions of orally administered Zn^{65} for 4 mammalian species

Species	No. animals	Retention function
Mice	12	$R_t^* = 86.82\, e^{-2.3423t} + 11.19\, e^{-0.0572t} + 1.84\, e^{-0.0043t}$
Rats	6	$R_t = 77.01\, e^{-1.4872t} + 15.83\, e^{-0.0447t} + 8.84\, e^{-0.0023t}$
Dogs	3	$R_t = 51.63\, e^{-1.2208t} + 10.06\, e^{-0.0743t} + 38.74\, e^{-0.0025t}$
Man (HMZn-1)		$R_t = 38.50\, e^{-0.5203t} + 11.36\, e^{-0.1549t} + 50.15\, e^{-0.00161t}$
Man (HMZn-2)		$R_t = 9.26\, e^{-0.7432t} + 24.37\, e^{-0.0277t} + 66.63\, e^{-0.00145t}$
Man (HFZn-3)		$R_t = 61.26\, e^{-0.5807t} + 7.79\, e^{-0.0288t} + 35.63\, e^{-0.00173t}$
Man (HMZn-4)		$R_t = 71.67\, e^{-1.1645t} + 4.80\, e^{-0.0797t} + 26.13\, e^{-0.00179t}$

* $R_t = \sum_{i=1}^{n} [a_i e^{-k_i t}]$, when t is in days. These values were derived by IBM-704 computer.

Table 3. Parameters of the components of effective retention functions for Zn^{65} administered orally to 4 mammalian species*

Species		a (%)	T (days)	$k + \lambda$ (days^{-1})	Area (% days†)	Area (% of total)
Mice	1	86.82	0.30	2.3449	37.5	8.09
	2	11.19	11.55	0.0600	186.4	40.22
	3	1.84	91.18	0.0076	239.6	51.69
Rats	1	77.01	0.47	1.4700	52.4	4.01
	2	13.83	14.56	0.0476	290.7	22.23
	3	8.84	75.33	0.0092	964.8	73.77
Dogs	1	51.63	0.57	1.2237	42.0	0.77
	2	10.06	8.96	0.0772	130.0	2.38
	3	38.74	94.93	0.0073	5292.0	96.84
Man (HMZn-1)	1	38.50	0.27	2.5230	16.0	0.14
	2	11.36	4.39	0.1577	72.0	0.64
	3	50.15	154.37	0.0045	11,167.0	99.22
Man (HMZn-2)	1	9.26	0.93	0.7481	13.0	0.08
	2	24.37	22.66	0.0306	797.0	4.89
	3	66.63	161.04	0.0043	15,480.0	95.03
Man (HFZn-3)	1	61.26	1.19	0.5835	105.0	1.29
	2	7.79	23.41	0.0296	263.0	3.23
	3	35.63	151.27	0.0046	7,776.0	95.48
Man (HMZn-4)	1	71.87	0.59	1.1673	61.0	1.07
	2	4.80	8.40	0.0825	58.0	1.01
	3	26.13	149.98	0.0046	5,654.0	97.93

* Effective retention function, Eff. $R_t = \sum_{i=1}^{n} [a_i e^{(-k_i - \lambda)t}]$.

† Total area under Eff. $R_t = \sum_{i=1}^{n} \left[\frac{a_i}{(k_i + \lambda)}\right]$.

equation obtained from intravenous administration experiments. The good agreement shown between columns 2 and 3 indicates an adequate mathematical description of the observed data. Also, the agreement between columns 2 and 4 suggests that retention of assimilated Zn^{65} is not influenced by the route of administration. Although the computation is given only for Zn^{65} retention by rats, similar results were obtained when oral and intravenous retention data for mice were analysed and compared in the same way.

Urinary and fecal samples were collected from all animals and were measured separately, after which the results were summed for each species and each collection period. Fecal excretion constituted the principal mode of Zn^{65} elimination for all 4 species. The average per cent of administered dose excreted per day in urine plus feces was compared with the first derivative of the biological retention function,

$$\frac{dR_t}{dt} = \sum_{i=1}^{n} [-k_i a_i e^{-k_i t}] \qquad (4)$$

in which $\frac{dR_t}{dt}$ is the rate of change of retention or total excretion rate. A comparison of $\frac{dR_t}{dt}$ for dogs with the observed average Zn^{65} urinary plus fecal elimination rate following oral administration is shown in Fig. 2. The whole-body activity on day 11 plus the total activity excreted to that time accounted for 96 per cent of the total administered activity. Material balance of Zn^{65} was comparable for the other species.

Data showing average Zn^{65} content of various tissues and organs of the rat as a function

Table 4. Comparison of observed and calculated retention of Zn^{65} in rats following oral administration

Time (days)	Retention observed after oral administration (% of dose)	Retention calculated from oral data* (% of dose)	Oral retention calculated from intravenous data† (% of dose)
0	100.00	100.00	99.69
1	39.99	39.90	39.77
2	25.54	26.11	25.48
3	24.06	22.84	21.72
4	21.95	21.43	20.41
7	18.23	19.00	18.59
9	16.96	17.65	17.60
11	16.26	16.47	16.71
15	14.26	14.51	15.12
17	13.96	13.70	14.41
23	11.94	11.77	12.59
30	10.47	10.21	10.93
38	8.99	8.98	9.49
46	8.40	8.16	8.38
53	7.76	7.51	7.62
60	7.26	7.02	7.00
67	6.55	6.60	6.49
75	6.05	6.18	5.99
81	5.61	5.89	5.67
86	5.42	5.66	5.43
95	5.13	5.29	5.05
102	4.76	5.02	4.79
109	4.69	4.76	4.55
128	4.27	4.14	3.99
164	3.46	3.17	3.15

* Derived from computer analysis.

† Calculated by substituting last 2 rate constants derived from intravenous data into equation for oral retention.

of time after oral administration are presented in Fig. 3 in which per cent of administered dose is plotted on a log scale against a linear scale of time in days.

DISCUSSION

Biological retention of Zn^{65}

Data for biological retention of orally administered Zn^{65} in mice, rats, dogs and man are accurately represented by multiple rate equations consisting of three exponential terms (Table 2). Deviations of the observed retention values from the calculated lines are quite small (Fig. 1). Individual components of the retention functions probably represent three gross rates of loss from a number of sites where zinc is bound by more than one mechanism rather than by loss from specific tissues or organs acting as separate compartments. It seems unlikely that additional components will be required to describe retention of Zn^{65} at times greater than those observed, since the human and dog data did not deviate from linearity after 100 days.

Effective retention of Zn^{65}

Effective retention functions in the four species were calculated from the biological retention data by intoducing the radiological decay constant for Zn^{65} (equation 2). The areas under the effective retention functions (equation 5) are proportional to the total number of Zn^{65} disintegrations occurring in the body and are, therefore, related to radiation dose. Evaluation of the integral from t_0 to t_∞

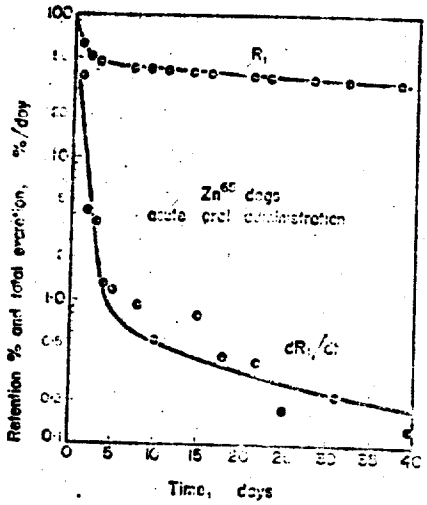

FIG. 2. Retention and excretion of orally administered Zn^{65} by dogs.

for each term of the retention equation gives the contribution of each component to total area (equation 3) and to total dose. The summed integrals also afford a prediction of the whole-body equilibrium level, in multiples of daily intake, under conditions of chronic exposure to a constant level of Zn^{65} intake. The average effective half-time of the third and last component of the retention equation for man is approximately 154 days. If equilibrium conditions were established in man under constant chronic exposure conditions, about 95–99 per cent of the Zn^{65} disintegrations

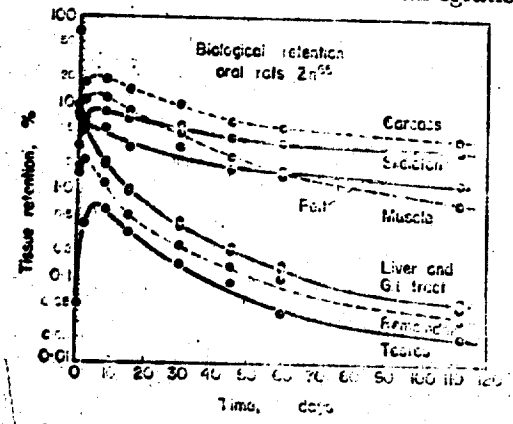

FIG. 3. Average Zn^{65} [illegible] of rat tissues as a function of time after [illegible] of Zn^{65}.

occurring in the body would be contributed by this component. Based on a 154-day half-time, an equilibrium level of about 112 times the daily intake could be established in man in about 1000 days. Miller and Steingraber[7] reported an effective half-time of 110 days for Zn^{65} in the Marshallese subjects exposed during the nuclear weapon test series in 1954. The 194 day effective half-time recommended by the ICRP[18] is based on measurements of the elimination rate of stable zinc.

Interspecies correlations in Zn^{65} metabolism

Cursory inspection of the parameters of the biological and effective retention functions suggests the possibility of some relationship between Zn^{65} metabolism and species size. The constants in the last terms of the equations

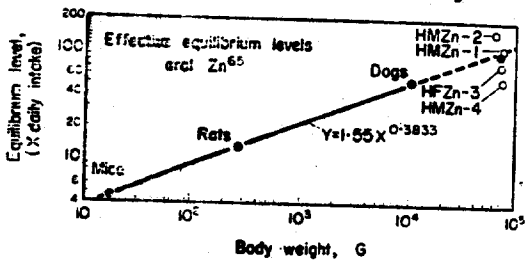

FIG. 4. Interspecies correlation between Zn^{65} equilibrium level and body weight. Regression line is based on mice, rats and dogs. Open circles represent experimentally determined values for human subjects.

show that more of the dose is retained for longer time as the size of the species increases. Likewise, the first terms indicate an inverse relationship between size and amount of Zn^{65} lost from the body at the fastest rate. Excreta analyses show that this rapid loss is largely via feces and is probably related to passage of unabsorbed Zn^{65} through the gastrointestinal tract. Although some material may be absorbed and re-excreted into the gut at early times, the administered dose minus the intercept of the first component of the retention function is assumed to be proportional to gastrointestinal absorption or uptake. The family of curves shown in Fig. 1 are a result, therefore, of combined effects of greater gastrointestinal absorption and decreased rate of excretion with increasing species weight.

An interspecific correlation between species weight and equilibrium level (derived from the areas under the effective retention functions) is shown in Fig. 4. Least squares analysis of the log transformation of equilibrium level and body weight gives,

$$\log A = 0.1903 + 0.3833 \log W, \quad (5)$$

or

$$A = 1.55\ W^{0.3833}$$

in which A is the equilibrium level in multiples of the daily intake, and W is species weight in grams. Gastrointestinal absorption also tends to increase with species weight.

For purposes of establishing interspecies correlations between biological parameters and body weight, the model of choice should be one containing only biological parameters. Using such a model the equilibrium level for stable zinc in man, extrapolated from smaller species, is about 190 times the daily intake. This value is about two-thirds the average value obtained for the human subjects. However, the radioactive decay factor must be introduced to calculate dose or to estimate radiation protection criteria. Thus, it is felt that the correlation as given in equation (5) has more practical usefulness to the health physicist.

A parabolic interspecies correlation between species weight and daily turnover of tritium water has been observed also.[19] Such correlations can be used to supplement sparse human data for derivation of radiation protection guides. ADOLPH[20] has pointed out many parabolic relationships among the anatomical and physiological constitutions of mammals and their body weights. It is quite possible that certain metabolic parameters such as uptake and retention may conform to similar relationships.

Zn^{65} *retention after oral and intravenous administration*

Although it was possible to measure Zn^{65} whole-body retention in rats and mice for reasonably long times after oral administration, Zn^{65} was also administered intravenously to these species to assess feasibility of substituting rate constants derived from intravenous administration in oral retention equations. Table 4 shows that such procedures may be valid and useful in studying retention of radionuclides which are not readily absorbed from the gastrointestinal tract and yield extremely limited retention data because of very low counting rates. Similar studies comparing retention of Sr^{85} and Ba^{133} in rats after both oral and intraperitoneal administration also validate the practice of substituting rate constants from intravenous data after normalization for oral uptake.

Excretion of Zn^{65} *after oral administration*

The good agreement found between measured daily excretion of Zn^{65} and the derivative of the retention function for dogs (Fig. 2) illustrates the accuracy and convenience of whole-body counting methods for the study of radioisotope metabolism. Activity levels of excreta samples soon fall to less than 1 per cent of the administered dose and present problems in measurement. The advantages of whole-body counting, when compared with excretion analyses for determination of retention functions (apparent in Fig. 2), include greater accuracy, less emphasis on excreta collections, and ability to make measurements of activity at longer times after administration. Agreement between total excretion and whole-body retention was equally good in mice, rats, and human subjects. Most of the Zn^{65} was excreted in the feces. It should be possible, therefore, to estimate fairly accurately the body burden of contaminated subjects solely from fecal analyses, if reliable estimates of the retention functions and date of exposure are available.

Distribution of Zn^{65} *in tissues and organs of the rat*

The data in Fig. 3 show that loss of Zn^{65} from various tissues and organs of the rat is governed by more than one rate process. Peak tissue concentrations were reached within 5 days after oral administration, and kinetics of loss were similar for all tissues other than bone and pelt. Bone and pelt hold Zn^{65} more tenaciously than do other tissues, and together they account for the fraction of administered dose which is lost from the rat at the slowest rate (Table 3). Others have found the highest Zn^{65} tissue concentrations in bone and hair of cattle[24] and rats.[21] It seems reasonable to assume that

radiozinc in tissues and organs of man would follow, at least qualitatively, the same general distribution pattern as observed in other species.

Radiation protection guides for Zn^{65}

The parameters of the effective retention function of Zn^{65} in man (Table 3) may be used to estimate the maximum permissible concentration for Zn^{65} in water or in food $(MPC)_w$ during continuous occupational exposure using the following basic equation taken from the *ICRP Report of Subcommittee II*:[18]

$$(MPC)_w = \frac{3.15 \times 10^{-4}\, q f_2}{T f_w (1 - e^{-0.693\, t/T})} \qquad (6)$$

in which q (the maximum permissible body burden) is 60 μc, f_2 (fraction of radionuclide in the critical organ of that in the total body) is unity, T is the effective half-time of the last component of the retention function, f_w is the intercept value for the third component of the retention function, and t is 50 years. Values for the 4 subjects are 2.4, 1.7, 3.4 and 4.7 $\times 10^{-4}$ μc-Zn^{65}/ml, respectively. The average value is 3.1×10^{-4}. Although Zn^{65} is concentrated largely in bone, the range of its hard electromagnetic radiation makes assumption of the whole body as the critical organ reasonable. Use of the parameters of the entire retention function in the denominator of equation (6) instead of those for the third term only makes essentially no difference in the calculated values for $(MPC)_w$. When the whole body is taken as the critical organ, the $(MPC)_w$ for continuous exposure may be estimated from the interspecies correlation data shown in Fig. 4, using,

$$(MPC)_w = \frac{q}{ES}$$

where q is 60 μc, E is the equilibrium level (112 times the daily intake) for man under conditions of constant chronic exposures, and S is 2200 ml (the daily water intake). Calculated in this manner, the $(MPC)_w$ of Zn^{65} for 40 and 168 hr/week exposure is 7×10^{-4} and 2.4×10^{-4} μc/ml, respectively. These values are approximately one-fourth of those recommended by the NCRP and ICRP.

SUMMARY

Retention of $Zn^{65}Cl_2$ in mice, rats, dogs and man following acute oral administration was measured using whole-body counting methods. Retention functions for all species were described by the sum of three exponential components. The average effective half-time of Zn^{65} in 4 human subjects was 154 days with a range of 149–161 days. The equation $A = 155X^{0.3833}$ relates area under the effective retention functions of the various species to body weight. This parabolic relationship between Zn^{65} uptake and retention and the body weight suggests that the allometric relationships among various physiological and anatomic parameters and body size may be reflected also in mineral metabolism.

Distribution studies of Zn^{65} in rats show that its loss from various organs and tissues is governed by more than one rate process. After oral administration, peak tissue concentrations are reached within 5 days. Bone and pelt together account for essentially all Zn^{65} making up the longest component of the retention function.

Using human data and the whole body as the critical organ, the average $(MPC)_w$ value for Zn^{65} for conditions of continuous occupational exposure was estimated to be 3.1×10^{-4} μc/ml. The comparable value derived from the interspecies correlation was 2.4×10^{-4} μc/ml.

Acknowledgments—The authors wish to express their appreciation to B. M. Cummins and D. W. Martin for their technical assistance during various phases of the reported work. Valuable counsel of R. K. Zeigler and W. L. LeStourgeon of the Theoretical Division (Group T-1) in programming and processing data for computer analysis is gratefully acknowledged.

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Poultry Sci. 39: 893-896 (1960)

The Tolerance of Growing Chicks for High Levels of Different Forms of Zinc[1,2]

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(Received for publication October 13, 1959)

RECENTLY, the zinc requirement of the growing chick was established at approximately twenty ppm. of zinc exclusive of that found in the isolated soybean protein basal diets (Roberson and Schaible, 1958; O'Dell *et al.*, 1958; Moeller and Scott, 1958).

Mehring *et al.* (1956) fed as high as 778 ppm. in the ration without harmful results on growth or feed efficiency of growing chicks.

The purpose of the experiments reported herein was to determine the upper level of zinc which can be tolerated by the chick without adverse effects. Three zinc compounds were used.

EXPERIMENTAL

Experiment 1. Five hundred White Leghorn male chicks, grown to one week of age on the basal diet (Table 1), were alloted on the basis of weight into five replicates of ten chicks for each of the ten treatments (Table 2). Graded levels of zinc ranging from none to 1,000 ppm, as zinc oxide, were added to the basal diet at the expense of corn.

The chicks were reared in electrically-heated batteries with raised wire floors. Feed and water were supplied *ad libitum*. Feed consumption and individual weights were determined at the end of the fifth week. Mortalities were recorded.

TABLE 1.—*Percentage composition of basal diets*

Ingredients	Exp. 1	Exps. 2 and 3
	%	%
Corn, grd. yellow	45.0	50.8
Soybean oil meal, 50% prot.	—	32.7
Soybean oil meal, 44% prot.	20.0	—
Menhaden fish meal, 60% prot.	—	2.0
Redfish meal, 58% protein	2.5	—
Alfalfa leaf meal, 20% prot.	—	2.0
Alfalfa leaf meal, 17% prot.	5.0	—
Oats, grd.	5.0	—
Wheat flour middlings	5.0	—
Yellow grease, stabilized	—	6.5
Wheat bran	5.0	—
Whey, product, dry, 50% lactose	—	0.5
Whey, dried cheese	2.5	—
Yeast, dried brewer's	1.5	0.5
Meat and bone meal, 50% prot.	5.0	—
Limestone, grd.	1.0	1.0
Bone meal, steamed	0.3	—
Dicalcium phosphate	—	1.0
Salt, iodized	0.3	0.5
DL-methionine	—	0.1
Trace mineral mix[1]	—	0.1
Manganese sulfate, 70% feed grade	0.02	—
Vitamin A & D feeding oil (2250A, 300D$_3$)	0.25	—
Vitamin supplement 249C[2]	—	0.07
Vitamin A (10,000 I.U./gm.)	—	0.05
Vitamin D$_3$ (1,500 I.C.U./gm.)	—	0.03
Choline chloride, 25% dry mix	0.15	0.07
Pro-Pen with B$_{12}$[3]	0.05	—
Vitamin B$_{12}$ Supplement (6 mg./lb.)	—	0.10

[1] Supplies in mg. per lb. of feed: manganese 27, iodine 0.54, iron 9, copper 0.9 and cobalt 0.09.
[2] Contains per lb.: 2 gm. riboflavin, 4 gm. pantothenic acid, 9 gm. niacin and 10 gm. choline.
[3] Contains 2 gm. procaine penicillin and 3 mg. vitamin B$_{12}$ per pound (Merck).

[1] Journal article 2508, Michigan Agricultural Experiment Station.
[2] Supported in part by a grant from Hardy Salt Co., St. Louis, Mo.
[3] Presented by the senior author in partial fulfillment of the Ph.D. degree.
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TABLE 2.—*The effect of feeding graded levels of zinc on growth and feed conversion by S. C. White Leghorn cockerels at five weeks of age*

Group	Zinc additions to the basal as the oxide	Number of chicks surviving (50 started)	Average weight	Feed/gain
	(p.p.m.)		(gm.)	
1	None	50	485	2.86
2	200	50	492	2.86
3	300	50	496	2.86
4	400	49	476	2.94
5	500	50	494	2.78
6	600	50	488	2.86
7	700	50	471	2.86
8	800	50	474	2.94
9	900	50	478	2.86
10	1,000	50	473	2.94

Experiment 2. One-day-old White Rock male chicks, obtained from a commercial hatchery, were allotted on the basis of weight into three replicates of ten chicks for each of the treatments. The birds were reared in electrically heated batteries with raised wire floors. Feed and water were supplied *ad libitum.*

Graded, high levels of zinc as the sulfate, oxide or carbonate were added to the basal diet at the expense of corn (Table 3). Individual chick weights were taken initially and at the end of the fourth week.

Experiment 3. One-day-old White Rock male chicks were allotted to each of the eight treatments and reared as in Experiment 2. Two high levels of zinc oxide or carbonate and three levels of zinc sulfate were added to the basal diet as shown in Table 4.

Body weights were analyzed by the analysis of variance according to Snedecor (1956), and significant differences between treatments were determined by Duncan's multiple range test (1955).

RESULTS AND DISCUSSION

The results of experiment 1 are given in Table 2. Dietary zinc levels up to 600 ppm. of the oxide did not adversely affect growth or feed conversion of chicks to the fifth week. The slight decreases at higher zinc levels were not significant, as will be seen in the next experiment.

The results of experiment 2 are given in Table 3. The 1,000 ppm. level of the three zinc compounds did not depress chick growth as compared with the basal group. The addition of the three zinc compounds at 2,000 ppm. resulted in a growth retardation as compared with their respective lower levels; however, there was no difference observed between the chicks receiving each of the three compounds at this higher level. Growth was depressed when each compound was included in the ration at

TABLE 3.—*The effect of feeding high levels of three zinc compounds upon growth, feed efficiency, and survival of White Rock chicks to four weeks of age*

Group	Zinc additions to the basal	Source of zinc	Average weight*	Number surviving chicks (30 started)	Feed/gain
	(p.p.m.)		(gm.)		
1	None	—	476^{abA}	29	1.85
2	1,000	ZnO	488^{aA}	29	1.72
3	1,000	$ZnSO_4$	464^{abA}	30	1.69
4	1,000	$ZnCO_3$	483^{aA}	30	1.72
5	2,000	ZnO	441^{bcAB}	30	1.89
6	2,000	$ZnSO_4$	419^{cB}	28	1.92
7	2,000	$ZnCO_3$	407^{cB}	29	2.08
8	3,000	ZnO	337^{dC}	29	2.27
9	3,000	$ZnSO_4$	282^{eD}	29	2.44
10	3,000	$ZnCO_3$	214^{fE}	23	3.45

* Values having the same letter are not significantly different from each other; small letters represent significance at the 5 percent level, capital letters at the 1 percent level.

TABLE 4.—*The effect of graded levels of three zinc compounds on growth, survival, and feed efficiency of White Rock chicks to four weeks of age*

Group	Zinc additions to the basal	Source of zinc	Average weight*	Number surviving chicks (40 started)	Feed/gain
	(p.p.m.)		(gm.)		
1	None	—	491[abA]	38	1.61
2	50	$ZnSO_4$	500[aA]	40	1.75
3	1,000	ZnO	497[abA]	38	1.67
4	1,000	$ZnSO_4$	470[bcAB]	39	1.69
5	1,000	$ZnCO_3$	490[abA]	40	1.79
6	1,500	ZnO	455[cB]	38	1.85
7	1,500	$ZnSO_4$	402[dC]	39	1.96
8	1,500	$ZnCO_4$	390[dC]	40	2.00

* Values having the same letter are not significantly different from each other; small letters represent significance at the 5 percent level, capital letters at the 1 percent level.

3,000 ppm. as compared with results at the lower levels. The growth depression varied with the compound fed. The zinc compounds were tolerated in the following decreasing order: oxide, sulfate, and carbonate.

The results of experiment 3 are given in Table 4. Growth was not affected by the addition of fifty ppm. of zinc as the sulfate to the basal diet. Thus, the basal diet was adequate in zinc and needed no further supplementation. The zinc level of 1,000 ppm. of each of the compounds was tolerated well by the growing chick. The 1,500 ppm. zinc level depressed growth when compared with the basal group. This level of the oxide was tolerated to a greater degree than that of the sulfate or carbonate.

Feed efficiency approximately paralleled growth in the three experiments. Mortality was severe only at 3,000 ppm. of the carbonate.

In each of the experiments, the oxide was tolerated at the higher level and to a greater extent than were the sulfate and carbonate. This was probably due in part to the difference in solubility of the compounds.

Since zinc as oxide, sulfate, and carbonate were well tolerated by the chick at levels up to 1,000 ppm. and since this is greatly in excess of the requirement for this mineral, all three compounds can be safely used to supplement practical chicken rations at the zinc levels normally recommended.

SUMMARY AND CONCLUSIONS

Three experiments were conducted to determine the tolerance of growing chicks to high levels of different forms of zinc. A total of 1,120 White Leghorn chicks was used. The experiments were conducted in batteries during the first four or five weeks of age of the chicks.

Levels of zinc up to a thousand ppm. as the oxide, sulfate and carbonate did not significantly influence rate of growth, feed efficiency or livability.

At 1,500 ppm. and above, the carbonate and sulfate adversely affected growth and feed efficiency.

Mortality was not severe, except at the highest level (3,000 ppm.) of zinc as the carbonate.

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PROPERTIES OF HUMAN PLASMIN[1]

EDWARD RONWIN[2]

Abstract

Several aspects of the properties and preparation of human plasmin are considered. It appears that glycerol activation of the proenzyme is a peculiar property dependent in part on the structure of the glycerol molecule since closely related compounds are without effect. Observations on the influence of ionic strength, on ion activation and inhibition of the enzyme, on substrate specificities, and on preparative procedures are recorded and comparisons with similar data on related bovine enzymes are made.

Introduction

The variation of the activity of human plasmin with changes in physical and chemical parameters has been studied. Although this type of approach with human plasmin is not new (1), the specific aspects investigated have apparently not been previously examined. In addition, some notes on the preparation of the enzyme receive comment as do comparisons with data on related enzymes from bovine sources. The material gathered here is considered to provide background information of use in a study of the active center of human plasmin.

Materials and Methods

Measurement of Enzyme Activity

Enzyme activity was in all cases determined by the null point titration method which has been described (2). Two-milliliter reaction volumes were employed; suitable controls were run. Enzyme concentrations are given in terms of the Th unit which has been previously defined (2) and slightly amended (3). The unit is as applicable to human plasmin as it is to the bovine tryptic enzymes. Experimental conditions are given in the diagrams and tables to which they pertain.

Substrates and Enzymes

The substrate TAME[3] was a product of H & M Chem. Co., Santa Monica, Calif. The substrates 3,5-DNBAME,[3] MNBAME,[3] and BAME[3] were all gifts from Dr. A. K. Balls. Beef trypsin was a twice-crystallized product and gift from Armour & Co. Human plasmin was prepared as described below from

[1]Manuscript received August 29, 1960.
Contribution from College of Pharmacy, University of Minnesota, Minneapolis 14, Minnesota. This work was done during the tenure of an Advanced Research Fellowship and Grant-in-aid from the American Heart Association.

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[3]TAME = N^{α}-*p*-toluenesulphonyl-L-arginine methyl ester; 3,5-DNBAME = N^{α}-3,5-dinitrobenzoyl-L-arginine methyl ester; MNBAME = N^{α}-*m*-nitrobenzoyl-L-arginine methyl ester; BAME = N^{α}-benzoyl-L-arginine methyl ester. All except BAME were initially in the form of the hydrochlorides. BAME was in the form of its carbonate and was converted to the hydrochloride prior to use.

aerated human blood used in heart surgery and made available through the courtesy of Dr. Newell R. Ziegler of the University Hospital. Beef trypsin was stored at 0–4° in HCl solution, pH 2.3. Human plasmin was stored at 0–4° in HCl solution, pH 2.7. Both enzymes are stable for many months under these conditions. Other comments on their stability receive attention elsewhere in this report.

The Preparation of Human Plasmin

Earlier approaches to the isolation of human plasminogen and its subsequent activation to plasmin rely essentially on isoelectric precipitation of the proenzyme (4–8) followed by its conversion to plasmin using any of several agents (9–14). In designing the preparative procedure described here, the acid and heat resistance of human plasminogen (15) and the solubility and stability of the proenzyme and enzyme in 50% aqueous glycerol solutions (15) were utilized.

One hundred and fifty NIH units of bovine thrombin produced a clot in the plasma from 3 liters of aerated human blood. The resulting 1.5 liters of serum was diluted 10-fold and adjusted to pH 5.3. After 12 hours at 0–4°, the precipitate was collected by centrifugation (1400×*g* in all cases in this report) and dissolved in 200 ml of 0.01 *N* HCl. The acid solution of plasminogen was heated at 60° for 20 minutes, diluted 10-fold, and adjusted to pH 5.3. After 12 hours at 0–4°, the precipitate was resubjected to solution in 0.01 *N* HCl, dilution, and isoelectric precipitation. The resulting precipitate was centrifuged off and suspended in 800 ml of 50% aqueous glycerol adjusted to pH 7.4. The suspension was left at room temperature for 5 days and then centrifuged to remove much undissolved protein. The supernatant was diluted fivefold and adjusted to pH 5.3; the rapidly formed precipitate was centrifuged off, dissolved in 75 ml of 0.01 *N* HCl, and kept at 0–4°. The solution lacked fibrinolytic activity.

The plasminogen solution was mixed with 10 ml of 0.6 *M* tris buffer, pH 8, and 85 ml of glycerol; the resulting 50% glycerol medium had a pH of 7.4. Twenty milligrams (=10 mg of enzyme) of bovine trypsin was added in solid form. The solution was occasionally shaken and left at room temperature for 10 hours. Dilution to 2 liters and adjustment to pH 5.3 yielded a white precipitate which was taken up in 35 ml of 0.005 *N* HCl. This solution possessed a total activity of 2540 Th units. The preparation was very fibrinolytic, but so, too, is bovine trypsin; however, the differences recorded elsewhere in this report, supported by the observations of others (14, 16–18), clearly demonstrate that the tryptic enzyme isolated here is not identical with the bovine trypsin used in its activation.

The active enzyme was further purified by isoelectric precipitation. A final solution of 1998 Th units was kept at 0–4°, pH 2.7.

Some experiments with an earlier preparation of human plasminogen warrant note. This preparation had been treated with $BaSO_4$ and later heated at 60°, pH 2, for 15 minutes, but a solution of the plasminogen fraction in tris buffer, pH 7.4, resisted activation by streptokinase. However, after 5 days at room temperature in glycerol – tris buffer (1:1), pH 7.8, the preparation was fully activated to 3000 Th units, which is in the range of the trypsin-activated preparation described above. The failure of this latter preparation to be activated by glycerol may have been due to insufficient incubation time which occasionally requires weeks (15).

Geiger (19) and later Troll and Sherry (1) reported the heat susceptibility of an entity in human serum or plasminogen preparations which, with streptokinase, produces an activator of human and other animal plasminogens. The conditions of the Troll and Sherry experiment (100°, pH 2, varying periods up to 1 hour) approximate, more closely than Geiger's, the acid–heat treatment of the plasminogen preparation described above. The more complete loss of proactivator observed here, as compared with Troll and Sherry's results, may be due to adsorption of the proactivator on $BaSO_4$ or to possible destructive effects by the O_2 used to aerate the blood.

These experiments were not undertaken to resolve the current conflict over the question of whether or not the proactivator is identical with plasminogen, but to produce very pure plasminogen and plasmin; however, the fact that the preparation, though not activated by streptokinase, was able to be converted by incubation in 50% glycerol, to the same level of activity as the trypsin-activated preparation described above, argues in favor of the concept that proactivator and plasminogen cannot be identical in every detail. Lassen's (20) alkali–heat studies lend added support to the non-identity theme.

Results and Discussion

Before human plasmin is discussed, it is necessary to establish its non-identity with bovine trypsin with which it possesses many properties in common and which was used to activate it. Earlier reports (14, 16–18) detail four different arguments for their non-identity and point to the enzymatic nature of the activation process. The significant difference in isoelectric point (10.4–10.8 for bovine trypsin (21) and approximately 5.3 for human plasmin) supports the hypothesis that the enzymes are not identical. Stability studies were previously used to attack the same problem with bovine plasmin (22). An identical approach was used here.

Stability of Human Plasmin and Bovine Trypsin

Although human plasmin and bovine trypsin are both stable for many months (seemingly indefinitely) when kept at 0–4° and pH's below 4 (2, 3, 15, 23, 24), it is apparent from Fig. 1A that human plasmin at pH 8, 38°, is more resistant to activity loss than bovine trypsin and is almost completely shielded from loss by glycerol (15) whereas trypsin enjoys no such protection. The results add support for the argument that the two enzymes are similar but not identical.

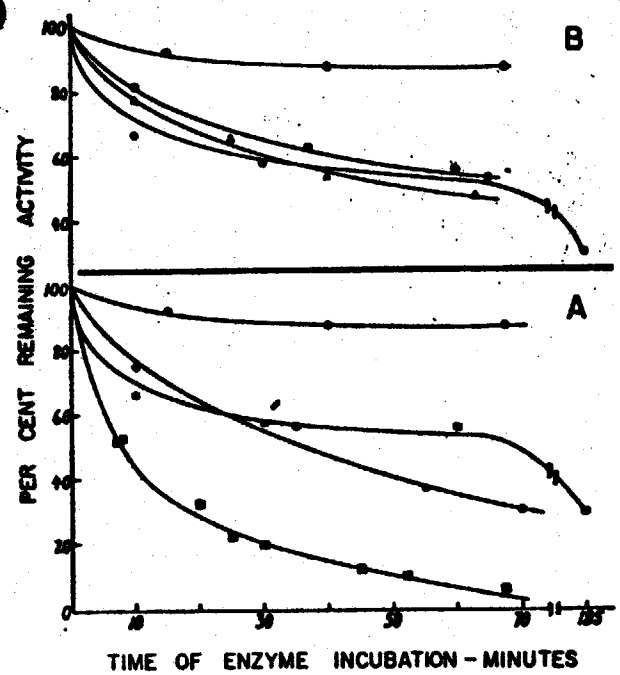

FIG. 1.(A)Stability of human plasmin and bovine trypsin. Each enzyme incubated in a concentration of 39 Th units/ml at 38°, pH 8.0, as follows: □ human plasmin in 0.01 *M* veronal buffer; ○ human plasmin in 0.01 *M* veronal buffer:glycerol (3:1); ■ bovine trypsin in 0.01 *M* veronal buffer; ● bovine trypsin in 0.01 *M* veronal:glycerol (3:1). Tests of activity conducted at 38°; pH 8.0; 0.01 *M* TAME as substrate; 0.01 *M* veronal buffer; enzyme concn. in tests: 3.90 Th units/ml.

(B)Effects of diols on the stability of human plasmin solutions. Human plasmin incubated in a concentration of 39.0 Th units/ml at 38°, pH 8.0, in the following solutions: ● 0.01 *M* veronal buffer; ○ 0.01 *M* veronal:glycerol (3:1); □ 0.01 *M* veronal:ethylene glycol (3:1); △ 0.01 *M* veronal:1,3-propanediol (3:1). Tests of activity conducted as indicated in legend to Fig. 1A.

Effects of Diols on the Stability of Human Plasmin Solutions

To explore the protective action of glycerol on human plasmin, the effect of ethylene glycol and 1,3-propanediol was investigated. The data are given in Fig. 1B. It is obvious that neither diol affords any protection from activity loss. Hence, it may be concluded that the protective action of glycerol very likely requires all three hydroxyl groups as well as the configuration of the glycerol molecule.

Rate of Hydrolysis as a Function of Initial Human Plasmin Concentration

Over the 2.5-fold range of human plasmin concentrations used, the data of Fig. 2 indicate a proportionality between enzyme concentration and activity. This permits extrapolations of enzyme activity in other experiments when desired.

Comparative Specificities of Human Plasmin and the Bovine Tryptic Enzymes

The data of Table I indicate that TAME is the most susceptible substrate for each enzyme and MNBAME is usually least susceptible. Nevertheless,

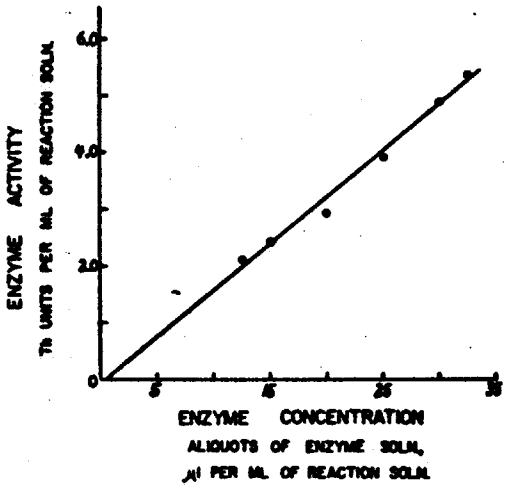

FIG. 2. Rate of hydrolysis as a function of initial human plasmin concentration. ● observed. The line was drawn by the "least-squares" method. Over its entire length there is a proportionate rate increase with increase in enzyme concentration. Tests of activity were conducted at 38°; pH 8.0; 0.01 *M* TAME as substrate; 0.01 *M* veronal buffer.

each of the arginine esters is a substrate for each enzyme. These observations illustrate the basic similarities of each enzyme with regard to substrate specificity and mode of action, but the data also mark their individual natures.

TABLE I

Comparative specificities of human plasmin and the bovine tryptic enzymes

Substrate (0.01 *M*)	Relative rates			
	Human plasmin*	Bovine trypsin*	Bovine plasmin†	Bovine thrombin‡
TAME	1.00	1.00	1.00	1.00
3,5-DNBAME	0.26	0.18	0.23	0.13
MNBAME	0.22	0.18	0.12	0.10
BAME	0.33	0.12	0.15	0.35

*At either 5 or 8 minutes of reaction. Conditions: substrate as given; 0.01 *M* veronal buffer; pH 8.0; 38°; human plasmin concn.: 3.90 Th units/ml; bovine trypsin concn.: 4.02 Th units/ml.
†At 15 minutes of reaction; see reference 22.
‡At 5 minutes of reaction; see reference 2.

Action of Cations on Human Plasmin and Comparison with Bovine Tryptic Enzymes

Although Cu^{++} totally inhibits the other three enzymes (Table II), bovine plasmin does possess a decidedly measurable activity in the presence of this ion but is strongly inhibited by it. Ni^{++} shows a fairly uniform rate of inhibition toward each enzyme, although its action on human plasmin is only partly identical with its action on the bovine enzymes (25). The failure of 0.01 *M* Ca^{++} to activate bovine thrombin, while the other three enzymes are activated,

TABLE II

The action of cations on human plasmin and comparison with bovine tryptic enzymes

Cation (as chloride)	Concentration (*M*)	Relative rates			
		Human plasmin*	Bovine trypsin*	Bovine plasmin†	Bovine thrombin‡
None		1.00	1.00	1.00	1.00
Cu	0.001	0.0	0.0	0.31	0.0
Ni	0.001	0.45	0.44	0.42	0.59
Zn	0.001	0.19	0.19	0.41	0.71
Ca	0.01	1.19	1.17	1.23	1.00

*At 5 minutes of reaction. Conditions: 0.01 *M* TAME as substrate; 0.01 *M* veronal buffer; pH 8.0; 38°; human plasmin concn.: 3.08 Th units/ml; bovine trypsin concn.: 2.93 Th units/ml.
†At 15 minutes of reaction; see reference 22.
‡At 5 minutes of reaction; see reference 2.

reflects the previously demonstrated difference in mode of activation of bovine thrombin by Ca^{++} as compared to bovine trypsin and plasmin (3, 23), which are joined by human plasmin in differing from bovine thrombin (25). The same active center difference is likely reflected by the results with Zn^{++}. Since thrombin is actually activated by lower concentrations of Ca^{++}, as are the other three enzymes, the broad import of the data mirrors the essential similarity of all four enzymes in that Ca^{++} activates, while the other three ions inhibit, each enzyme.

Effect of Ca^{++} Concentration on Human Plasmin and Bovine Trypsin Activity

In marked distinction to the case of bovine thrombin (2), the results in Fig. 3 show that the activation of human plasmin and bovine trypsin by Ca^{++} is relatively unaffected by the ion's concentration.

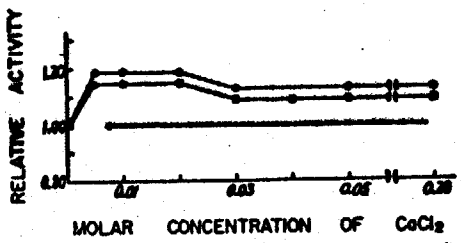

FIG. 3. Effect of variations in Ca^{++} concentration on the activity of human plasmin and bovine trypsin. ● human plasmin; ◻ bovine trypsin. Tests of activity conducted at 38°; pH 8.0; 0.01 *M* TAME as substrate; 0.01 *M* veronal buffer; human plasmin concn.: 3.90 Th units/ml; bovine trypsin concn.: 4.02 Th units/ml.

Effect of Ionic Strength on Human Plasmin Activity

Because the effect with Ca^{++} might be due to the known interaction of this ion with an active center group (3, 23), the probable lack of influence of ionic strength on human plasmin (and bovine trypsin) activity was further checked with NaCl as ionic strength agent. The data of Fig. 4 indicate that ionic strength is without practical effect on human plasmin activity. The value of

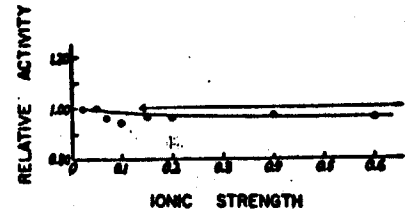

FIG. 4. Effect of ionic strength on human plasmin activity. Tests of activity conducted at 38°; pH 8.0; 0.01 *M* TAME as substrate; 0.01 *M* veronal buffer; enzyme concn.: 4.14 Th units/ml. NaCl used as ionic strength agent. The zero point at 0.02 ionic strength is due to the contribution to this factor by the substrate and the buffer.

0.6 ionic strength in Fig. 4 is the same level of this factor as the 0.2 *M* concentration of Ca^{++} in Fig. 3; thus, the data of each curve is mutually supporting. These results contrast pointedly with the observations on bovine thrombin (2) whose activity is significantly influenced by this physical parameter and further reflects the fundamental differences in the active center group composition and function between bovine thrombin and the other three enzymes.

Conclusion

The data allow the conclusion that human plasmin is very similar to bovine plasmin and bovine trypsin yet quite distinct from either. Further, it is joined by the two bovine enzymes in its similarities and dissimilarities to bovine thrombin. The various properties that were probed extend our knowledge of the enzyme's behavior which, in turn, permits a more reasoned approach to the question of the chemical identity and physical action of its active center.

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THE EFFECT OF INTAKE OF ZINC ON METABOLISM

PART A

It was pointed out earlier[1] that the intake of zinc exerted an influence on the fat content of liver in the rat when it was supplemented to a high fat, low protein diet and that irrespective of the nature of the diet, zinc supplements adversely affected the development of the bones. Prabhu[2] records that zinc is used to cure infantile cirrhosis of liver in the indigenous systems of medicine. It was therefore of interest to find out if zinc influenced the metabolism of nitrogen, phosphorus and sulphur in rats fed on a stock diet and high fat, low protein diet.

Table I presents the results of experiments conducted on three groups of four rats each with a view to studying the effect of zinc (supplied as zinc oxide at 0·5 and 1·0 per cent. levels) on the metabolism of N, P and S in rats on a stock diet.

The results show that supplements of zinc affect metabolism of all the constituents studied. While urinary excretion of nitrogen is affected only at the higher level of zinc, fæcal excretion and retention are altered to a very appreciable extent at all the levels of zinc tried. Its influence on the metabolism of phosphorus and sulphur appears to be more marked. Thus there is a considerable decrease in urinary excretion and retention and a corresponding increase in fæcal excretion of these constituents as a result of the supplements of zinc. These observations lead to the conclusion that zinc in some manner affects the assimilation of these constituents in the intestines, so that the reserves of the body are mobilized in order to maintain their concentration in the body fluids. This appears to be the reason for the retarded growth rate and poor development of the bones of rats fed on a diet supplemented with zinc and possibly also for other toxic symptoms reported by Sutton and Nelson[3] and Smith and Larson.[4]

TABLE I

Metabolism of nitrogen, phosphorus and sulphur in rats fed on a stock diet supplemented with zinc

Diet	Urinary* mg.	Fæcal* mg.	Retention* mg.
Nitrogen :			
Stock diet alone	338·71	892·76	2646·63
Do. *plus* 0·5% ZnO	355·08	1091·17	2186·35
Do. *plus* 1·0% ZnO	536·20	1121·65	1790·75
Phosphorus :			
Stock diet alone	41·37	143·79	526·44
Do. *plus* 0·5% ZnO	4·29	477·79	239·52
Do. *plus* 1·0% ZnO	4·59	573·23	54·78
Sulphur :			
Stock diet alone	23·94	86·90	−53·15
Do. *plus* 0·5% ZnO	15·76	129·25	−83·51
Do. *plus* 1·0% ZnO	16·84	155·59	−121·04

* Total for a period of fifteen days and average for the group consisting of four rats.

In experiments conducted with high fat, low protein diets also, observations substantially similar to the above were made.

Table II shows that zinc causes an increase in the urinary excretion of both uric acid and total creatinine when the basal diet consists of the stock ration. This may be because of the general wastage especially of the muscle tissues. On a high fat, low protein diet, uric acid excretion shows a slight tendency to decrease. As far as the excretion of creatinine is concerned, the composition of the diet appears to make some difference in that while zinc tended to increase the urinary excretion of creatinine in rats on a stock diet, it lowered its excretion in those fed a high fat, low protein diet. The reason for this difference is not clear.

The manner in which zinc lowers the fat content in the liver of rats fed on a high fat, low protein diet is also not quite clear, but it

TABLE II

Excretion of uric acid and total creatinine in the urine of rats fed on a stock diet and a high fat, low protein diet supplemented with zinc

Diet	Three-day periods 1	2	3	4	5	Total
	Milligrams					
Uric Acid:						
Stock diet alone	0·242	0·762	0·778	0·929	1·182	3·893
Do. *plus* 0·5% ZnO	0·378	0·843	0·971	1·036	1·056	4·284
Do. *plus* 1·0% ZnO	0·504	1·033	1·139	1·381	1·390	5·447
High fat, low protein diet	0·202	0·170	0·532	0·278	0·429	1·611
Do. *plus* 0·5% ZnO	0·311	0·418	0·446	0·141	0·259	1·575
Do. *plus* 1·0% ZnO	0·227	0·211	0·238	0·139	0·191	1·006
Total Creatinine:						
Stock diet alone	1·700	1·373	2·064	2·173	5·303	12·613
Do. *plus* 0·5% ZnO	2·178	2·218	3·041	2·984	3·976	14·397
Do. *plus* 1·0% ZnO	2·116	2·909	4·291	4·822	6·473	20·611
High fat, low protein diet	0·958	1·078	1·075	2·773	5·964	11·848
Do. *plus* 0·5% ZnO	0·958	1·312	2·382	1·822	2·259	8·733
Do. *plus* 1·0% ZnO	1·208	1·466	1·594	1·227	1·373	6·868

that in rats fed a diet supplemented with zinc, there should be an appreciably greater excretion of fat in the fæces than in the control. But this appears not to be the case. Further investigation is in progress.

PART B

In an experiment designed to find out the metabolism of fat in rats fed on a high fat, low protein diet with and without the supplement of zinc, it was observed that fæcal fat did not show any appreciable increase in the zinc-fed group during the period of fifteen days (Table III).

These results show that fat is assimilated without impairment even when the assimilation of phosphorus from the intestines is poor and thus support the contention of Frazer[3] who has adduced some evidence to show that phosphorylation of fat is not a necessary intermediate step in its assimilation from the intestines. The explantion for the lipotropic action of zinc has, therefore, to be sought for elsewhere.

It is a pleasure to thank Major-General Sir S. S. Sokhey and Dr. K. Ganapathi for their interest in this work.

Haffkine Institute, Bombay, V. SADASIVAN.

January 16, 1950.

TABLE III

Metabolism of fat in rats fed on a high fat, low protein diet supplemented with zinc

Diet	Three-day periods 1	2	3	4	5	Total
	Grams					
Fat in Diet:						
High fat, low protein diet	6·248	6·075	5·889	5·745	5·380	29·328
Do. *plus* 0·5% ZnO	4·820	3·940	4·205	4·205	4·145	21·315
Fæcal Fat:*						
High fat, low protein diet	0·1483 (2·375)	0·1982 (3·263)	0·1197 (2·036)	0·1125 (2·010)	0·2656 (4·937)	0·8443 (2·879)
Do. *plus* 0·5% ZnO	0·1523 (3·159)	0·1207 (3·067)	0·0775 (1·367)	0·2201 (5·234)	0·1376 (3·319)	0·7082 (3·323)
Fat Assimilated:						
High fat, low protein diet	6·0997	5·8768	5·7692	5·6295	5·1144	28·4897
Do. *plus* 0·5% ZnO	4·6677	3·8193	4·1275	3·9849	4·0074	20·6068

* Figures in brackets indicate fæcal fat as percentage of dietary fat.

is likely that zinc, by inhibiting the phosphorylation of fat to form phospholipids, prevents its migration from the intestines to the liver. In this event, it is to be expected

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Biochem. J. Vol. 48 : 527-530, 1951

Studies on the Biochemistry of Zinc

1. EFFECT OF FEEDING ZINC ON THE LIVER AND BONES OF RATS

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(*Received* 11 *October* 1950)

The indispensability of zinc as an element in nutrition has now been fairly established. Todd, Elvehjem & Hart (1934), Stirn, Elvehjem & Hart (1935), Day & McCollum (1940) and Day & Skidmore (1947) were able to show that when animals were fed a diet deficient in zinc they grew subnormally and developed various symptoms which were cured by supplementing the diet with small amounts of zinc, but attempts to elucidate the mechanism of its action have not been very successful. Hove, Elvehjem & Hart (1937) showed that deficiency of zinc resulted in the impairment of intestinal absorption. Other changes brought about by a deficiency of this element have been reported by Gorter (1939) and Day (1942). Scott (1934) and Scott & Fisher (1935, 1938*a*,*b*) showed that even the purest preparations of insulin contain zinc and that this element is somehow associated with the hypoglycaemic action of insulin. Keilin & Mann (1939) reported the presence of this element as a component of the enzyme, carbonic anhydrase, but Hove, Elvehjem & Hart (1940*a*,*b*) and Day & McCollum (1940) could find no change in the carbonic anhydrase activity in cases of zinc deficiency. Davidson (1942) and Holmberg (1939) reported the presence of zinc in their preparations of uricase, but Wachtel, Hove, Elvehjem & Hart (1941) could not observe any difference in the uric acid content of the blood of normal and deficient animals. Further work is therefore necessary to find out how this element functions as an indispensable constituent of food of animals.

In this and the following papers, the problem has been attacked from a standpoint somewhat different from that of the investigations mentioned above. Here, the changes brought about by zinc when fed in concentrations much higher than the optimum have been investigated since it was observed in the preliminary experiments of the author that in broad outline, the changes brought about by either a deficiency of zinc or by toxic concentrations of it were almost identical. Moreover, since the preparation of diets completely deficient in this element without affecting any of the other constituents in the diet is very laborious and not quite satisfactory, it was thought that the changes brought about by the element when fed in toxic concentrations would be easier to follow and would lead eventually to a better elucidation of the biochemical functions of

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this element. The process may be regarded as analogous to the elucidation of the nature of activity of an enzyme by an investigation of its activation and inhibition.

Investigations on the changes brought about by diets containing excess zinc have been few. Grimmett, McIntosh, Wall & Hopkirk (1937) reported that, on a high-zinc diet, pigs showed toxic symptoms in about 2 months. This toxicity syndrome was characterized by loss of appetite and stiffness of the legs. On autopsy, the liver showed necrosis and the head of the humerus was affected. They also reported that zinc accumulated in the pancreas, kidney, liver and humerus. Sutton & Nelson (1937) observed that high-zinc diets brought about anaemia, subnormal growth and reproductive failure in rats. This was confirmed by Smith & Larson (1946) who investigated the nature of the anaemia caused by zinc. According to them, it was microcytic and hypochromic, and the condition could be cured by additional supplements of copper while supplements of liver or liver extracts caused resumption of growth.

During the present investigation, it was observed that the high-zinc diets brought about a reduction in the weight and also a reduction in fat content of the liver. Zinc appeared to have a lipotropic effect. Development of the bones was also adversely affected. A preliminary report of this work has already appeared (Sadasivan, 1950).

EXPERIMENTAL

Albino rats of the Haffkine inbred strain were divided into groups of six, with an equal distribution between the sexes in each group. All the rats were fed a basal diet consisting of the following (parts by weight): wheat (*Triticum vulgare*), 58; gram (*Cicer arietinum*), 12; peas (*Pisum sativum*), 12; rice (*Oryza sativa*), 6; green leaves (*Amaranthus* sp.), 6; milk, 6. The constituents, excepting the green leaves and milk, were ground to a powder and well mixed. Weighed quantities of the powder were worked into a dough with the proportionate quantities of milk and green leaves, steamed for 30 min. and fed to the animals *ad lib.* On this diet, rats have been observed to thrive well. The supplements of Zn were made as pure grade ZnO (Merck) at levels of 0·5 and 1·0% in groups 2 and 3 respectively. The experiment extended over a period of 15 days only, since a longer period resulted in the death of rats in group 3. The food intake for each 3-day period was noted and growth measurements made at the end of each 3-day period, and, at the close of the experiment, the animals were killed by ether and liver, kidneys and femurs removed for assay. The fresh weight of liver and kidneys was noted after superficial drying with filter paper. The livers were minced and extracted with acetone in a Soxhlet extractor to determine the fat content. The results are presented in Table 1.

The femurs were cleaned of adhering flesh by rubbing with cloth, extracted with ether-ethanol mixture and dried to constant weight. They were ashed in a muffle furnace after preliminary heating with a gas burner and the ash weight noted. The ash was dissolved in pure dil. HCl, made up to a volume of 100 ml. and Ca and P determined in measured portions of the solution. Ca was determined by precipitating it as the oxalate and titrating it against standard permanganate solution. P was determined by the method of Fiske & Subbarow (1925) and the results are summarized in Table 2.

It will be observed from these tables that, on a stock diet, supplements of ZnO lowered the growth rate, the weight of liver and its fat content. The addition of ZnO to the diet at an 0·5% level did not affect the food intake, but the higher

Table 1. *Analysis of livers of rats fed on a stock diet supplemented with zinc*

(Figures in brackets represent standard deviation.)

Group	Diet	Initial wt. (g.)	Final wt. (g.)	Average daily food intake (g.)	Fresh wt. of liver (g.)	Wt. of liver (as % of final wt.)	Fat in liver (g.)	Fat in liver (as % of liver wt.)	Fresh wt. of kidney (g.)
1	Stock diet	96·0	138·0	21·4 (±0·59)	5·585 (±0·13)	4·050 (±0·10)	0·3844 (±0·010)	6·89 (±0·58)	1·230 (±0·03)
2	Stock diet +0·5% ZnO	102·0	139·5	21·7 (±0·81)	4·823 (±0·17)	3·485 (±0·18)	0·2935 (±0·016)	5·88 (±0·24)	1·195 (±0·04)
3	Stock diet +1·0% ZnO	103·0	116·0	19·0 (±0·64)	4·458 (±0·04)	3·865 (±0·17)	0·2707 (±0·030)	6·07 (±0·64)	1·160 (±0·014)

Table 2. *Analysis of femurs of rats fed a stock diet supplemented with zinc*

(Figures in brackets represent standard deviation.)

Group	Diet	Final body wt. (g.)	Dry wt. of femurs (g.)	Ash wt. (g.)	Ash (%)	Ca (mg.)	P (mg.)	Ca/P ratio
1	Stock diet	138·0	0·3875 (±0·009)	0·1990 (±0·0065)	51·35 (±0·94)	68·28 (±2·14)	40·35 (±0·80)	1·692 (±0·066)
2	Stock diet +0·5% ZnO	139·5	0·3218 (±0·028)	0·1553 (±0·0153)	47·52 (±1·27)	54·51 (±5·39)	33·30 (±2·81)	1·637 (±0·030)
3	Stock diet +1·0% ZnO	116·0	0·2608 (±0·011)	0·0998 (±0·0098)	38·09 (±2·65)	32·63 (±3·52)	25·05 (±1·91)	1·303 (±0·046)

Table 3. *The analysis of livers of rats fed on a high-fat, low-protein diet supplemented with zinc*

(Figures in brackets represent standard deviation.)

Group	Diet	Initial wt. (g.)	Final wt. (g.)	Average daily food intake (g.)	Fresh wt. of liver (g.)	Wt. of liver (as % of body wt.)	Fat in liver (g.)	Fat content of liver (%)
1	10% casein, 30% fat diet	50	63	7·7	5·26 (±0·43)	8·35 (±0·56)	1·2775 (±0·296)	24·29 (±3·90)
2	As group 1 +0·5% ZnO	47	53	6·3	4·04 (±0·16)	7·62 (±0·22)	0·5727 (±0·118)	14·18 (±2·27)
3	As group 1 +1·0% ZnO	45	47	5·1	3·03 (±0·16)	6·45 (±0·19)	0·3687 (±0·080)	12·17 (±1·96)
4	10% casein, 10% fat diet	53	76	9·6	6·08 (±0·27)	8·00 (±0·15)	1·0367 (±0·127)	17·05 (±1·60)
5	As group 4 +1·0% ZnO	48	47	6·9	2·78 (±0·23)	5·96 (±0·23)	0·1420 (±0·039)	5·11 (±1·24)

Table 4. *Analysis of bones of rats fed on a high-fat, low-protein diet supplemented with zinc*

(Figures in parentheses represent standard deviation.)

Group	Diet	Final body wt. (g.)	Dry wt. of femurs (g.)	Ash wt. (g.)	Ash (%)	Ca (mg.)	P (mg.)	Ca/P ratio
1	10% casein, 30% fat diet	63	0·2430 (±0·008)	0·0898 (±0·0075)	36·95 (±0·36)	34·8 (±0·79)	14·70 (±1·58)	2·53 (±0·31)
2	As group 1 +0·5% ZnO	53	0·2195 (±0·009)	0·0643 (±0·0027)	29·29 (±0·61)	24·4 (±1·21)	11·00 (±1·07)	2·22 (±0·20)
3	As group 1 +1·0% ZnO	47	0·1863 (±0·009)	0·0512 (±0·0022)	27·48 (±1·34)	18·6 (±0·84)	8·55 (±0·44)	2·21 (±0·16)
4	10% casein, 10% fat diet	76	0·2477 (±0·009)	0·1120 (±0·0028)	45·22 (±0·53)	40·8 (±0·99)	19·52 (±0·32)	2·09 (±0·04)
5	As group 4 +1·0% ZnO	47	0·1563 (±0·011)	0·0502 (±0·0055)	32·12 (±1·32)	17·6 (±2·30)	8·74 (±1·07)	2·01 (±0·04)

level of supplement markedly decreased the intake of food. The analysis of the femurs of rats showed that Zn appreciably reduced the dry weight and ash content of the bones. Evidently, the supplement interfered with development and mineralization.

The analysis of livers presented in Table 1 shows that Zn may act as a lipotropic agent though this fact is not unequivocally demonstrated by the results obtained on rats fed a stock ration. In order to confirm this point, Zn was added to a high-fat, low-protein diet on which rats normally developed fatty livers in about 15 days. Rats about a month old and of weight ranging between 40 and 60 g. were divided into five groups of six rats each. The first three groups were fed a diet consisting of ethanol-washed casein (choline content 1·5 mg./100 g.) 10 parts, hydrogenated groundnut oil 30, salt mixture (McCollum's) 4, sucrose 55 and shark-liver oil (12,000 i.u. vitamin A/g.) 1 part. In addition, the diet contained 15 μg. each of aneurin, riboflavin, and pyridoxine hydrochloride, 50 μg. of nicin and 20 μg. of calcium pantothenate/g. Groups 4 and 5 were fed a diet similar to the above but with only 10 instead of 30% fat, the difference being made up with sucrose. Groups 2, 3 and 5 received supplements of ZnO at 0·5, 1·0 and 1·0% levels, respectively. The low-fat groups (groups 4 and 5) were included in the series in order to find out the extent to which Zn proved toxic or lipotropic, as the case may be, when the fat content of the diet was low. The rats were weighed every 4th day and the food intake for each 3-day period was noted. At the end of the 15 days, the animals were killed and analysis of livers and femurs carried out as described earlier. These results are presented in Tables 3 and 4.

DISCUSSION

The results presented in this paper show that zinc in comparatively high concentrations causes a reduction in the weight and the fat content of rat livers. The reduction is marked in rats fed on a high-fat, low-protein diet and the results suggest that zinc possesses lipotropic activity.

Zinc brings about other changes also. On a stock diet, the 0·5% level of supplementary zinc oxide does not appear to lessen food intake, though it affects growth rate appreciably. On the stock ration, normal rats showed an increase in weight of 42 g. during an experimental period of 15 days. When, however, zinc oxide was added to the diet, the corresponding increases in weights were 37·5 and 13 g. for 0·5 and 1·0% levels of zinc oxide, respectively. The fresh weight of the kidneys also showed a corresponding decrease. At the 1% level zinc oxide caused a lowered food intake.

On a high-fat, low-protein diet, these changes were more pronounced. However, the decrease in the weight of the livers and their low fat content was particularly striking when the fat content of the diet was 10%. The content of fat in the diet thus appears to influence the changes brought about by the element.

Tables 2 and 4 show clearly that the development of bones is affected adversely in both the series. The observations of Smith & Larson (1946) that high-zinc diets bring about an anaemia, retarded growth rate and lowered food intake in rats, and the results presented in this paper show that zinc probably affects metabolism in general and assimilation of phosphorus in particular.

SUMMARY

1. The effect of supplements of zinc at levels of 0·5 and 1·0% on the livers and bones of rats has been investigated.

2. Zinc supplements caused a marked lowering of the fat content of livers of rats fed on a diet on which control rats developed fatty liver.

3. Zinc appears to act as a lipotropic factor in the concentrations studied. This element also interferes with development and mineralization of bones.

My thanks are due to Major-General S. S. Sokhey, Director, and Dr K. Ganapathi, Assistant Director, for their interest in this investigation.

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Studies on the Biochemistry of Zinc

3. FURTHER INVESTIGATIONS ON THE INFLUENCE OF ZINC ON METABOLISM

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(*Received 4 May 1951*)

Changes in the liver and bones and in the metabolism of nitrogen, phosphorus and sulphur in rats maintained on a stock diet supplemented with zinc have already been reported (Sadasivan, 1951*a*, *b*). It was also observed that on a high-fat, low-protein diet, zinc supplements caused a lowering in fat content of rat livers. In this paper, results are presented of investigations on the metabolism of nitrogen, phosphorus and sulphur and the urinary excretion of uric acid and total creatinine (creatine and creatinine) in rats fed on a high-fat, low-protein diet supplemented with 0·5 and 1·0% of zinc oxide. Results of experiments on the influence of the element on the assimilation of fat from the intestines and on the activity of the phosphatases and lipase in the system are also included.

EXPERIMENTAL

Metabolism experiments. The details of the metabolism experiments have already been described in the previous paper in this series. In the present experiments, the high-fat, low-protein diet described in Part 1 was fed to rats. The daily food intake was recorded and calculated for each 3-day period. The collection of urine, faeces, etc., and their analyses were carried out in the same manner as described in Part 2 (Sadasivan, 1951*b*). The first three groups of young rats weighed about 40–50 g. each and were about a month old, while groups 4 and 5 were older ones weighing between 90 and 100 g. and 2–2·5 months old. Younger and older rats were compared because preliminary observations suggested that younger rats are probably more susceptible to the effects of zinc than older ones.

For the experiment on fat metabolism, each group consisted of six rats equally distributed between the sexes and litters. Both the groups were fed the high-fat diet, the second group receiving, in addition, ZnO at 0·5% level. The faeces were collected for each 3-day period over the experimental period of 15 days and the fat content of the dried faeces was determined by extraction with ether in a Soxhlet extractor. For the determination of fat in the liver, the animals were killed at the end of the experiment. The liver was removed, freed of blood by pressing lightly against blotting paper and ground with a small amount of acetone in a mortar. Fat was then extracted in a Soxhlet extractor with acetone.

Determination of phosphatase activity. Two groups of six rats, each weighing between 60 and 80 g., were fed the stock diet described in Part 1. The second group received, in addition, ZnO at 1·0% level of diet. At the end of 15 days, the animals were killed and liver, small intestines, kidneys and femurs were removed for the determination of phosphatase activity. The tissues were freed of blood by washing once with distilled water and femurs cleaned of adhering flesh. Portions of the tissues and the whole of the femurs were prepared for determination of activity according to the method of Bodansky (1937) and of Martland & Robison (1929). Activity was measured in sample portions of the material suspended in 0·033M-veronal-acetate buffer adjusted to a pH of 9·1. Sodium β-glycerophosphate at concentrations of 1·0% served as substrate and the mixture was incubated at 37° for 1 hr. at the end of which inorganic P was determined by the method of Fiske & Subbarow (1925) with due correction for the presence of phosphate in the crude enzyme preparations by running parallel controls in all cases.

Determination of lipase activity. An active preparation of pancreatic lipase of rats was prepared by treating fresh, minced pancreas from healthy rats with an excess of pure acetone, grinding in a mortar and filtering after contact with acetone for about half an hour. The process was repeated twice and the residue dried *in vacuo*. This powder was extracted with glycerol for 24 hr., filtered through muslin and the extract used as the source of the enzyme. To test the activity, 2 ml. portions of a 5% extract were added to 5 ml. of 0·033M-veronal-acetate buffer adjusted to the pH required and 4 ml. of pure olive oil added as substrate. After the additions had been made, the final volume was made to 15 ml. with water and the mixture incubated at 37° for 3 hr. after which 25 ml. of neutralized ethanol were added and the acid liberated by the enzyme determined by titration against 0·1N-NaOH using phenolphthalein as the indicator. $ZnSO_4$, NaCN, $CaCl_2$ etc., used in the experiments, were of Merck's purest grade.

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RESULTS AND DISCUSSION

Metabolism on a high-fat diet. Table 1 summarizes the results of metabolism of nitrogen, phosphorus, etc., obtained for the duration of the experiment when the animals were fed a high-fat, low-protein diet supplemented with zinc. It may be concluded from these results that on a high-fat, low-protein diet, the changes in metabolism of the various constituents studied are similar to those on a stock diet reported in the previous paper. The adverse effect of zinc supplements on the assimilation of phosphate from the intestines does not appear to be due to the precipitation of phosphate in the insoluble form as in beryllium rickets (Kay & Skill, 1934). The figures in Table 2 show that the quantities of zinc ingested in the animals in the different groups were much less than required to precipitate the phosphate completely. Thus, though a fairly good proportion of the phosphates in the synthetic diet

Table 1. *Influence of dietary zinc on the excretion and retention of N, P and S and on the excretion of uric acid and total creatinine by rats fed on a high-fat, low-protein diet*

(Summary of results for the whole period of experiment. All values expressed as mean ± S.E.)

	Young rats			Older rats	
	Control	Zinc fed at 0·5% level as ZnO	Zinc fed at 1·0% level as ZnO	Control	Zinc fed at 0·5% level as ZnO
Food intake (g.)	57 ± 8·3	37 ± 2·0	30 ± 1·7	69 ± 0·7	67 ± 1·1
Change in body weight (g.)	+5 ± 4·4	−5 ± 4·7	−11 ± 1·3	+0·5 ± 2·1	+0·5 ± 1·9
Metabolism of nitrogen (mg.):					
Urinary excretion	62 ± 4·5	71 ± 14·1	78 ± 11	142 ± 31	100 ± 11
Faecal excretion	63 ± 8·4	93 ± 4·3	97 ± 6	103 ± 8	151 ± 5
Retention	578 ± 91	311 ± 34	105 ± 18	660 ± 64·3	384 ± 58
Metabolism of phosphorus (mg.):					
Urinary excretion	30 ± 3·6	20 ± 3·9	16 ± 3·7	70 ± 12·2	36 ± 3·5
Faecal excretion	21 ± 1·5	49 ± 7·8	71 ± 9·4	32 ± 3·0	125 ± 11
Retention	232 ± 37·9	114 ± 7·1	61 ± 6·2	241 ± 28	155 ± 16·1
Metabolism of sulphur (mg.):					
Urinary excretion	10·2 ± 0·17	9·6 ± 0·2	8·8 ± 0·14	8·8 ± 1·43	8·12 ± 1·1
Faecal excretion	15·4 ± 0·55	18·2 ± 0·55	19·4 ± 0·65	18·5 ± 0·6	26·3 ± 1·8
Retention	−1·3 ± 3·1	−12·0 ± 0·8	−15·0 ± 1·0	2·8 ± 2·6	−4·9 ± 0·6
Uric acid in urine (mg.)	1·61 ± 0·23	1·57 ± 0·54	1·0 ± 0·79	4·58 ± 1·7	4·9 ± 0·6
Total creatinine in urine (mg.)	11·85 ± 7·1	8·73 ± 3·8	6·87 ± 0·9	12·6 ± 2·9	10·83 ± 0·9

Table 2. *Quantities of zinc and phosphorus in the diet of rats supplemented with zinc*

(High-fat, low-protein diet in all groups.)

Group no.	Supplement to diet	Intake of food during 15 days (g.)	Intake of ZnO in diet (g.)	Phosphorus in diet (g.)	Amount of P precipitable by ZnO in diet (g.)
2	0·5% ZnO	36·65	1·80	1·83	0·75
3	1·0% ZnO	29·70	3·05	1·48	0·48
5	0·5% ZnO (older rats)	67·00	3·18	3·35	0·86

remained in an assimilable form, the quantity actually assimilated appeared small.

Zinc and phosphatase activity. The decrease in the assimilation of phosphorus from the intestines and the poor development of the bones consequent on the intake of zinc in the concentrations studied suggest that zinc affects adversely some process of phosphorus metabolism. To check this point, the phosphatase activity of the tissues of zinc-fed animals was compared with that of normal ones. The results (Table 3) show that the intake of zinc at the level investigated brings about a significant decrease in phosphatase activity of the intestine while increasing it in the liver and kidneys. The difference in the activity of the bones does not appear to be significant (cf. Hove, Elvehjem & Hart, 1940).

Table 3. *Phosphatase activity of tissues of rats fed on a stock diet supplemented with zinc*

(For method see text. Results as mean ± S.E.)

	P liberated (mg.)	
	Control	Zinc-fed
Intestines	0·615 ± 0·04	0·499 ± 0·01
Liver	0·089 ± 0·005	0·116 ± 0·005
Bones (femur)	0·103 ± 0·007	0·133 ± 0·019
Kidneys	0·253 ± 0·01	0·477 ± 0·02

Zinc and fat metabolism. The importance of phosphorylation in the absorption of triglyceride fat from the intestines has been investigated by several workers, and the literature on the subject has been reviewed by Frazer (1946). If assimilation of fat is closely related to and dependent on its phosphorylation in the intestines, the impairment of phosphate assimilation from the organ should result in an increase in the faecal fat with or without a simultaneous decrease in the fat intake. In our experiments no appreciable difference in the faecal excretion of fat could be observed, it being 0·84 g. ± 0·05 and 0·71 g. ± 0·05 per rat in the control and zinc-fed groups respectively for the experimental period of 15 days. The corresponding figures for fat intake during the period were 29·3 g. ± 1·06 and 21·3 g. ± 1·36 and for fat content of liver, 1·69 g. ± 0·1 and 0·68 g. ± 0·09.

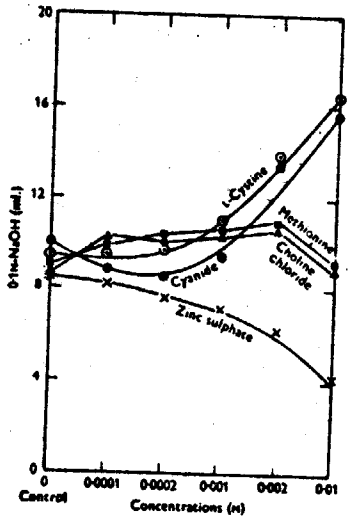

Fig. 1. Influence of zinc, cystine, NaCN, etc., on the lipolytic activity of pancreatic lipase of rats at pH 8·2 and in the presence of Ca^{++} ions.

Zinc and lipolytic activity of pancreatic lipase. The influence of zinc on the fat content of liver was further investigated by testing its effect on the lipolytic activity of pancreatic lipase, and the results of experiments in which zinc sulphate, sodium cyanide and L-cystine were added at a concentration of 0·01 M to pancreatic lipase with and without prior addition of calcium chloride and at two different pH values (6·8 and 8·2) are presented in Table 4. In another experiment, the effect of different concentrations of these substances and also of the two well known lipotropic factors, choline and methionine, on the activity of the enzyme at a pH of 8·2 in the presence of Ca^{++} ions was tested. The results, presented in Fig. 1, show clearly that both cystine and cyanide increase the lipolytic activity of the enzyme under the conditions specified. This effect of cystine may be related to its known influence on the accumulation of fat in the liver of rats fed on a diet low in lipotropic factors (Beeston & Channon, 1936; Tucker & Eckstein, 1937; Mulford & Griffith, 1942). The significance of results obtained when zinc and cyanide were added is not at present clear and merits further investigation.

Table 4. *Influence of zinc and cystine on the lipolytic activity of pancreatic lipase of rats*

(For method see text.)

	(ml. 0·1 N-NaOH)	
	pH 8·2	pH 6·8
Control	11·4	7·8
Control + 0·01 M-NaCN	15·0	14·4
Control + 0·01 M-$ZnSO_4$	12·0	4·8
Control + 0·01 M-L-cystine	15·9	7·5
Control + 20 mg. $CaCl_2$	20·7	5·4
Control + 20 mg. $CaCl_2$ + 0·01 M-NaCN	30·9	14·1
Control + 20 mg. $CaCl_2$ + 0·01 M-$ZnSO_4$	13·2	8·4
Control + 20 mg. $CaCl_2$ + 0·01 M-L-cystine	29·4	6·0

SUMMARY

1. The changes in the metabolism of nitrogen, phosphorus and sulphur and in urinary excretion of uric acid and total creatinine (creatine and creatinine) in rats fed on a high-fat diet supplemented with zinc have been shown to be similar to those observed for a stock diet. It has also been shown that the decreased assimilation of phosphate from the intestines is not due to its being precipitated as insoluble zinc phosphate.

2. Zinc supplements at the levels investigated affected phosphatase activity of the tissues. While the activity of intestinal alkaline phosphatase was lowered, there was an increase in activity in the liver and kidneys in the supplemented groups.

3. Though zinc supplements lowered the fat content of the liver and the assimilation of phosphate from the intestines, there appeared to be no change in the excretion of fat in the faeces.

4. The influence of zinc and other substances such as choline, methionine, L-cystine and cyanide on the lipolytic activity of pancreatic lipase of the rat was also investigated.

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Am. J. Physiol. 125: 24-30 (1939)

EFFECT OF ZINC ON INSULIN AND ITS MECHANISM

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Received for publication September 28, 1938

Maxwell and Bischoff (1) were first to show that the addition of ferric ions to insulin causes an augmentation of its physiologic effect as measured by blood sugar changes in animals. Since then several investigators (2, 3, 4, 5) have demonstrated that the addition of zinc to insulin causes a retardation and a prolongation of its hypoglycemic effect on blood sugar. No attempt has been made to explain the mechanism of this prolongation.

Clear solutions of insulin for clinical or experimental purposes are usually prepared in a slightly acidified medium. When such a solution is injected subcutaneously the buffer action of the tissues adjusts the acidity of the material injected to about neutrality. Thus the insulin must pass through various stages of hydrogen ion concentration until the reaction at the site of injection is in equilibrium with that of the tissue fluid, whereupon absorption takes place. In order to demonstrate this phenomenon a simple experiment may be performed. One adds a few drops of brom-thymol blue to an acidified solution of insulin and injects a small amount into a clean-shaven area of a rabbit's ear. In about 10 minutes the color of the injected area changes from orange to green and finally to blue. Once the color has become permanently blue, diffusion becomes noticeable which probably corresponds to the initial action of insulin on blood sugar.

In this investigation we demonstrate *A*, the effect of crystalline and amorphous insulin on the blood sugar of rabbits; *B*, the effect of small amounts of zinc added to zinc-free insulin on the blood sugar of rabbits, and *C*, the precipitation of insulin protein by the addition of zinc.

Experimental. *A*. Suitable dilutions of crystalline and unmodified insulin were made so as to contain 2.5 units per 1 cc. In order to present a true picture of the effect of each preparation the "cross-over" method was used; half of the animals received a dose of crystalline insulin and the other half received a dose of the unmodified insulin. One week later, those animals which had received unmodified received crystalline insulin and vice versa. In each instance 1 unit of insulin per kgm. was given subcutaneously. Thirty-three rabbits were used for the test. Blood was withdrawn at

0, 1.5, 3 and 5 hours. The averages of the data obtained are found in figure 1.

B. The preparation of a zinc-free insulin has been previously described (6). From a stock solution of this preparation containing 100 units per 1 cc. a 2 units per 1 cc. dilution was made. This was used as a control. Similar dilutions were then made containing 1, 2 and 4 mgm. of zinc per 1000 units. They were kept in a refrigerator when not in use.

Rabbits that had been starved for 24 hours were used for the test. Five-tenths unit per kilogram of each of the above samples was given subcutaneously to each of 24 rabbits. Blood was withdrawn before and 1.5, 3, 4 and 5 hours after the injection. Averages of blood sugars were then made. The results are shown in figure 2.

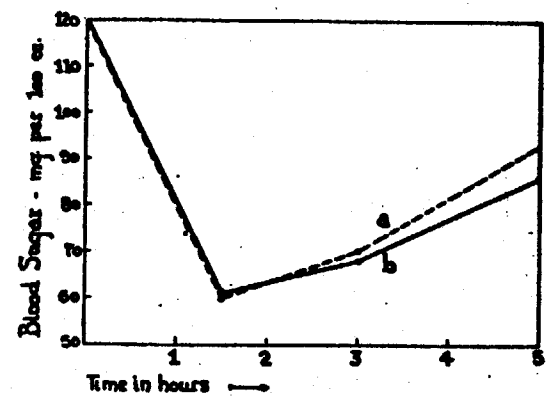

Fig. 1. The effect of crystalline and unmodified insulin on the blood sugar of fasting rabbits. Thirty-three animals were used for each material. One unit per kilogram was given subcutaneously. *a:* amorphous (unmodified) insulin; *b:* crystalline insulin.

C. The use of zinc as zinc hydroxide for the precipitation of proteins has been widely used (7). In this experiment we studied the precipitation of insulin protein with zinc at different acidities. One thousand units of zinc-free insulin were introduced into each of a series of 50 cc. cone-shaped centrifuge tubes. One cubic centimeter of a zinc chloride solution containing 9.5 mgm. zinc was added to each, followed by 2 cc. of sodium acetate buffer. The volume of each sample was brought to exactly 25 cc. The samples were then thoroughly mixed, allowed to stand at room temperature for 2 hours and centrifuged. Nitrogen and zinc determinations were then made on both the supernatant liquids and the precipitates. The nitrogen and zinc in the supernatant liquid were considered to represent the soluble fraction and the amounts of nitrogen and zinc in the precipitate to represent the insoluble fraction of insulin and zinc respectively. The results are recorded in table 1.

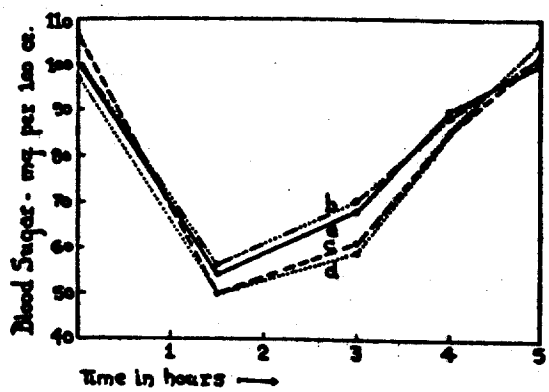

Fig. 2. The effect of added zinc in varying amounts on the blood sugar of 24 fasting rabbits. Five-tenths unit per kilogram was given subcutaneously. *a:* Control, no zinc; *b:* 1.0 mgm. zinc per 1000 units; *c:* 2.0 mgm. zinc per 1000 units; *d:* 4.0 mgm. zinc per 1000 units.

TABLE 1

Precipitation of insulin protein in the presence of zinc, in acetate buffer

MATERIAL	pH	INSULIN		ZINC	
		Soluble	Insoluble	Soluble	Insoluble
		per cent	*per cent*	*per cent*	*per cent*
Insulin + Zinc	4.5	14.5	85.5	92.6	7.37
Zinc	4.5			98.5	1.58
Insulin + Zinc	5.2	9.68	90.4	94.8	5.26
Zinc	5.2			98.0	2.10
Insulin + Zinc	5.8	4.84	95.02	73.8	26.38
Zinc	5.8			99.0	1.05
Insulin + Zinc	6.5	6.45	93.5	73.8	26.38
Zinc	6.5			72.1	27.85
Insulin + Zinc	7.0		100.0	42.1	57.99
Zinc	7.0			44.2	55.8
Insulin + Zinc	7.6	3.22	96.9	36.8	63.01
Zinc	7.6			39.0	61.0

TABLE 2

Adsorption of insulin protein on zinc hydroxide at pH 7.0

Amount of added zinc per 1000 units insulin	Insulin adsorbed
mgm.	*per cent*
1.0	2.0
1.5	12.0
2.0	75.0
2.5	85.0
3.0	90.0
5.0	95.0
10.0	99.0

In the second experiment of this series we studied the minimum amount of zinc required to cause a complete precipitation of 1000 units of insulin protein at pH 7.0. The details are as in the foregoing experiment. The amount of zinc added in each instance and the relative amount of insulin adsorbed at about pH 7.0 are shown in table 2.

Discussion. Since it has been shown that insulin with added zinc has a more prolonged hypoglycemic effect on the blood sugar of animals than insulin without zinc, an attempt was made to determine the minimum amount of zinc required to produce a definite prolongation, and to offer an explanation of the mechanism involved. The data presented in figure 2 show that the insulin containing 1 mgm. of zinc per 1000 units does not exert any greater effect on the blood sugar of rabbits than the same insulin without added zinc. However, when 2 or 4 mgm. of zinc per 1000 units are added to insulin there is a noticeable change in the blood sugar curve (fig. 2). It is to be remembered that the amount of insulin injected was only 0.5 unit per kgm. Had a larger dose been administered the prolonged effect of the added zinc might have been more noticeable but it was our aim to avoid convulsions and permit the animals to return to normal within the 5 hour period.

The prolongation of insulin action by zinc is probably due to the adsorption of insulin protein on insoluble basic zinc salts. In the introductory paragraph we presented a simple experiment in which it was shown that the buffering action of the tissue fluid effects a neutralization of the insulin injected. This is in accord with Donnan's theory of equilibrium and diffusion of ions. Thus when equilibrium is reached at the site of injection the soluble zinc present is transformed to its insoluble form of basic zinc salts. The data presented in tables 1 and 2 show that if zinc is present in sufficient amounts the insulin protein is completely adsorbed at pH 7.0 or thereabout. Thus from a chemical point of view, in order to produce the most efficient and prolonged hypoglycemic curve about 10 mgm. of zinc are required for every 1000 units and any extra zinc need not be added. However, from a physiological point of view, an exchange of ions at the site of injection must be taken into consideration. Since the addition of 1 mgm. zinc per 1000 units does not apparently effect any appreciable precipitation of insulin protein at about neutrality one therefore cannot expect any noticeable change in the blood sugar curve. However, when 2 mgm. of zinc are added to 1000 units of insulin, as the data presented in table 2 show, about 75 per cent of the insulin protein precipitates at pH 7.0. The blood sugar curves of rabbits injected with insulin containing these amounts of added zinc agree with these chemical data.

Kirsner and Bernstein (8), basing their opinion on results obtained with a few animals, have recently reported that in rabbits the effect of crystalline insulin is not only in general more prompt but of less duration than the commercial preparation of insulin. In figure 2 we present the effect of

crystalline and unmodified insulin on the blood sugar curves of 33 rabbits using the "cross-over" method. The crystalline insulin used is identical with the material furnished by this laboratory to Kirsner and Bernstein. Nevertheless we do not propose to draw any conclusion other than that the duration of the blood sugar curve of rabbits injected with crystalline insulin is at least as prolonged as that of rabbits injected with an equal amount of unmodified insulin. Kirsner and Bernstein used their few animals at intervals of two days, switching from one kind of insulin to another. The customary technique is to use rabbits that have been starved for 24 hours, at intervals of one week, and to administer doses of insulin proportional to the body weight. Blatherwick and co-workers (5) using the same crystalline insulin in rats noted a significant difference in the duration of hypoglycemic effect of crystalline and unmodified insulin, with a slight prolongation of the hypoglycemia following crystalline insulin.

Significant are the results obtained by the various laboratories (9) who coöperated on the standardization of the Crystalline Insulin Standard. Although the averages of their results have no bearing whatever on the question of whether or not crystalline insulin has a more prolonged effect on the blood sugar of animals than unmodified insulin, nevertheless one notes a considerable difference between the value of the Crystalline Insulin Standard obtained by the rabbit method and the mouse method of assay. By the rabbit method, 1 mgm. of the Crystalline Insulin Standard assayed at 21.7 International Units and by the mouse method, 25.2. The 14 per cent difference between the two methods of assay is beyond any experimental error. In the mouse method of assay one relies on the incidence of convulsions while in the rabbit method the criterion is the fall in blood sugar. In evaluating the Crystalline Insulin Standard an amorphous insulin standard of 8 units per 1 mgm. was used. Since the comparison was made between a crystalline and an amorphous preparation of insulin, the difference in results may be attributed to a difference in chemical properties, or to the presence of unknown substances in the amorphous insulin. Anyone familiar with the properties of amino acids knows that the solubility of certain amino acids in water is quite different when dissolved singly than when dissolved in the presence of other amino acids. Proteins behave in a similar manner. The solubility of crystalline insulin is not identical with that of unmodified insulin as can be seen from the curve in figure 3. Owing to its solubility and to its freedom from associated proteins crystalline insulin possibly exerts a different effect on blood sugar of animals and of humans.

In their clinical investigations, Rabinowitch and co-workers (10) observed that crystalline insulin has a more prolonged hypoglycemic action than unmodified insulin. This prolonged action was explained on the basis of

the zinc associated with crystalline insulin. The amount of zinc in the crystalline material they used in their investigation was at no time in excess of 0.9 mgm. zinc per 1000 units. Altshuler and Leiser (11) who had previously noted a prolonged action of crystalline insulin investigated clinically the effect of the addition of 1 mgm. of zinc to insulin and were unable to confirm Rabinowitch and co-workers' theory. The chemical data and our results on rabbits, presented in this paper, clearly demonstrate that the addition of 1 mgm. of zinc per 1000 units does not augment the physiological response of animals to insulin. Since crystalline insulin as it is actually prepared does not contain more than 0.04 mgm. zinc per 100 units, it is highly improbable that its hypoglycemic effect on blood sugar is influenced by this small amount of zinc. On the other hand any difference in physiologic response that may be observed between crystalline and

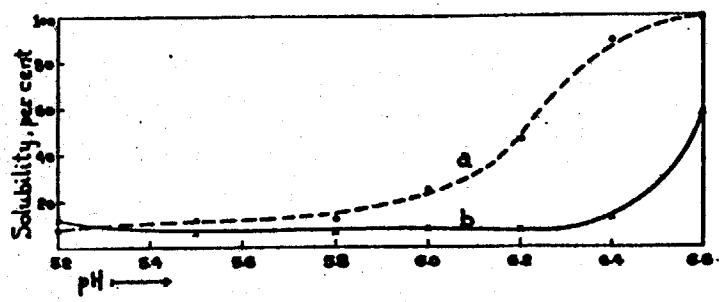

Fig. 3. The solubility of crystalline and amorphous (unmodified) insulin in the pH range 5.2–6.6 *a:* amorphous (unmodified) insulin; *b:* crystalline insulin.

amorphous insulin could be logically attributed to a difference in solubility, and to the purity of crystalline insulin.

SUMMARY

Studies were made of the effect on the blood sugar of fasting rabbits of crystalline insulin, unmodified insulin and insulin to which known amounts of zinc had been added. The data presented show that 1, the hypoglycemic effect of crystalline insulin is at least as prolonged as that of the unmodified; 2, the addition of 1 mgm. zinc per 1000 units of insulin does not augment the physiological response, while 3, the addition of 2 mgm. zinc or more produces a pronounced augmentative effect.

The experimental evidence presented shows that insulin protein is adsorbed on basic zinc salts at about neutrality. This probably explains the prolongation of hypoglycemia produced by insulin with added zinc.

Crystalline insulin is shown to produce a different solubility curve from that of amorphous insulin.

The author wishes to thank the following for assistance with the experimental work: Rolland Feldkamp, Harlan Davis, Arthur Nixon and Molly Goodell.

He is also indebted to Dr. E. M. K. Geiling for valuable suggestions and criticism.

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THE JOURNAL OF INDUSTRIAL HYGIENE 2: 72-78, 1920.

THE PHARMACOLOGY OF HEAVY METALS*

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THE behavior of heavy metals has long been the subject of inquiry by pharmacologists and toxicologists—inquiry resulting in the growth of an extensive literature on the subject. Indeed, a bibliography of the action of heavy metals would fill many pages including such investigations as their manner of absorption, their fate in the body, their effects on metabolism and their influence on different organs. There is, nevertheless, a striking paucity of data concerning the action of some of the heavy metals, while the behavior of others in the body is scarcely known. This is true notwithstanding the fact that many cases of poisoning have occurred as a result of presence of these metals in food products and notwithstanding the fact that a number of them are known to play an important rôle in industrial hygiene.

The extent to which some of the heavy metals are present in food products is indicated in the following reports. According to analyses published recently by Birckner (1) the quantity of zinc in oysters may reach 1.15 gm. per kilo, in baker's yeast 0.414 gm. per kilo. Dried eggs, according to analyses made in the Bureau of Chemistry, may contain as much as 2.4 gm. per kilo; while Hiltner and Wichmann (2) reported 2.9 gm. of zinc per kilo in oysters. The results of similar analyses for copper are also interesting. The quantities in oysters varied between 52 and 539 mg. per kilo, but in one instance, with drained oysters, 1487 mg. per kilo were found. According to Leach (3), from traces up to 2.75 gm. of copper per can were found in canned vegetables. The same writer states that the quantity of tin in canned vegetables may amount to about a third of a gram per kilo. According to Harrington (4), 0.25 gm. of nickel sulphate may be present in green peas. Tin has been found in a number of foods in amounts of 10 to 450 mg. per kilo, the quantity even reaching so high a figure per kilo as 1.5 gm. Lead also is present in very small quantities but in view of the clinical and experimental evidence concerning this metal, as shown by the work of Straub (5), Erlenmeyer (6), Legge and Goadby (7), and Oliver (8), very small doses cannot be considered safe when taken into the body habitually — a contingency which must occur if it is present in foods.

The effect of heavy metals on health is therefore a matter of prime importance — a fact which emphasizes the need for more extensive investigation — for only by obtaining incontestable evidence of their poisonous effects can preventive methods be adopted. But a better understanding of the action of heavy metals is needed in other connections. Heavy metals are also known to cause injurious effects of a serious character in persons exposed daily and for years to their action in many important industries. It is well known that lead poisoning occurs in a large number of trades. Oliver (8), Legge and Goadby (7) in England, and Alice Hamilton (9) in this country, who have for years been directing attention to industrial lead poisoning, state that lead is employed in about 150 industries. Zinc is also known to be an important etiological factor in the causation of certain industrial diseases. The symptoms of poisoning observed in braziers and brassworkers may be mentioned as some of the abnormal changes produced by this metal. Copper is not commonly considered an industrial poison, but according to Hayhurst (10) its astringent and caustic salts

* Presented before the New York Academy of Sciences, Section of Astronomy, Physics and Chemistry, Feb. 2, 1920. Received for publication March 3, 1920.

may produce contact poisoning in workers handling them. Cases of poisoning with manganese have been reported within recent years by several observers. Casamajor (11) reported ten cases with peculiar neurological symptoms and a number of cases have been described by Emden (12), by von Jaksch (13), and very recently by Edsall and his collaborators (14). Nickel in metallic form was considered to be without toxic effect, but experiments carried out by Armit (15) indicate that it is by no means free from poisonous properties. This is especially the case when nickel is in combination with carbon monoxide forming nickel carbonyl, the fumes of which when inhaled produce serious and even fatal symptoms. The effect, according to Armit, is not due to the carbon monoxide present but to nickel in the form of a slightly soluble compound or in a fine state of subdivision. Other heavy metals, such as mercury and certain compounds of iron, vanadium and chromium may also be mentioned in this connection as causative agents in the production of occupational diseases.

Further and wholly different reasons from these suggest also the need of further inquiry into the mode of action of heavy metals. In the absence of accurate information on this subject, federal and state officials may, and sometimes do, impose unnecessary restrictions on the manufacture and sale of various food products, leading to unnecessary waste of foodstuffs and to needless disturbance of important industries with consequent economic loss, which in these days of the high cost of living is a matter for serious consideration.

To aid in safeguarding the public health and in preventing economic losses to the community and to the individual, the writer several years ago planned extensive investigations into the action of heavy metals. Some of the substances in question have already been studied by him and his collaborators, but much more remains to be done. Owing to circumstances over which he had no control, the work was interrupted, but the writer hopes to resume these urgently needed investigations. Meanwhile, preliminary observations on the subject are presented in the following report.

Experiments with Zinc

Zinc in our experiments was given to different animals, usually in the form of malate but sometimes as the chloride. The salts were fed to animals in their food and tests were made of the effects on different organs. The feeding experiments were carried out on rats for periods of two to four months, and on cats for from ten days to two months. No effects were noticed on the general health, although considerable quantities of the metal were given, much more than could possibly be taken with food by a human being in the course of a day. Rats weighing 150 to 200 gm. which received 10 to 15 mg. of zinc acetate daily for four months kept their weight, though in some cases there seemed to be some disturbance of renal function. Cats received daily 50 mg. of zinc in the form of malate. Some of them gained in weight 23 to 26 per cent.; one cat gained 4 per cent.; three cats showed a loss of 3 to 5 per cent. The literature on this subject shows contradictory results. According to Sacher (16), amounts up to 34 mg. of zinc per kilo given daily in their food to rabbits for from five to thirty days proved harmful, and 6 to 28 mg. proved harmful to cats. Sacher reported loss of appetite, albuminuria, congestion of the kidney, and hemorrhage into the gastric mucosa. On the other hand, it was reported by Lehmann (17) that 0.5 gm. of zinc per day, fed daily for 242 days, failed to cause any untoward effects. Brandl and Scherpe (18) fed various amounts of zinc salts to different animals. Dogs, rabbits, and guinea pigs which received daily 1 to 2

mg. of zinc as malate for seventy-seven to eighty days failed to show any effects. Young dogs that were fed 8 to 9 mg. daily for 127 days continued to grow and gain weight. In another experiment on two dogs, increasing doses of zinc were administered for fifteen days until in one case a dose of 420 mg. of zinc per kilo was given, but no symptoms of injurious action of the metal were observed. Rabbits, however, seemed to be less resistant. Up to 8 mg. per day were borne without any apparent injury, but when the dose was increased to 437 mg. per kilo daily and given for some time, there was loss of appetite and albuminuria. On autopsy the kidneys were found congested and showed the presence of cloudy swelling; there was also hemorrhage into the mucous membrane of the stomach.

Although the action of zinc when taken by mouth, even in comparatively large amounts, is not very injurious, the case is quite different when zinc is introduced directly into the circulation and comes in contact with various organs. The writer and Miss Connet (unpublished experiments) have shown that when a nutrient solution to which a small quantity of zinc is added is perfused through the isolated frog heart, a change in its activity is at once noticed. Heart action becomes weak, the amplitude becomes visibly smaller with each beat, and the action becomes irregular, auricles and ventricles ceasing to work in harmony. This may go on as long as the heart is exposed to the solution; not infrequently the change caused by the zinc may continue even after the perfusion has been discontinued. A second treatment, after a considerable interval of time, produces more marked effects, thus suggesting cumulative action. In heart-perfusion experiments, carried out by me with the collaboration of Miss Connet, with a solution of 1:50,000 of zinc malate in Ringer's solution or one part of zinc to about 200,000, one minute was sufficient time to produce very marked cardiac depression. The injury to the heart was very striking when stronger solutions of zinc malate were used. One-twenty-fifth of 1 per cent. caused depression of heart action which tended to be permanent, while in other cases recovery was very slow. With a concentration of 1:1000 of zinc malate in Ringer's solution, permanent arrest of the heart was observed.

The depressing effect of zinc is not confined, however, to the heart. Mitchell and I (19) carried out experiments with zinc salts on different animals and found that smooth muscle is likewise affected by zinc. Segments of the intestines, a few centimeters long, removed from animals under deep anesthesia and suspended in Locke's solution, ceased their movements when a sufficient amount of zinc malate was added. A solution of N/2000 of zinc malate produced a very distinct diminution in the force of rhythmic contraction, and when the concentration of zinc was doubled the contractions became very weak and irregular at the end of forty-five or fifty minutes. When the solution containing zinc was removed and another solution without zinc substituted, there was only partial recovery of function. When the amount of zinc was increased still more, there was complete abolition of function, which in some cases was permanent.

That zinc is a general protoplasmic poison is further indicated by the fact that it has a decided influence on other organs than the heart. According to Harnack (20) zinc caused in higher animals symptoms of nervous depression, muscular paralysis, and paralysis of respiration and of the circulation. According to the observations of several investigators, zinc, like some of the other metals such as mercury, uranium and chromium, may produce serious injury to the kidney. This, as shown by D'Amore, Falcone and Marmaldi (21) may be associated with glycosuria. Recently Dr. Wise and I (22) carried out experiments with

zinc malate. We found that a gram of zinc malate fed to animals may be followed by albuminuria. The effect was more constant and the damage more pronounced if a small quantity of zinc was introduced directly into the circulation. Ten mg. of zinc per kilo injected intravenously caused very marked injury to the kidney; a large amount of albumin and many casts were present in the urine. In a large proportion of cases death occurred within a few days. Associated with these renal changes was the presence of sugar in the urine as well as an increased concentration of sugar in the blood, amounting at times to 30 or 35 per cent. The amount of sugar in the urine of rabbits did not exceed 0.3 per cent., but in cats it reached 2.75 per cent. after subcutaneous injections. Glycosuria was also observed in dogs after subcutaneous injections of zinc salts, but the quantity was less than in the urine of cats.

The elimination of zinc after it reaches the circulation has been studied by several workers, the results obtained being somewhat contradictory. In co-operation with Rieger and Treuthardt (23), I was able to obtain interesting data on this subject. We found that zinc is absorbed from the intestine and that the intestinal canal is also the main channel of elimination, the kidney playing a very subordinate rôle in ridding the body of the metal. No zinc was found in the stomach of animals which received zinc salts intravenously. A study of its distribution in the body indicated that it was stored up in considerable amounts in the liver; it was almost always found in the skin and in the muscles, but none was present in the brain.

Experiments with Tin

Another metal which is of frequent occurrence in foods is tin. The enormous development of the canning industry makes it particularly desirable that accurate information regarding the physiological effects of tin should be obtained. I carried out feeding experiments with the tartrate and chloride on cats, administering daily 10 to 50 mg. of tin in the form of these salts, mixed with the food of the animals. Ten mg. of tin per kilo, fed for twenty-three days and followed by 20 mg. per kilo for three months failed to show any harmful effects. There was, on the contrary, a gain in weight of 100 to 400 gm. But when the dose was increased to 30 mg. per kilo and fed for about two and a half months there was a loss of weight amounting to 10½ per cent. in two cats. The decrease was much faster in these two cases when the dose was increased to 50 mg. of tin per kilo. At the end of four weeks' feeding of such a dose, the loss in weight of one cat amounted to 10 per cent.; in the other it was 16 per cent. Three other cats similarly treated were more resistant, as they lost only 5 per cent. of their weight during the four weeks. But a sixth cat which weighed 1400 gm. lost 450 gm. and died at the end of the period. It is evident, therefore, that moderately large amounts of tin given daily with food for fairly long periods may prove harmful to health. It might be added that the chloride of tin is probably more injurious than the tartrate, since the marked emaciation occurred in the cat which received the chloride. It is possible, however, that the decreased resistance to tin was due to some abnormal condition in this cat, since two other cats belonging to the same series of experiments received the same amounts of tin chloride and survived with a loss of weight amounting to only 5 per cent.

Rieger, Treuthardt and I (24) also studied the fate of tin in animals. After the subcutaneous injection of some of its salts, the metal was present in the urine and in the contents of the stomach and of the intestine. Smaller amounts, however, were eliminated by the kidneys than by the intestines, tin resembling zinc in this respect. It is of interest to note that the rate of

excretion of these two metals was somewhat different, the amount of tin excreted by the kidney being greater than that of zinc. In some experiments almost as much tin was eliminated by the kidneys as by the intestines, but this was never the case with zinc. That tin is very slowly absorbed from the intestines was shown by the fact that after feeding considerable quantities daily and for weeks, very small amounts only were found in the urine. This is not at all surprising in view of the fact that the salts of tin are so insoluble. When they are fed for a long time, however, absorption may take place, for small quantities were recovered from animals that had received the tartrate for from three to four months. It is quite probable that after tin has been fed for a certain time changes in the mucosa occur which increase its permeability to the metal. This is supported by observations by Buchanan and Schryver (25), who detected tin in the urine of dogs during the second and third weeks of the feeding of tin salts, but not during the first week of the experiment.

The results of experiments carried out on other heavy metals are of interest in this connection. Miss Connet and I (unpublished results) made a number of tests with copper sulphate on the isolated heart of the frog. A solution of 1:10,000 produced prompt depression of cardiac action. At the end of one minute's exposure to the salts, the heart became very feeble and remained permanently in this condition. Much more pronounced was the effect of cadmium. A solution of 1:1,000,000 of the acetate in Ringer's solution caused a very noticeable diminution in the force of the heart, without changing the rate to any appreciable extent. A solution of 1:100,000 produced about the same effect as a concentration of copper sulphate that was ten times as strong.

Experiments with nickel acetate were made on the heart as well as on the isolated intestine. The effects were different from those obtained with the other heavy metals. When the frog heart was perfused with 1:10,000 nickel acetate in Ringer's solution, the contractions became less frequent but more forcible, and recovery occurred after perfusion with nickel acetate was discontinued. A more detailed study of the influence of nickel, copper, cadmium, and zinc on the isolated heart will appear shortly in the *Journal of Pharmacology and Experimental Therapeutics*. The report will also include experiments with cobalt, iron, manganese and uranium. The reaction of the isolated intestine to nickel acetate, which was studied by Mitchell and myself (19), indicated a very pronounced depression with strong solutions only. Weak solutions caused primary depression followed by stimulation.

Of the other heavy metals to which attention might be called, lead is the most toxic. Straub and his pupil, Erlenmeyer, have shown that less than 1 mg. of lead per kilo when present in the circulation is sufficient to cause bulbar paralysis in cats within sixty days. In experiments on the isolated intestines of animals the writer and Levine (unpublished experiments) found that small quantities are markedly toxic, which is particularly interesting in view of the fact that lead has been shown to be present in small quantities in certain food products. Baking powder contains about 10 mg. of lead per kilo, but it is doubtful if even this amount should be permitted in articles intended for human consumption. According to Professor Rubner and Dr. Smith, 0.36 mg. of lead is the minimum of safety quoted by Oliver. According to Oliver (8), 0.2 to 1.5 mg. of lead in a liter of water cause lead poisoning when taken for several months. A note of warning may therefore be sounded to those who are in a position to decide whether the public should be permitted to use food products that contain any lead at all.

In conclusion, it may be pointed out that some of the heavy metals considered are very poisonous, producing degenerative changes in some organs and causing functional disturbances in others. At the same time it is necessary to recognize the very important fact that the heavy metals are apparently well borne for a considerable length of time when taken with food. At least no injurious effects can be traced to their ingestion, which indicates that the intestinal canal probably exerts a protective action against the poisonous action of these metals and their salts. It is necessary to bear in mind, however, that disturbance of function of the intestinal canal or structural changes of the mucous membranes of the stomach and intestines may occur, in which case the protective action of these organs may cease and the metals may thus gain access to the different organs causing serious and even irreparable damage. It is advisable, therefore, that notwithstanding the apparent harmlessness of some of the heavy metals when taken with foods, caution should be exercised in permitting the manufacture and sale for human consumption of foods containing even small amounts of such metals. The public is entitled to the benefit of the doubt in the case of those metals, the harmlessness of which has not been firmly established.

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The Effect of Emetics on Canine Gastric Secretion

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Further parameters on the effects of emesis on gastric secretion were investigated in dogs with denervatd gastric pouches. In adequate concentrations, copper sulfate, zinc sulfate, and Veriloid caused salivation, retching, and vomiting which was followed, after a latent period, by an increase in the gastric volume and the acid concentration. This latent period corresponded to the gastric inhibitory phase that follows emesis. Apomorphine, which also induced salivation, retching, vomiting, and an inhibition of gastric secretion, did not cause an augmented gastric secretion.

VOMITING is an ancient biologic protective function that appears to be designed to rid the body of orally ingested noxious materials. It can be produced by the administration of certain pharmacologic agents.[1] A review of the literature indicates that gastric secretion is inhibited by vomiting produced by pharmacologic agents,[2] by duodenal distention,[3] and by stimulation of the vestibular apparatus.[4] Recently, it was reported[5] that the intravenous administration of nitrogen mustard in unanesthetized dogs produced retching and vomiting that was followed, after a latent period, by increased gastric secretion. The present report is concerned with the action of other emetic agents on gastric secretion in the dog.

MATERIALS AND METHODS

A total of 40 studies were carried out on 11 unanesthetized dogs weighing 10–12 kg. Each animal was prepared with a vagally denervated gastric (Heidenhain) pouch of the stomach, and a 12- to 14-day recovery period was allowed before the experiments were begun. Control fasting samples of gastric juice were collected at 15-min intervals for 60–120 min. The emetic agents were then administered, and samples of gastric juice were again collected at 15-min intervals for a 4-hr period. The volume of each sample of gastric juice

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Supported in part by Grant GB-7687 from the National Science Foundation.

The investigators wish to acknowledge the library assistance of D. L. House, Jr., MSLS (supported by US Public Health Service Grant LM-00102).

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*US Public Health Service Part-time Student Fellowship (Grant 21301 1675R).

American Journal of Digestive Diseases
14(7): 470-476, 1969.

was measured, and its acid concentration was determined by titration with 0.1 N NaOH by using a Topfer's reagent.

The emetics used were copper sulfate, zinc sulfate, Veriloid,* and apomorphine. In nine experiments on 6 dogs, 50 mg/kg, and in five studies on 4 dogs, 25 mg/kg, of a 0.5–1.0% aqueous solution of copper sulfate was instilled into the main stomach through a Levin tube during 2 min. In five studies on 4 dogs, 75 mg/kg of a 0.75–1.5% aqueous solution of zinc sulfate was instilled into the main stomach through a Levin tube during 2 min. Veriloid was also instilled through a Levin tube into the main stomach. In five experiments on 3 dogs, 0.2 mg/kg, and in four studies on 2 dogs, 0.1 mg/kg, of a 0.01–0.02% aqueous solution was administered. Finally, in 12 studies, apomorphine was injected intravenously during a 30-sec interval. In four of these studies on 3 dogs 0.6 mg/kg was used; in three experiments on 2 dogs 0.3 mg/kg was given; in three studies on 3 dogs 0.1 mg/kg was injected; and in two studies on 1 dog 0.05 mg/kg was given. The dogs were rested a minimum of 5 days between experiments.

In another series of studies, 16 experiments were carried out on 4 unanesthetized dogs previously prepared with a Heidenhain pouch. These dogs previously had responded to an intragastric instillation of 50 mg of copper sulfate per kilogram. A subcutaneous injection of 0.05 mg of histamine (base) was made every 15 min until a gastric secretory plateau was maintained. In three studies on 3 dogs, 50 ml of 0.9% NaCl was given intragastrically, in seven studies on 3 dogs 50 mg of copper sulfate per kilogram was given through a Levin tube, and in six studies on 4 dogs 0.3 mg of apomorphine per kilogram was injected intravenously.

RESULTS

In nine experiments on 6 dogs the mean basal gastric volume was 0.8 ml/hr (range 0.4–1.3 ml/hr; SD 0.28). During this basal period no acid was demonstrated. The intragastric administration of copper sulfate (50 mg/kg) caused, with 5–15 min, salivation, retching, and vomiting that lasted for a 10- to 20-min period. The gastric secretion remained unaltered from control values for 60–90 min. After this time, both the gastric volume and the acid concentration of the denervated gastric pouch increased and remained elevated for 120–150 min (Table 1). During the first hour of the increase, the gastric volume was elevated 86% to a mean of 5.8 ml/hr (range 4.6–8.3 ml/hr; SD 1.25) and the acid output increased to 0.56 mEq/hr (range 0.47–0.74 mEq/hr). During the second hour, the gastric volume increased 85% above the basal value to a mean of 5.5 ml/hr (range 4.3–7.8 ml/hr; SD 1.18) and the acid output increased to 0.58 mEq/hr (range 0.47–0.75 mEq/hr). An example of one experiment is given in Fig 1.

*Riker Laboratories, Northridge, Calif.

TABLE 1. ACTION OF SOME EMETIC AGENTS ON MEAN BASAL AND HISTAMINE-STIMULATED GASTRIC SECRETION OF UNANESTHETIZED DOGS

Dogs (No.)	Experiments (No.)	Emetic agent	Dose (mg/kg)	Route of administration	Less than 1 hr: Gastric vol (ml/60 min)	Less than 1 hr: Acid output (mEq/60 min)	More than 1 hr: Latent* or inhibitory† period (min)	More than 1 hr: Gastric vol (ml/60 min)	More than 1 hr: Acid output (mEq/60 min)	More than 2 hr: Gastric vol (ml/60 min)	More than 2 hr: Acid output (mEq/60 min)
MEAN BASAL GASTRIC SECRETION											
6	9	$CuSO_4$	50	PO	0.8	0	80*	5.8	0.56	5.5	0.58
4	5	$CuSO_4$	25	PO	0.7	0	—	0.6	0	0.9	0
4	5	$ZnSO_4$	75	PO	0.9	0	55*	3.8	0.35	3.5	0.32
3	5	Veriloid	0.2	PO	0.9	0	80*	3.1	0.28	2.6	0.25
2	4	Veriloid	0.1	PO	0.8	0	—	0.8	0	1.1	0
3	4	Apomorphine	0.6	IV	0.9	0	—	0.9	0	0.8	0
2	3	Apomorphine	0.3	IV	0.9	0	—	1.1	0	0.6	0
3	3	Apomorphine	0.1	IV	0.7	0	—	0.9	0	0.9	0
1	2	Apomorphine	0.05	IV	0.3	0	—	0.2	0	0.3	0
HISTAMINE-STIMULATED GASTRIC SECRETION											
3	3	0.9% NaCl	50	PO	10.2	2.92	0†	11.1	3.04	10.6	3.12
3	7	$CuSO_4$	50	PO	13.4	1.83	60†	15.4	2.10	17.5	2.25
3	6	Apomorphine	0.3	IV	8.9	2.48	75†	7.8	2.04	8.2	2.22

* Latent period.
† Inhibitory period.

The mean basal gastric volume in five studies on 4 dogs was 0.9 ml/hr (range 0.8–1.1 ml/hr; SD 0.14). No acid was demonstrated during this basal period. The intragastric administration of zinc sulfate (75 mg/kg) caused, within 10–20 min, salivation, retching, and vomiting that persisted for 15–20 min. The gastric secretion remained unaltered from control values for 45–60 min. The gastric volume and the acid concentration then increased and remained elevated for 120–180 min (Table 1). During the first hour of the increase the gastric volume was elevated 76% to a mean of 3.8 ml/hr (range 3.3–4.4 ml/hr; SD 0.45) and the acid output increased to 0.35 mEq/hr (range 0.31–0.40 mEq/hr). During the second hour, the gastric volume increased 74% above the basal value to a mean of 3.5 ml/hr (range 3.1–4.1 ml/hr; SD 0.40) and the acid output increased to 0.32 mEq/hr (range 0.27–0.38 mEq/hr).

In five experiments on 3 dogs the mean basal gastric volume was 0.9 ml/hr (range 0.7–1.1 ml/hr; SD 0.14). During this basal period no acid was demonstrated. The intragastric administration of Veriloid (0.2 mg/kg) resulted, within 5–20 min, in salivation, retching, and vomiting that lasted for 20–40 min. Gastric secretion remained unaltered from control values for 75–90 min. After this time, the gastric volume and acid concentration increased and remained elevated for 120–150 min (Table 1). During the first hour of the increase, the gastric volume was elevated 77% to a mean of 3.1 ml/hr (range

2.9–3.6 ml/hr; SD 0.26) and the acid output increased to 0.28 mEq/hr (range 0.23–0.35 mEq/hr). During the second hour, the gastric volume increased 65% above the basal value to a mean of 2.6 ml/hr (range 2.2–3.1 ml/hr; SD 0.32) and the acid output increased to 0.25 mEq/hr (range 0.21–0.30 mEq/hr).

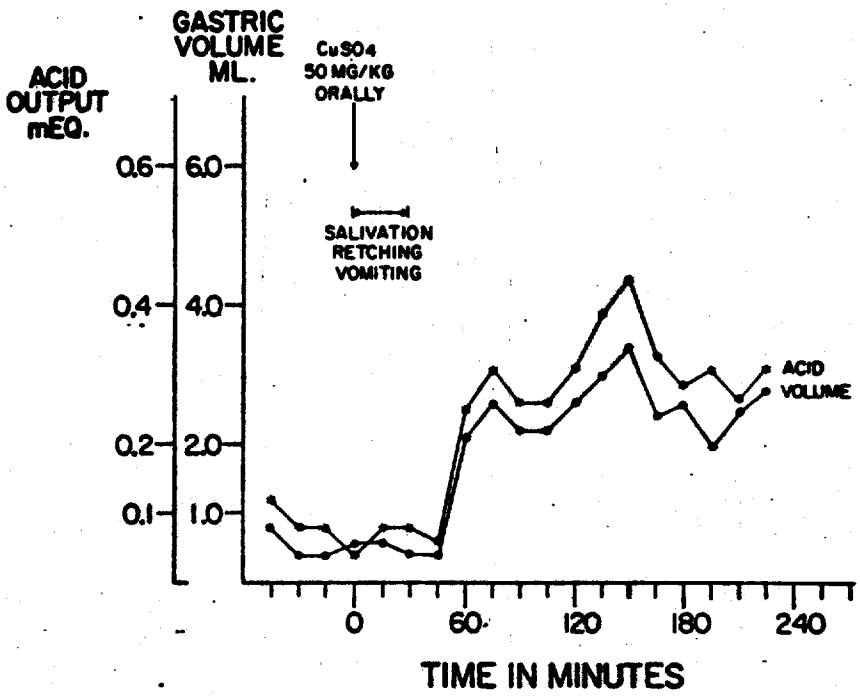

Fig 1. Action of $CuSO_4$ emesis on basal gastric secretion.

In five experiments the intragastric instillation of copper sulfate was reduced by 50% to 25 mg/kg, and in four studies the intragastric administration of Veriloid was reduced by 50% to 0.1 mg/kg. Although there was little or no salivation, retching and vomiting persisted for 10–30 min. There was, however, no increase in the gastric volume or the acid concentration over the control levels. Also, in 12 studies apomorphine (0.05–0.6 mg/kg) always resulted in salivation, retching, and vomiting, but no increase in the basal gastric secretion was noted. These dogs previously had responded to the intragastric instillation of copper sulfate (50 mg/kg) with an increased gastric secretory response.

In a final series of studies, a gastric secretory plateau was established by regular subcutaneous injections of histamine. In three experiments on 3 dogs the mean histamine-induced volume was 10.2 ml/hr (range 9.1–11.3 ml/hr; SD 0.9) and the acid output was 2.92 mEq/hr (range 2.82–3.01 mEq/hr; SD 0.08). The intragastric instillation of 50 ml of 0.9% NaCl did not alter this

plateau (Table 1). During the first hour, the mean gastric volume was 11.1 ml/hr (range 9.7–12.5 ml/hr; SD 1.0) and the mean acid output was 3.04 mEq/hr (range 2.92–3.15 mEq/hr; SD 0.08). During the second hour, the mean gastric volume was 10.6 ml/hr (range 9.9–11.3 ml/hr; SD 0.6) and the acid output was 3.12 mEq/hr (range 2.94–3.34 mEq/hr; SD 0.17).

The mean histamine-induced gastric volume in six studies on 3 dogs was 8.9 ml/hr (range 8.0–10.1 ml/hr; SD 0.77) and the acid output was 2.48 mEq/hr (range 2.37–2.60 mEq/hr; SD 0.08). This gastric secretory response was reduced for a 30- to 90-min period after the intravenous injection of apomorphine, and then it approximated its original level (Table 1). During the first hour after the inhibition, the mean gastric volume was 7.8 ml/hr (range 6.9–9.2 ml/hr; SD 0.84) and the mean acid output was 2.04 mEq/hr (range 1.92–2.17 mEq/hr; SD 0.10). During the second hour, the mean gastric volume was 8.2 ml/hr (range 7.4–9.0 ml/hr; SD 0.55) and the mean acid output was 2.22 mEq/hr (range 2.05–2.34 mEq/hr; SD 0.10).

Finally, in seven experiments on 3 dogs the mean histamine-induced volume was 13.4 ml/hr (range 12.4–14.2 ml/hr; SD 0.66) and the mean acid output was 1.83 mEq/hr (range 1.71–1.91 mEq/hr; SD 0.07). This gastric secretory output was sharply reduced for a 45- to 75-min period after the oral administration of copper sulfate (50 mg/kg), after which time the response increased above the original plateau (Table 1). During the first hour of the increase, the mean gastric volume was elevated by 15% to 15.4 ml/hr (range 14.8–16.3 ml/hr; SD 0.55) and the mean acid output also was raised by 15% to 2.10 mEq/hr (range 1.98–2.22 mEq/hr; SD 0.08). During the second hour, the mean gastric volume was increased by 23% to 17.5 ml/hr (range 16.6–18.7 ml/hr; SD 0.71) and the acid output was raised by 18% to 2.25 mEq/hr (range 2.14–2.37 mEq/hr; SD 0.07). This increase in the gastric volume and acid concentration, as tested by the *t* test, was significant to the $p < 0.01$ value. An example of one experiment is given in Fig 2.

DISCUSSION

In 1938, Atkinson and Ivy[2] studied the effects of emetics on the gastric secretion of innervated canine gastric pouches and on duodenal ulcer patients. Fixed doses of histamine were used as the secretory stimulus. The subcutaneous injection of 6.0 mg of apomorhpine combined with 36 mg of codeine sulfate produced vomiting accompanied by a reduction in the gastric secretory response to histamine. The intravenous administration of 120 mg of emetine hydrochloride produced vomiting and diarrhea and stopped gastric secretion completely. Wolf[1] induced nausea in 3 normal subjects and in a patient with gastric fistula by caloric vestibular stimulation; gastric secretion and motility were inhibited for 20–40 min. Finally, in 1945, Grossman and co-workers[2] induced salivation and retching by distention of a balloon in the duodenum, while gastric pouches, deprived of all connections with the central nervous

system, were secreting continuously in response to repeated injections of histamine. These secretions were inhibited for 30- to 160-min periods, after which time they returned to their secreting levels.

These initial studies were carried out on denervated gastric pouches for

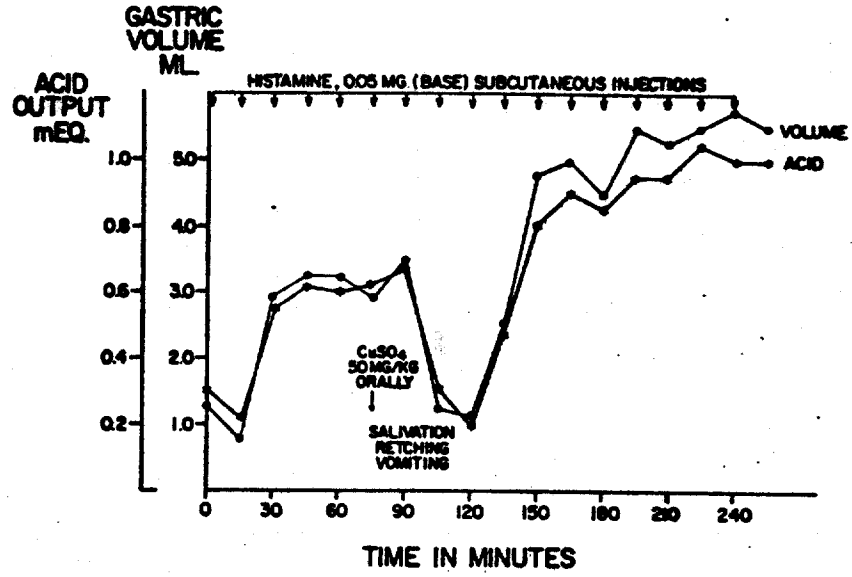

Fig 2. Action of $CuSO_4$ emesis on histamine-stimulated gastric secretion.

two reasons. First, the present project was a continuation of our work with nitrogen mustard emesis[5] and a similar experimental model was used. Secondly, uncontaminated gastric juice samples were readily obtained from these pouches.

The data in this study do not contradict previous observations. Rather, they add to the overall picture by demonstrating three specific periods following the administration of some emetic agents in adequate concentrations. The first period occurred soon after the emetic agent was given and consisted of salivation, retching, and vomiting. This period lasted 10–40 min. The second period corresponded to the gastric secretory inhibition that was reported by previous investigators[2-6] and which corresponded in time to the latent periods recorded in Table 1 (55–80 min) and to the inhibition of gastric secretion illustrated in Fig 2. In the third period, a gastric hypersecretion was noted that lasted longer than 2 hr. This gastric hypersecretion after emesis previously had been reported to have occurred after the administration of nitrogen mustard.[5]

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STUDIES ON THE PANCREAS AND LIVER OF NORMAL AND OF ZINC-FED CATS

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Received for publication July 29, 1937

During the past few years the relation between zinc and insulin has interested us greatly in these Laboratories. It was shown (Scott, 1934) that many commercial preparations of insulin contained zinc, and that this metal was a constituent of insulin crystals prepared by the methods then available. When this small quantity of zinc was removed from the insulin, crystallization could no longer be effected unless zinc, nickel, cobalt or cadmium was added at some stage of the process. Based on these observations, a practical method for the preparation of large quantities of insulin crystals was described. Essentially this method differed from previous ones in that it involved the addition of small amounts of one or more of the metals. It was found that the metal-content of crystals of insulin was constant for each metal and was uniformly proportional to the atomic weight of the metal used in the process of crystallization (Scott and Fisher, 1935). In biological experiments it was shown that solutions of insulin containing added zinc produced a more retarded hypoglycemic effect in rabbits and mice than did similar solutions containing no added zinc (Scott and Fisher, 1935). More recently we have demonstrated that the presence of minute quantities of zinc in suspensions of protamine and insulin not only greatly increases the stability of these preparations but also prolongs their blood-sugar lowering effect (Scott and Fisher, 1936).

Although zinc is present in all tissues and organs of the body (Itallie, 1907), and in amounts almost as great as that of iron (Lutz, 1926), its physiological function has not as yet been clearly demonstrated. It has been shown by Bertrand and Bhattacherjee (1934) and by Stern, Elvehjem and Hart (1935), that zinc is a necessary constituent of the diet for the normal growth of mice and of rats. Thompson, Marsh and Drinker (1927) fed large amounts of zinc to albino rats. They observed that such animals produced offspring which grew normally during their early life. In similar experiments these authors (Drinker, Thompson and Marsh, 1927) showed that dogs and cats could be maintained on a high zinc diet without impairing the health of the animals. On this regimen the concentration of zinc was increased in the liver, gall bladder and bile, gastro-intestinal tract and kidneys—that is, in the excretory organs. In three cats receiving large doses of zinc over a long period of time, fibrotic changes in the pancreas were observed. Otherwise these authors report that histological examination of the organs of such dogs and cats showed no damage attributable to zinc.

Because of the close chemical relationship between insulin and zinc, and because of the fibrotic changes in the pancreas noted by Drinker et al. in certain cats receiving zinc, it seemed possible that this metal might be a factor in the liberation, utilization or storage of insulin. Accordingly the experiments reported herein were undertaken to determine if there were any significant relations between the insulin content and zinc content of the pancreatic glands of cats maintained on a basal diet or on a diet containing added zinc. Since the liver also is concerned with carbohydrate metabolism, it was decided to estimate the zinc in this organ as well as in the pancreas.

EXPERIMENTAL. Thirty healthy male cats were selected. They were divided into two groups of fifteen each. The average weight per cat in each group was approximately 3500 grams. Each cat was placed in a cage approximately 2 feet by 2 feet by 1 foot and the cages numbered.

The diet of the cats consisted mainly of minced ox heart or canned salmon. On five days of each week, each cat was fed 100 grams of the heart per day, and on the other two days 100 grams of fish per day. Each cat received approximately 75 cc. of milk per day. In addition one group of cats (nos. 16–30) received a weighed amount of highly purified zinc oxide daily. This compound was thoroughly mixed with the meat. At the beginning of the experiment each of these cats was given 0.50 gram of zinc oxide per day. However, it was found that certain cats either did not eat their food containing the metal or vomited the greater part of their meal. Accordingly the amount of zinc oxide given to each cat per day was decreased to 0.25 gram. The cats ate their food normally for about one month. An attempt was then made to increase the amount of zinc oxide to 0.40 gram per day, but again difficulties in eating were encountered, and after a few days the amount of metal was decreased to 0.30 gram per day. This amount of the compound was added daily to the diet until the completion of the experiment, a period of from three to four months. During the last few weeks of the experiment it was observed that certain cats receiving the zinc did not eat all their food. This was particularly noticeable in cats 18, 19, 26 and 28. Moreover, the general condition of this group of cats was not as good as was that of the cats in the control group (nos. 1–15).

At the end of three months, one or two cats were killed each day. Such cats were deprived of all food for sixteen hours. They were then weighed. Each cat was stunned by a sharp blow on the head and the carotid artery immediately severed. A sample of blood was collected and sugar estima-

tions were made in duplicate. The sugar was determined by the modified Shaffer-Hartman method (DeLong, 1927) and the values are recorded for each cat in tables 1 and 2. After a cat had bled out an autopsy was immediately performed and the pancreas and liver were removed. These organs were each weighed and minced with stainless steel scissors. Zinc estimations were made on minced portions of the pancreas and of the liver. Insulin estimations were made on a portion of the pancreas.

Insulin estimations. Three grams of pancreas were weighed on a watch glass and finely minced by means of scissors. (In certain cats receiving the zinc diet the pancreas was small and consequently a smaller amount of pancreas and extraction fluid was used for the estimations.) This material was then transferred to a 50 cc. centrifuge thimble and to it were added 12 cc. of an acid alcoholic solution. (Several litres of this extraction fluid were prepared and portions of it used for extracting the pancreas of each cat. It consisted of 750 cc. absolute alcohol, 15 cc. concentrated hydrochloric acid, and 250 cc. water.) After standing over night the mixture was filtered through a double layer of cheese-cloth and pressed until nearly dry. The solid material was again minced and re-extracted with 12 cc. of the acid alcohol for two hours. The alcoholic extract was again filtered through cheese cloth and the two extracts combined. This filtrate was made ammoniacal and the volume measured. The mixture was filtered through filter paper and two 9.0 cc. quantities pipetted into each of two 50 cc. centrifuge thimbles. To each tube were now added 15 cc. of absolute alcohol and 25 cc. of ether. The mixtures were shaken and placed in the refrigerator over night. They were then centrifuged, the supernatant alcohol-ether discarded, and the tubes allowed to drain for one hour. The insulin in each tube was then dissolved in 25 cc. of isotonic saline (pH 2.5). The potency of these solutions was then estimated by the mouse method of assay (Scott, 1936). Generally six tests were conducted involving at least 200 mice. From the average of these potency values the number of units of insulin per gram of pancreas was calculated. The results for each pancreas are recorded in tables 1 and 2.

Zinc estimations. All samples of pancreas and of liver for zinc estimations were digested with 5N HCl. A quantity of this acid was prepared by distilling 20 per cent hydrochloric acid (CP) in pyrex glassware, and diluting the distillate to the desired strength with distilled water. A spectroscopic analysis of this solution gave a negative test for zinc.

In the control group a 10 gram sample of liver and a 3 gram sample of pancreas were used for the estimation of the zinc in these organs of each cat. These samples were cut into small pieces by means of stainless steel scissors and put into 100 cc. pyrex digestion flasks. To the sample of liver and of pancreas were added 50 cc. and 15 cc. respectively of the acid digestion liquid. These mixtures were refluxed for 1 hour and were then allowed to

stand over night. The following morning the volume was measured and each mixture filtered through acid washed filter paper. The zinc content of aliquots of each solution was estimated spectroscopically and the amount of zinc per gram of tissue calculated. These results are shown in table 1.

In the group of cats maintained on the zinc diet a similar procedure was followed. It was necessary, however, to use smaller samples of glands or to dilute the digestion mixture after hydrolysis, on account of the high zinc content. When the pancreas was small, the former procedure was used and a 1 gram sample was digested with 15 cc. of the 5N HCl solution.

TABLE 1

The insulin and zinc content of cat pancreas, and the zinc content of the liver. Basal diet

CAT NUMBER	INITIAL WEIGHT	FINAL WEIGHT	BLOOD SUGAR	LIVER WEIGHT	PANCREAS WEIGHT	Zn PER GRAM PANCREAS	TOTAL Zn PER PANCREAS	Zn PER GRAM LIVER	TOTAL Zn PER LIVER	INSULIN PER GRAM PANCREAS	TOTAL INSULIN PER PANCREAS
	grams	*grams*	*per cent*	*grams*	*grams*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*units*	*units*
1	3,830	3,925	0.110	75	7.00	0.077	0.53	0.053	3.98	1.91	13.37
2	3,355	3,475	0.100	80	6.45	0.065	0.42	0.058	4.68	1.46	9.42
3	3,825	3,860	0.103	76	9.00	0.050	0.45	0.041	3.14	0.80	7.20
4	3,005	3,320	0.107	60	8.75	0.073	0.64	0.066	3.96	1.34	11.73
5	3,135	3,810	0.141	78	6.50	0.085	0.55	0.050	3.91	2.22	14.41
6	3,500	3,610	0.117	91	8.20	0.032	0.26	0.100	9.12	1.63	13.36
7	3,815				4.70					1.90	9.00
8	4,380	4,670	0.095	118	7.45	0.071	0.53	0.059	7.02	1.24	9.24
9	3,470	3,800	0.103	76	7.10	0.084	0.60	0.034	2.69	2.31	16.40
10	3,030	3,520	0.102	77	9.95	0.111	1.10			0.80	7.96
11	4,100	4,470	0.107	59	4.83	0.087	0.42	0.050	2.96	2.90	14.01
12	2,500	3,600	0.099	81	8.12	0.072	0.59	0.041	3.35	1.56	12.67
13	3,430	3,310	0.092	73	6.00	0.092	0.55	0.036	2.63	2.01	12.06
14	2,710	3,500	0.099	73	5.52	0.046	0.25	0.089	6.46	1.50	8.29
15	3,800	3,400	0.094	64	8.20	0.061	0.50	0.071	4.53	1.67	13.69
Average	3,459	3,734	0.108	77.2	7.18	0.072	0.53	0.058	4.49	1.68	11.52

Cat 7 developed a severe infection and was killed thirty days after commencing the experiment.

In other cases, a 3 gram sample was used and after hydrolysis an aliquot was diluted with the 5N HCl. In estimating the zinc in the livers of this group of cats sometimes 3.3 gram samples were digested with 50 cc. of the 5N HCl, in other cases 10 gram samples were digested with 50 cc. of the 5N HCl and then further diluted with the digestion liquid. The amount of zinc in the various digests was estimated spectroscopically as in the normal group of cats and the results are recorded in table 2.

The spectroscopic analyses were conducted by Dr. S. Bateson of the Department of Physics of this University. Details of the method, limit of error ±3 per cent, will be published elsewhere.

Pathological findings. All the cats used in this study, cat 29 excepted, were autopsied immediately after death. Sections from the heart, thyroid, lymph gland, spleen, pancreas, liver, kidney, adrenal, duodenum and terminal ileum were examined. The samples were fixed in formol saline, embedded in paraffin and stained with hematoxylin and eosin.

In the case of all the cats receiving the zinc diet, there was evidence of various degrees of damage to the pancreatic glands. Degenerative changes in the acinar tissue seemed to occur first. These were followed by an influx of lymphocytes and reticulo-endothelial cells, and later fibroblasts.

TABLE 2

The insulin and zinc content of cat pancreas, and the zinc content of the liver. Zinc diet

CAT NUMBER	INITIAL WEIGHT	FINAL WEIGHT	BLOOD SUGAR	LIVER WEIGHT	PANCREAS WEIGHT	Zn PER GRAM PANCREAS	TOTAL Zn PER PANCREAS	Zn PER GRAM LIVER	TOTAL Zn PER LIVER	INSULIN PER GRAM PANCREAS	TOTAL INSULIN PER PANCREAS
	grams	*grams*	*per cent*	*grams*	*grams*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*units*	*units*
16	2,850	2,540	0.100	62	2.60			1.22	76.10	2.57	6.68
17	3,495	2,480	0.126	61	2.95	0.39	1.14	0.94	57.34	2.90	8.55
18	3,275	Died		92	1.80	0.70	1.25	1.00	91.49		
19	3,615	2,675	0.110	96	5.50	0.52	2.86	1.04	100.32	1.61	8.86
20	3,625	2,460	0.112	72	6.20	0.49	3.05	0.88	63.36	1.63	10.11
21	3,660	3,100	0.097	104	6.20	0.44	2.70	0.59	61.88	2.83	17.55
22	3,325	2,970	0.105	61	5.20	0.56	2.93	0.96	58.38	1.62	8.43
23	5,355	4,855	0.094	133	6.30	0.54	3.40	0.77	102.81	3.63	22.87
24	3,530	2,500	0.111	60	2.12	0.41	0.87	1.27	75.90	6.49	13.75
25	3,465	2,000	0.189	72	7.05	0.44	3.08	1.19	85.82	1.11	7.83
26	2,660	2,050	0.107	47	3.45	0.44	1.51	0.61	28.84	2.31	7.97
27	4,110	2,635	0.147	62	2.62	0.44	1.15	1.00	61.68	2.31	6.05
28	4,320	3,080	0.098	69	3.85	0.30	1.14	0.35	26.15	2.93	11.28
29	3,100	Died		101	3.70	0.61	2.26	1.14	115.14		
30	3,625	2,510	0.191	107	3.70	0.44	1.63	0.52	55.64	1.40	5.18
Average	3,600	2,758	0.121	79.9	4.22	0.48	2.07	0.90	70.72	2.50	10.40

Cats 18 and 29 died 91 and 90 days respectively after the zinc-feeding commenced. All cats were autopsied with the exception of no. 29. Marked fibrotic changes in the pancreas of all cats were observed.

The end result commonly seen in this group of cats consisted of small nodular pancreatic glands which gross examination showed to be of a gritty nature. In the microscopic sections one could see bands of fibrous tissue separating or replacing areas of acinar tissue and resulting in a lobulated appearance. These changes are evident in figures 1 and 2, which are photomicrographs of glands of cats 23 and 30 (zinc diet). The control group receiving no zinc revealed none of these changes and all the pancreatic glands examined were normal in appearance (fig. 3). No differences in the islet tissue of the pancreas were observed between the cats on the basal diet and those on the high zinc diet.

In addition, however, to the striking changes in the pancreas, it should be noted that the spleen of many cats receiving the zinc diet often showed collections of degenerated cells and small amounts of amyloid deposits. In two animals in this group the adrenals were found to be hard and areas resembling calcification (or zincification?) occurred in the cortex.

The livers and kidneys of both groups appeared to have undergone varying degrees of intoxication as evidenced by failure to stain properly. Vacuolization, and loss of cell substance, was also apparent, particularly in the kidneys. Frozen sections stained with scharlach-R showed that a large amount of fat was present. Since such changes occurred in both groups one cannot say at this time that the zinc-feeding was specifically concerned. No changes deemed significant in this experiment were found in the other tissues examined.

Figs. 1 and 2 are photomicrographs of sections of the pancreases of cats 23 and 30. Both these cats were maintained on a high zinc diet for approximately 15 weeks, eating their food regularly. Bands of fibrous tissue separating or replacing areas of acinar tissue and resulting in a lobulated appearance are in evidence.

Fig. 3. A photomicrograph of a section of the pancreas of a cat (no. 11) maintained on the basal diet.

Discussion. The object of the present experiment was to determine the relation, if any, between the ingestion of large amounts of zinc and the storage of insulin and of the metal in certain organs. Such conclusions as can be drawn are made as a result of observations with two groups of fifteen cats each, one group being maintained on a basal diet, and the other group on a similar diet to which zinc oxide was added.

From the average values recorded in tables 1 and 2 it can be seen that the ingestion of large quantities of zinc caused no significant decrease in the total insulin content of the pancreas of the cats. Although the average quantity of insulin per pancreas was 10 per cent less in those cats on the zinc diet than in those of the control group, this difference is not much greater than the error in the biological assay of insulin content of the various solutions. It is probably of greater importance, however, to note

that although the average *total* amount of insulin per pancreas did not vary greatly from one group to the other, there was considerable variation (45 per cent) in the average amount of insulin per *gram* of pancreas. From a consideration of these facts and in view of the comparatively small size of the pancreatic glands of the cats maintained on the zinc diet, we are inclined to believe that, while the ingestion of zinc may *appear* to increase the amount of insulin *per gram* of pancreas, the *total* insulin content of the gland remains unaltered for the reasons set forth above.

The changes in the pancreatic glands which were observed at autopsy are interesting. It will be noted that there was a marked difference in the size of the pancreases obtained from the two groups of cats. In addition the pancreatic glands from all the cats which received zinc were of a hard, gritty nature, showing marked fibrotic changes. (Cat 29 was not autopsied.) Microscopic sections showed bands of fibrous tissue separating or replacing areas of acinar tissue (figs. 1 and 2). No difference in the islet tissue of the pancreases was observed between the cats on the basal diet and those receiving added zinc. The changes in the size and the nature of the pancreatic glands of the cats on the high zinc diet can be attributed, in our opinion, only to the ingestion of the zinc oxide. Care was taken to select a grade of such purity (arsenic less than 0.0005 per cent; heavy metals less than 0.01 per cent) that the ingestion of other metals was not significant. There remains the possibility that some of the degenerative phenomena observed in this group could be due to malnutrition since certain cats did not eat regularly during the last month and there was an average decrease of several hundred grams in weight during the experiment. From the results of the control group it is evident that the food, if ingested, was totally adequate to permit maintenance of body weight. Although, in the early stages of the experiment, we made many changes in the diet and the quantity of metal, we were not able to attain the success reported by Drinker, Thompson and Marsh (1927), who reported that all their cats ate heartily, even when ingesting a larger amount of zinc than was used in the present work. These authors, however, observed in their experiments, with 10 cats and 3 dogs on a high zinc diet, that the pancreas of three cats showed marked fibrotic changes.

In the cats on the basal diet the average zinc concentration per gram of liver and of pancreas was 0.058 mgm. and 0.072 mgm. respectively (table 1). The value for liver agrees with that found by Lutz (1926) and by Drinker, Thompson and Marsh (1927). The concentration of zinc in the pancreas, however, is approximately three times that found by these investigators. Our results indicate that the pancreas is a richer organ in zinc than the liver. From table 2 it will be seen that the zinc concentration in the pancreas and the liver was increased in the cats on a high zinc diet 7 and 15 times respectively above their normal content.

In summarising these experiments the great variation amongst even the control group allows only general conclusions such as have been made. For example, in table 1 it is recorded that there were two cats whose pancreatic glands were approximately the same weight as the average value recorded for the zinc-diet cats. Until a satisfactory explanation for this and similar variations can be offered, conclusions can only be drawn from the average of a large number of experiments.

SUMMARY

Cats fed on a high zinc diet for from 12 to 16 weeks showed a loss in weight averaging approximately 900 grams per cat. The amount of zinc per gram of pancreas and of liver was about 7 and 15 times, respectively, as great as that found in similar organs in the control group of cats. The average weight of the liver in each group of cats was approximately the same. The average weight of the pancreas of the cats receiving the zinc diet was only approximately half that of the control group. The total insulin in the pancreas of each group of cats was approximately the same, although on account of the great difference in size, the insulin content per gram of pancreas was higher in the cats receiving the zinc diet than in the control group. Marked fibrotic changes in the pancreas of all the cats on a high zinc diet were observed.

The authors are indebted to Dr. D. L. MacLean, who conducted the autopsies and prepared the pathological report recorded in this paper.

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In vivo Effect of Zinc on Iron Turnover in Rats and Life Span of the Erythrocyte [1,2,3]

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ABSTRACT A series of experiments was conducted with rats to characterize further the effects of high dietary zinc (0.75%) on iron metabolism. It was found that the red blood cells from animals fed a diet high in zinc were irregularly shaped and microcytic and hypochromic. The osmotic fragility of the red blood cells from the zinc-fed rats was greater than that of the control rats. The zinc content of the blood of the former group was more than twice as high as that of the controls. Blood cells of rats fed the diet high in zinc had a life span (utilizing glycine-2-^{14}C) one-third of that of the controls. By means of ^{59}Fe studies, it was shown that iron incorporation into heme was not affected by zinc toxicity. ^{59}Fe studies showed that the greater turnover of red blood cells for the animals fed the zinc-supplemented diet brought about a greater loss of red blood cell iron via the feces. From the results of this study and those of a previous report, a hypothesis was developed rationalizing the effects of zinc toxicity on iron metabolism.

High zinc intakes have been reported to result in the development of anemia (1) which has been suggested, in part at least, to be the result of an effect of zinc on ferritin (2).

In vitro incubations with zinc have been shown to alter the properties of normal red blood cells (3), and other metals have been shown to affect their synthesis (4, 5). The present study was initiated to determine whether the anemia which develops in rats fed high zinc diets was associated with changes in other properties of the red blood cell.

EXPERIMENTAL

Male rats were maintained with the experimental diets as outlined in a previous publication (6).

Red blood cell stains were prepared according to the procedure of Cook (7). Osmotic fragility of the red blood cells was determined by the procedure of Siverd [illegible]. Samples of whole blood, removed from the tail vein of rats were prepared for iron and zinc analyses by wet-ashing in nitric and perchloric acids. All metal analyses conducted in this study were made with an atomic absorption spectrophotometer.[5] Life span of the red blood cells was followed by using the in vivo labeling procedure of Shemin and Rittenberg (9). Nine rats fed the control diet and 8 rats fed a 0.75% zinc diet were injected with 15 μCi of glycine labeled in the methylene carbon. A 0.15-ml sample of blood was removed from the tail vein of a rat at alternate sampling periods to minimize the reduction in activity due to the sampling procedure. Hemin was isolated from the blood (9), and the amount of radioactivity present determined by use of a proportional counter.[6] After counting, the hemin was wet-ashed and an iron analysis made.

The incorporation of radioactive iron into hemin was followed by injecting 10 μCi ^{59}Fe intraperitoneally. Blood samples were collected daily from each rat, the hemin isolated and radioactivity present

Received for publication December 19, 1966.

[1] Contribution from the Department of Biochemistry, School of Agriculture and Life Sciences, and School of Physical Sciences and Applied Mathematics, Raleigh, North Carolina. Published with the approval of the Director of Research, North Carolina Agricultural Experiment Station, as Paper no. 2311 of the Journal Series.

[2] Supported in part by grants from the Herman Frasch Foundation and Public Health Service Research Grant no. AM-03631-03 from the National Institute of Arthritis and Metabolic Diseases.

[3] This report constitutes a portion of a thesis submitted by T. Settlemire in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[5] Model 303 Atomic Absorption Spectrophotometer, The Perkin-Elmer Corporation, Norwalk, Connecticut.

[6] Model Number PC-4B Gas Flow Proportional Counter, Nuclear Measurements Corporation, Indianapolis, Indiana.

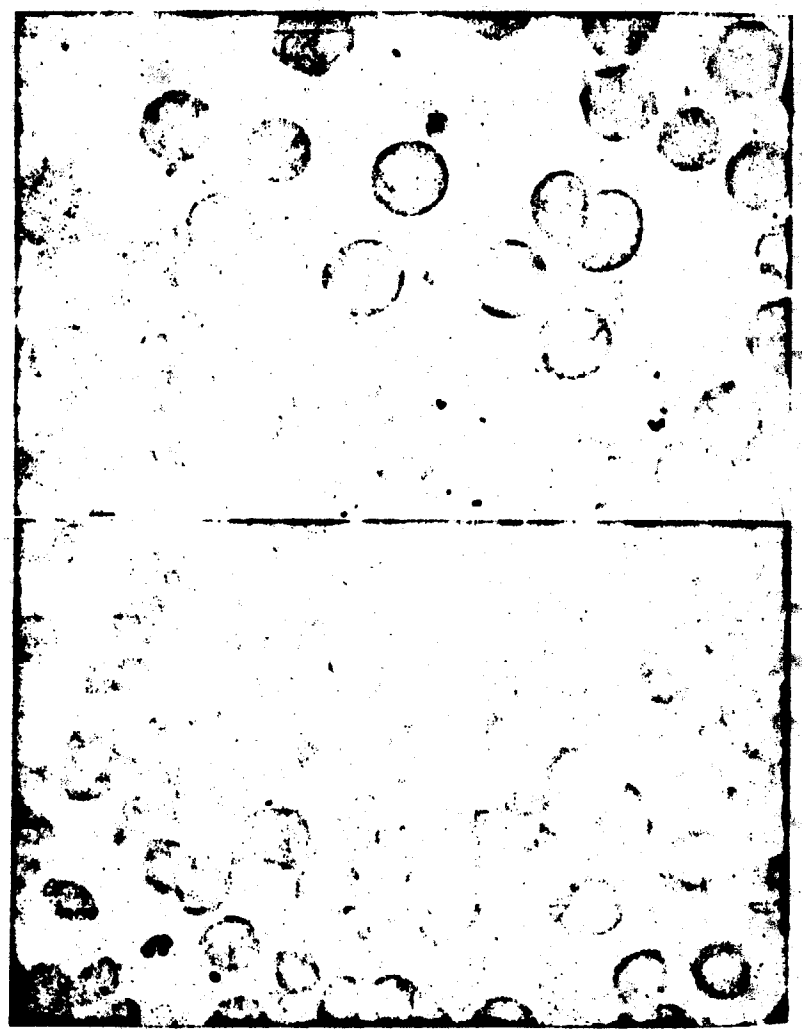

Fig. 1 Blood stains from a control rat (upper) and a rat high zinc (lower).

was determined by use of a proportional counter.[7] The iron content of the hemin was determined as in the life span study.

Measurement of excretion of iron was carried out by intraperitoneal injection of the experimental animals with 10 μCi of ^{59}Fe and counting the radioactivity excreted daily in the feces and urine. The amount of radioactivity present was determined by use of a gamma scintillation counter.[8]

RESULTS AND DISCUSSION

The effect of a high zinc intake on appearance of red blood cells is illustrated in figure 1. The cells of the control rats were uniform in shape and show the even staining of hemoglobin typical of a normal cell. However, the red blood cells of rats fed a 0.75% zinc diet were irregular in shape and are typical of a microcytic-hypochromic cell.

The osmotic fragility of the red blood cells was determined in the next study. With blood from the control rats, hemolysis first appeared in a salt concentration of 0.40% NaCl, whereas with blood from the zinc-fed rats, hemolysis first appeared in the 0.32% solution. This change in the hemolysis pattern indicates a very definite decrease in osmotic fragility of the red cells. The cause of such a decrease is not known; however, it has been reported that when erythrocytes are incubated in vitro with solutions containing 65 μg of zinc/ml, an increase in osmotic fragility is obtained (3, 10) and is attributed to a large irreversible leakage of potassium from the cell.

The zinc concentration of whole blood was determined on blood removed from the tail vein of the rat. The results of this study are shown in table 1. The amount of zinc in whole blood of the zinc-fed rats increased approximately 90 μg/ml above that of controls, offering the possibility that the in vitro effect reported previously (3) may occur under the in vivo conditions of this study.

Since the red blood cells of the rats fed the zinc diet were shown to be abnormal in appearance and exhibited changes in osmotic fragility, another study was undertaken investigating the effect of high zinc intakes on the life span of the red blood cells. The results of this study are presented in figure 2. The red blood cell life span of the rats fed a high zinc diet was much shorter than that of the controls, as indicated by the sharp decline in specific activity of the hemin between 10 and 20 days, whereas the control rats followed a normal cycle (11). The high zinc intake shortened the life span of the red blood cell approximately one-fifth to one-fourth that of the control animals.

In the next study the effect of high zinc intakes on the rate of incorporation of radioactive iron into heme was investigated since the other metals have been shown to result in a decreased rate of iron incorporation into heme (4, 5). The results are shown in figure 3. The specific activity increased in both the control and high

[7] See footnote 6.

[8] Gamma Scintillation Counter, Nuclear-Chicago Corporation, Des Plaines, Illinois.

TABLE 1

Concentration of iron and zinc in the blood of control and high zinc-fed rats

Rat	Hemoglobin	Weight	Blood Zn conc	Blood Fe conc
	g/100 ml blood	g	μg/ml	μg/ml
Control				
C-12	14.03	289	67.5	1,550.0
C-16	13.95	356	52.5	1,312.5
C-17	14.55	303	60.0	1,575.0
Mean	14.18	316	60.0	1,479.2
0.75% Zn				
Z-19	6.42	143	155.0	675.0
Z-20	7.03	110	152.0	975.0
Z-21	6.34	150	153.0	825.0
Mean	6.60	134	154.0	825.0

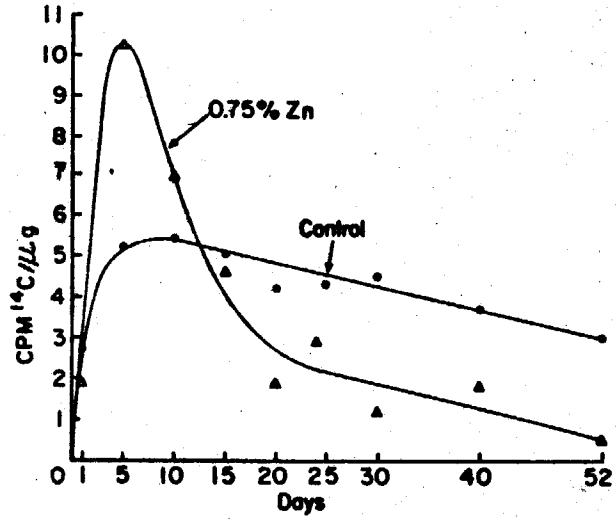

Fig. 2 Carbon-14 activity in hemin following injection of 15 μCi of methylene-labeled glycine ^{14}C; ○ = control (each point is mean of 5 rats); △ = rats fed high zinc (each point is mean of 4 rats).

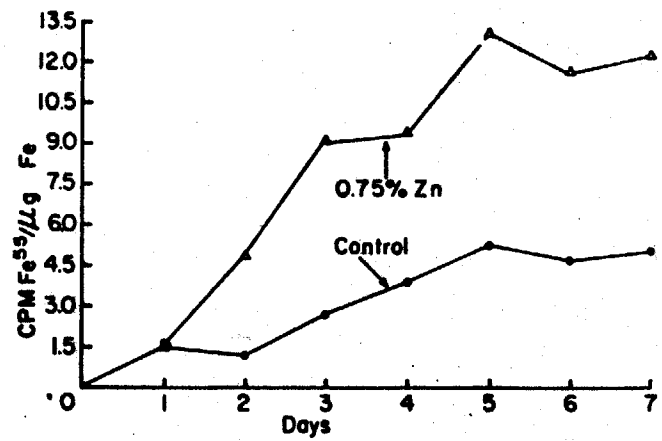

Fig. 3 Incorporation of ^{59}Fe into hemin.

zinc-fed rats during the first 5 days. This follows the pattern reported by Hahn et al. (12). The rate of incorporation was considerably higher for the zinc-fed rats. This greater rate is attributed to the fact that the rats were anemic (13). In any case these results suggest that incorporation of iron into heme was not impaired.

As previously described, the rats fed a high zinc diet have a shortened red blood cell life span or, in other words, a faster turnover of red blood cell iron. Since a portion of the red blood cell iron is excreted with each turnover (14), the decrease in tissue iron, which is observed in the high zinc-fed (2) may in part be a result of this faster turnover rate. To determine whether, in fact, iron excretion is affected, ^{59}Fe was injected (intraperitoneally) into control rats and rats fed the high zinc diet, and feces and urine were collected. The urine was found to contain no radioactivity. The average excretion of ^{59}Fe in the feces is shown in figure 4.

The pattern of ^{59}Fe excretion in the feces obtained from the control rats is similar to that reported by Dubach et al. (15) and by Thirayothin and Crosby (16). The average excretion of ^{59}Fe by the zinc-fed rats followed closely the pattern observed for control rats during the first 8 days after injection. Thereafter, a significant increase in ^{59}Fe excretion was observed in contrast with the decrease observed in the feces of the control animals.

This increase in ^{59}Fe excretion by the zinc-fed rats occurs 10 to 12 days after injection; this period of time corresponds to the length of the life span of the red blood cells of these rats. Thus, these results support the hypothesis that the decrease in body iron levels observed when rats are fed high zinc diets is in part associated with a more rapid turnover of the red blood cells.

The mechanism by which a high zinc intake reduces the life span of the red blood cell is not known. Copper is known to be involved in some manner in red blood cell formation (17), and zinc at high dietary levels has been shown to interfere with normal copper metabolism resulting in an abnormal red blood cell synthesis (18). The red blood cell may also be affected by the increased zinc content of the blood of the rats fed high zinc resulting in an abnormal red blood cell, as shown by the in vitro studies of Vincent and Blackburn (3).

The combination of the concepts developed in a previous paper (2) concerning the effect of a high zinc intake on ferritin and the results reported in this study indicate that these levels of zinc affect iron

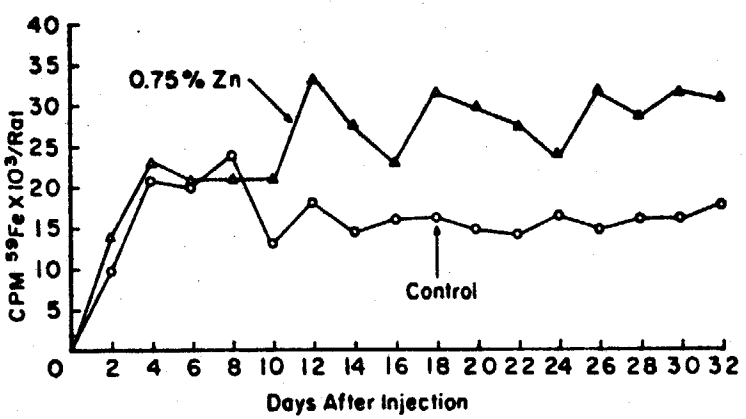

Fig. 4 Excretion of ^{59}Fe in feces of control and high zinc-fed rats each point is mean of 3 rats

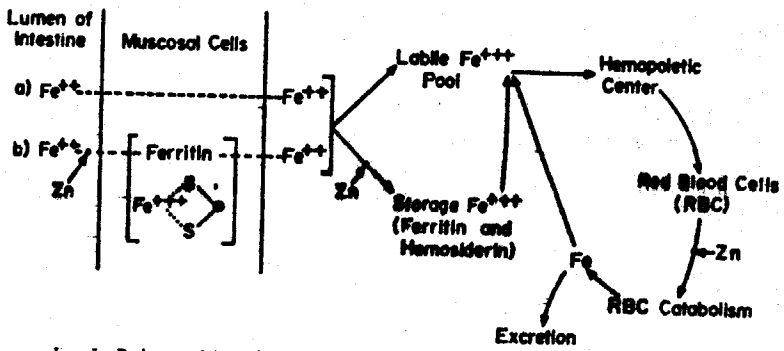

Fig. 5 Pathway of iron through the body with proposed sites of interference by zinc.

metabolism basically in 2 ways. These include (a) an effect of zinc on ferritin such that the incorporation of iron into or release from ferritin is impaired. This would, as discussed in the previous paper (2), impair iron absorption and limit the tissue storage of iron as ferritin. Evidence to support these concepts was presented. And (b) as presented in this paper, the feeding of high zinc diets was observed to result in a shortened life span of the red blood cells resulting in a faster turnover of iron. The points at which zinc is postulated to interfere with iron metabolism are summarized in the scheme shown in figure 5.

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J. Biol. Chem 153: 627 (1944)

THE PEPTIDASES OF INTESTINAL MUCOSA

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(Received for publication, February 28, 1944)

Of the many and diverse peptidase activities present in intestinal mucosa, the most frequently studied were the enzymes hydrolyzing leucylglycine and leucylglycylglycine. Earlier investigators of the enzymatic splitting of leucylglycine, alanylglycine, and glycylglycine derived from their observations the impression that a single dipeptidase was being studied. Linderstrøm-Lang (1), however, demonstrated that the two dipeptides, leucylglycine and alanylglycine, were split by distinct enzymes which could be differentiated on the basis of different stability and pH optima. One of these enzymes, designated leucylpeptidase, was assumed to hydrolyze leucylglycylglycine and leucyldiglycylglycine as well as leucylglycine. Thus, the older theories of peptidase specificity relating to a dipeptidase, an aminopeptidase, and an occasional carboxypeptidase as the sole components of the intestinal erepsin were shown to be erroneous. Since then evidence has been brought forward indicating the existence of additional types of enzymes in intestinal mucosa such as prolinase (2) and prolidase (3).

Most of these earlier results were obtained by the use of enzymes, which were purified by the adsorption-elution technique and with the help of all too few substrates. More recently it was discovered that the activity of various peptidase preparations obtained from glycerol extracts of intestinal mucosa by precipitation with acetone was decreased on dialysis or absorption and could be partially or totally restored by the addition of various metal ions (4). Moreover, it was demonstrated that the restoration of the activity towards leucylglycine, as produced by the combination of the activating metal ion with the inactivated peptidase, is a time reaction (5). Thus, one of the intestinal peptidases was shown to be a conjugated protein, although in a sense somewhat different from Willstätter's frequently cited theory which postulated that each enzyme consists of a colloidal carrier and an active group regarded as responsible for the enzymatic specificity. It is shown below that prolidase is also a manganese protein compound.

The experiments reported in this communication were undertaken with the intention to differentiate, characterize, and purify several of the peptidases present in intestinal mucosa. Because of the present war emergency, this work had to be terminated before it had attained its goal.

l-Leucine-aminoexopeptidase

The enzyme discussed in this section corresponds to Linderstrøm-Lang's leucylpeptidase.

Preparation and Specificity—The activity of leucine-aminoexopeptidase may be easily differentiated from most of the other activities present in crude aqueous extracts of mucosa by a precipitation with acetone followed by a fractionation with ammonium sulfate.

345 gm. of mucosa from twenty-five 2 to 3 foot lengths of the upper end of hog duodenum were ground with 35 gm. of sand and 350 cc. of water. The extract was centrifuged and the mucosa reextracted with 350 cc. of water. The combined yellowish opalescent extracts (Preparation A) consisted of 650 cc. and contained 3.98 mg. of protein N per cc. The proteolytic coefficient for *l*-leucylglycine, C_{LG}, was 0.062. Preparation A represented 80,500 enzyme units, as defined earlier (6), corresponding to 31 leucine-aminopeptidase units per mg. of protein N.

The precipitate obtained by mixing chilled Preparation A with an equal volume of cold acetone was collected and thoroughly washed with acetone.[1] The dried powder (16.8 gm.) was twice extracted with water at 40°, and the solution clarified by filtration with Celite. This solution (Preparation B) consisted of 360 cc., and contained 1.30 mg. of protein N per cc. C_{LG} was 0.35. Preparation B contained 82,000 enzyme units, corresponding to 175 units per mg. of protein N.

To 350 cc. of Preparation B were added 84.7 gm. of ammonium sulfate to bring about a 40 per cent saturation. The precipitate was discarded and to the clear solution (Celite filtration) were added 48 gm. of ammonium sulfate to give a 60 per cent saturation. The precipitate was collected and dissolved in water. After dialysis in the cold against distilled water, the inactive precipitate was filtered off and discarded. 66 cc. of a clear, faintly yellowish solution (Preparation C) were obtained. It contained 0.360 mg. of protein N per cc. C_{LG} was 2.1. Preparation C corresponded to 25,000 enzyme units and contained 1050 units per mg. of protein N. A 34-fold purification was thus obtained and a yield of 31 per cent of the original activity of Preparation A. The purified preparation was stable for many weeks when kept in the cold under toluene. A lyophilized preparation kept for several months in the dry state in the cold had not lost any of its activity when redissolved and tested in the presence of Mn^{++}.

Table I indicates that the bulk of the activities present in the crude mucosa extract, Preparation A, was removed in the course of further purification. Several conclusions may be drawn from the data presented in

[1] The crude, dry solid may be kept in the desiccator for months with no apparent loss of activity.

Table I. For example, 99 per cent of the activities towards *l*-alanylglycine and glycylglycine has been removed and, therefore, must be due to an enzyme or enzymes distinct from leucine-aminoexopeptidase. However, the small residual activities towards these substrates may or may not be due to the leucine-aminoexopeptidase. It will be noted that the activity towards *d*-leucylglycine has been reduced from one-eighty-second in Preparation A to less than 0.00005 in Preparation C of the activity towards the *l* isomer.

TABLE I

Activities of Crude and of Purified l-Leucine-aminoexopeptidase Preparations towards Various Substrates

For the crude extract (Preparation A), no activator was added for the tests, since no activation was detectable. For the other tests, the enzyme was incubated with 0.02 M $MnSO_4$ buffered at pH 8.0 at 40° for 3 to 4 hours. A test aliquot was then added to the buffered peptide solution.

Substrate	Proteolytic coefficient, *C*			Yield*
	Preparation A	Preparation B	Preparation C	
				per cent
l-Leucylglycine	0.062	0.35	2.1	31
d-Leucylglycine	0.00076	0.0010	<0.0001	
l-Leucylglycylglycine	0.11†	0.55†	2.2	18
l-Leucinamide	0.022		1.0	42
Glycylglycine	0.025		0.0065	0.24
Benzoyl-*l*-argininamide	0.0005		<0.0001	
Glycyl-*l*-leucine	0.034		0.012	0.32
l-Alanylglycine	0.26	0.042	0.17	0.60
Glycyl-*l*-proline	0.015		0.035	2.1
l-Prolylglycine	0.035		0.027	0.71
Glycyl-*l*-alanine			0.021	
l-Leucyl-*l*-leucylglycine			1.6	
Benzoyl-*l*-leucylglycine			<0.0001	

* Yields are given as $\frac{100 \times \text{total activity of Preparation C}}{\text{total activity of Preparation A}}$.

† Initial *C* values.

This appears to be conclusive evidence that distinct enzymes cause the splitting of the two stereoisomeric peptides.

The kinetics of *l*-leucine-aminoexopeptidase are complicated by the fact that the enzyme, in the course of purification, becomes increasingly responsive to activation by Mn^{++} or Mg^{++}. Moreover, when samples of the purified enzyme are incubated at pH 7.8 to 8.0 with Mn^{++} ions, before the substrate is added, the enzymatic activity increases during the first few hours. Longer incubation, however, produces a considerable decrease of

enzymatic activity and after an incubation period of 24 hours about 95 per cent of the activity is lost (Table II).

This behavior is the same whether the substrate is *l*-leucylglycine, *l*-leucylglycylglycine, or *l*-leucinamide. The fact that both the reaction of Mn^{++} with enzyme and the subsequent inactivation run approximately parallel for the three substrates suggests that all three substrates are hydrolyzed by the same enzyme.

The enzyme which is responsible for the activity of Preparation C towards *l*-leucylglycine cannot be called a dipeptidase, since it splits *l*-leucylglycylglycine, *l*-leucyl-*l*-leucylglycine, and *l*-leucinamide as fast as *l*-leucylglycine. The splitting of leucinamide indicates that the enzyme does not require a

TABLE II

Effect of Incubation of l-Leucine-aminoexopeptidase with $MnSO_4$ upon Its Activity towards Several Substrates

The enzyme (protein N = 36 γ per cc.) was incubated at 40° and pH 8.0 with 0.02 M $MnSO_4$ before the respective substrate was added. The hydrolyses were carried out at 40° and pH 7.8 to 8.0. Protein nitrogen for the 3 hour test was 2.88 γ per cc.; for the 25 hour test, 5.76 γ per cc.

Time of preceding incubation	Action on *l*-leucylglycine			Action on *l*-leucylglycylglycine			Action on *l*-leucinamide		
	Time	Hydrolysis	*C*	Time	Hydrolysis	*C*	Time	Hydrolysis	*C*
hrs.	*min.*	*per cent*		*min.*	*per cent*		*min.*	*per cent*	
3	30	33	2.0	30	32	1.9	30	20	1.12
	60	59	2.2	45	48	2.2	60	31	0.94
	75	64	2.1	60	60	2.3	120	55	1.01
							150	64	1.03
25	180	25	0.12	180	28	0.14	150	18	0.100
	300	35	0.11	300	44	0.15	180	21	0.100
	330	39	0.11	330	46	0.14	300	29	0.087
							330	32	0.088

free carboxyl group. A free amino group seems to be necessary for the action of this enzyme, since no splitting of benzoyl-*l*-leucylglycine could be observed. The data at present available make it therefore appear that the enzyme should be classified as *l*-leucine-aminoexopeptidase, in accordance with a previous suggestion (7, 8). *l*-Leucine-aminoexopeptidases have previously been found to be present in extracts of beef spleen, beef kidney, and swine kidney. The kinetics of the intestinal *l*-leucine-aminoexopeptidase were studied extensively with the expectation that it might become possible to establish on a quantitative basis the homospecificity (Bergmann (9) p. 49) of the leucine-aminopeptidases from intestinal mucosa, on the one hand, and from spleen and kidney, on the other. However, the instability

of the manganese-activated intestinal enzyme mentioned above has hitherto frustrated all such efforts.

Berger and Johnson (10) have reported that the addition of cysteine enhances the effect of Mn^{++}. We have found that our leucine-aminopeptidase is partially inactivated when incubated with cysteine before Mn^{++} and substrate are added. Table III shows the result of such an experiment. The first order velocity constants are calculated in order to illustrate the changes which take place. Control Experiment A shows that incubation

TABLE III

Effect of Incubation of Protein of l-Leucine-aminoexopeptidase with Cysteine and with $MnSO_4$

Incubations were carried out at 40° and pH 7.9. The test substrate was *dl*-leucylglycine. Protein N = 1.97 γ per cc. in the test solution and 24.7 γ per cc. in the incubation mixture.

Experiment A. Control incubation of enzyme in absence of $MnSO_4$; test solution contained 0.01 M $MnSO_4$		Experiment B. Incubation of enzyme with 0.01 M cysteine, in absence of $MnSO_4$; test solution contained 0.01 M $MnSO_4$		Experiment C. Incubation of enzyme with 0.01 M $MnSO_4$; test solution contained 0.0008 M $MnSO_4$ introduced with protein	
Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis
Immediate test of incubation mixture before incubation					
min.	*per cent*	*min.*	*per cent*	*min.*	*per cent*
60	18	60	18	60	12
90	26	90	27	90	21
180	71	150	68	180	60
240	83	240	82	240	78
360	92	360	91	360	87
Test after 3 hrs. incubation					
60	19	60	2	60	33
120	38	120	10	120	67
180	70	180	19	150	82
210	80	210	24	180	88
				210	92

of the inactive protein alone for 3 hours at 40° effects no appreciable change. The instantaneous addition of cysteine in Experiment B gives results identical with the control. However, after 3 hours, a large inactivation is shown. Experiment C shows the results of incubating Mn^{++} and enzyme. Initially, the test hydrolysis shows a slightly lower rate which is the result of the somewhat lower Mn^{++} concentration in the test solution. After 3 hours, the initial splitting of the substrate is considerably higher.

Leucine-aminoexopeptidase is activated by Mg^{++} as well as Mn^{++}.

However, higher concentrations of Mg^{++} are necessary and, in addition, still longer times are required for maximal activation. As with the Mn^{++} activations, the data run in parallel manner for the three different substrates when tested with the Mg^{++} enzyme.

Leucylglycine was introduced about 15 years ago as a substrate for the characterization of ereptic dipeptidase. The fact is now sufficiently established that the intestinal "leucylpeptidase" responsible for the manganese-activatable hydrolysis of *l*-leucylglycine and *l*-leucylglycylglycine can be neither a dipeptidase nor a carboxypeptidase but must be an aminoexopeptidase of a specificity adapted to the side chain and configuration of *l*-leucine. It is to be expected that intestinal mucosa contains other aminoexopeptidases[2] of different side chain and antipodal specificities and that each of these aminoexopeptidases also acts upon dipeptides that fulfill the side chain and antipodal requirements of the enzyme. Consequently, the occurrence in intestinal mucosa and elsewhere of dipeptidases which are specifically and exclusively adapted to the hydrolysis of dipeptides must be regarded as doubtful until new evidence for their existence is brought forward.

Prolidase

In 1937, it was reported (3) that glycyl-*l*-proline is split by an enzyme distinct from the previously known peptidases. This enzyme was named "prolidase." It was shown that prolidase is only slightly inhibited by cyanide in concentrations which strongly inhibit the splitting of *l*-leucylglycine and *l*-alanylglycylglycine. In contrast to other proteolytic enzymes, prolidase is capable of hydrolyzing a peptide bond that does not contain peptide hydrogen. In order to facilitate further studies of prolidase, an attempt was made to improve its separation from other enzymes of the intestinal mucosa.

Prolidase remains in the mother liquor when *l*-leucine-aminoexopeptidase is prepared from a crude aqueous extract of intestinal mucosa by precipitation with acetone. The mother liquor was lyophilized. The material thus obtained was extracted several times with ether and the residue after drying *in vacuo* dissolved in water. The aqueous solution was dialyzed and subsequently tested for its activity towards various substrates. The findings reported in Table IV indicate that the predominant activity was found to be prolidase.

The enzymatic activities present in the crude mucosa extract towards glycylglycine, *l*-prolylglycine, and glycyl-*l*-leucine are destroyed by the

[2] Some of these will be components of the enzyme fraction usually designated intestinal aminopolypeptidase; some may occur in other enzyme fractions obtainable from intestinal mucosa.

acetone treatment and are not found either in the acetone precipitate or in the mother liquor and, therefore, cannot be identical with prolidase. The residual activity towards glycyl-*l*-alanine, which accompanies the acetone-purified prolidase, is apparently distinct from the extremely high activity towards *l*-alanylglycine present in the crude mucosa extract. That glycyl-*l*-proline (GP) and glycyl-*l*-alanine (GA) are split by different enzymes is indicated by the varying values of the quotient C_{GP}/C_{GA} obtained by different methods of treatment; *e.g.*, the acetone-purified prolidase shows $C_{GP}/C_{GA} = 10.3$, while a lead acetate-treated prolidase (see below) gives $C_{GP}/C_{GA} = 29.4$. The data of Table IV show that prolidase

TABLE IV

Activities Present in Crude Prolidase Preparations (Acetone Method)

Hydrolyses were carried out at 40° and pH 7.8 to 8.0. Enzyme concentration, 0.165 mg. of protein N per cc. of test solution.

Substrate	Hydrolysis		
	No Mn^{++} added to test solution	Test solution contained 0.001 M $MnSO_4$	
	26 hrs.	2 hrs.	26 hrs.
	per cent	*per cent*	*per cent*
l-Alanylglycine	1	1	8
Glycylglycine	−1	−1	3
l-Leucylglycine	−1	1	12
l-Leucylglycylglycine	1	1	9
l-Prolylglycine	0	−1	4
Carbobenzoxyglycyl-*l*-proline	0		0
Glycyl-*l*-leucine	0		0
Glycyl-*l*-phenylalanine	0	0	2
Glycyl-*l*-alanine		13	59
Glycyl-*l*-proline	47 (2 hrs.)	78	

is a highly specific enzyme with little or no action on peptide bonds containing a peptide hydrogen, as already suggested by Bergmann and Fruton (3).

These data also show that prolidase is activated by Mn^{++} and may be added to the list of metal-activated peptidases. Unlike leucine-aminopeptidase, which is activated by both Mn^{++} and Mg^{++}, prolidase is not activated by Mg^{++}. Other metal ions tested, such as Co^{++}, Cu^{++}, and Zn^{++}, strongly inhibited the enzyme. For the splitting of glycyl-*l*-proline by prolidase in the presence of Mn^{++} satisfactory pseudo first order reaction velocity constants are obtained (Table V). This behavior may be used to advantage in future attempts to define the specificity of prolidase in terms of the classification of proteolytic enzymes suggested previously (8).

Because only small quantities of enzyme were available from the acetone-

containing mother liquor, another method of preparing prolidase was developed. This latter method possesses the advantage that larger quantities of much more active material can be easily prepared. This material simultaneously contains large amounts of a second enzyme which hitherto was regarded as an aminopolypeptidase (Johnson and Berger (9)

TABLE V

Kinetics of Prolidase Action on Glycyl-l-Proline

The enzyme was prepared by the acetone method. Hydrolyses were carried out at 40° and pH 8.0. The test solutions contained 0.01 M $MnSO_4$.

Protein N per cc. test solution	Time	Hydrolysis	C_{GP}	C_{GP} average
mg.	*min.*	*per cent*		
0.149	30	27	0.031	
	60	49	0.033	
	75	57	0.033	
	90	68	0.037	
	120	78	0.037	
	150	81	0.032	0.034
0.0992	30	23	0.038	
	60	35	0.032	
	75	42	0.032	
	90	51	0.035	
	120	64	0.037	
	150	72	0.037	0.035
0.0496	30	13	0.041	
	60	22	0.036	
	90	30	0.035	
	120	39	0.036	
	150	47	0.037	
	180	55	0.039	0.037
0.0198	60	9	0.035	
	120	18	0.036	
	150	21	0.035	
	180	26	0.036	0.036
0.149 (No $MnSO_4$ added)	30	17	0.018	
	45	25	0.018	
	60	34	0.020	
	90	42	0.018	
	120	47	0.015	0.018

p. 69, (11)). The specificity of this enzyme will be discussed in the following section.

An aqueous extract of hog intestinal mucosa is prepared as described above for leucine-aminoexopeptidase. With an aliquot of this crude extract, the amount of a saturated lead acetate solution necessary for maxi-

mal precipitation is determined. The calculated quantity is then added to the bulk of the solution and the precipitate removed by centrifuging. To the supernatant solution one adds an amount of disodium phosphate in excess of that necessary for the removal of the lead. After 30 minutes, the precipitate is removed by filtration with Celite and the clear filtrate dialyzed overnight against distilled water in the cold. Solid ammonium sulfate is then added to 40 per cent saturation, and the slight precipitate is discarded. Additional ammonium sulfate is added to bring to 60 per cent saturation and the precipitate is collected. The precipitate is dissolved in water, filtered clear, and dialyzed against distilled water until free of sulfate ions. This solution, in the presence of 0.001 M Mn^{++}, has a prolidase activity, C_{GP}, of 0.47, while the crude extract had a C_{GP} of about 0.015. Thus, the purified prolidase contained about 30 times as much activatable prolidase per mg. of protein N as the crude extract. The purified prolidase has, in the absence of added Mn^{++}, a C_{GP} of 0.075. Thus, the increase in activity produced by Mn^{++} is greater than 6-fold. With other preparations, the increase was even greater; in one case, more than 8 times.

The crude extract and the purified solution also cause a splitting of glycyl-*l*-hydroxyproline. This hydrolysis is also activated by Mn^{++} ions (in one instance, as high as 16 times). The splitting of the proline compound (GP) by the activated enzyme is about 8 times more rapid than that of the hydroxyproline compound (GHP). The proteolytic quotient C_{GP}/C_{GHP} is practically identical in the presence of Mn^{++} for the various enzyme preparations presented in Table VI. It will be noted, however, that the proteolytic quotient of the purified prolidase, Preparations 8 and 9 in Table VI, is higher in the absence of added manganese.

These data can be interpreted in two ways. Either we are dealing with a single enzyme which shows different affinities for the substrates at different manganese concentrations or the two substrates are hydrolyzed by two extremely similar enzymes both of which are activated by Mn^{++} and are affected in exactly the same manner by several purification procedures: acetone, lead acetate, ammonium sulfate, and inactivation at 60°. The latter assumption is highly improbable. It appears, therefore, most likely that the first hypothesis is the correct one, since the ratio C_{GP}/C_{GHP} after maximal activation is the same as for the crude extract when the addition of Mn^{++} has no effect.

In Table VII are presented some experiments with various proline and hydroxyproline compounds. It is apparent that as compared with glycyl-*l*-proline and glycyl-*l*-hydroxyproline, the amino- and carboxyl-substituted compounds are split rather slowly. In fact, the slight activity towards these latter substrates may be due to different enzymes. In particular, the hydrolysis of carbobenzoxyglycyl-*l*-prolinamide and carbobenzoxyglycyl-*l*-hydroxyprolinamide cannot be ascribed to prolidase or prolinase, but must be attributed to one or several endopeptidases. No splitting was observed of the two diketopiperazines.

l-Prolylglycine and *l*-hydroxyprolylglycine were hydrolyzed very slowly by the purified extract. Comparison with the experiments reported in the lower section of Table VII shows that these two substrates are split more rapidly than the respective reciprocal peptides by the crude extract. Apparently, the major part of the activity towards prolylglycine and hydroxyprolylglycine has been removed during the purification of the extract.

TABLE VI

Proteolytic Quotients for Glycyl-l-Proline and Glycyl-l-Hydroxyproline

The hydrolyses were carried out at pH 8.0 and 40°. Preparations 1 to 7 contained 0.001 M $MnSO_4$ in test solutions.

Preparation	C_{GP}	C_{GHP}	C_{GP}/C_{GHP}
1. Crude extract of mucosa	0.012	0.0015	8.0
2. " " " "	0.015	0.0019	7.9
3. Lead acetate-purified *l*-prolidase	0.29	0.034	8.5
4. " " " from Preparation 2	0.47	0.065	7.2
5. Preparation 4 heated to 60° for 10 min., 90% of activity destroyed	0.0036*	0.00047*	7.7
6. *l*-Leucine-aminoexopeptidase	0.028	0.0034	8.2
7. Preparation 6 reprecipitated with 50% acetone and dialyzed	0.027	0.0035	7.7
Average			7.9
8. Preparation 3, no added Mn^{++}	0.039	0.0029	13.4
9. " 4, " " "	0.075	0.0040	18.8

* These are *K* values, since no nitrogen determinations were performed on the heated solution.

Of particular interest is the relatively rapid hydrolysis by the crude extract of *l*-hydroxyprolylglycine, a substrate not previously studied.

Other Enzymes

The preparation of prolidase by the lead acetate procedure described in the preceding section is a rich reservoir of other enzymatic activities responsible for a rapid splitting of tripeptides such as *l*-leucylglycylglycine, *l*-alanylglycylglycine, diglycylglycine, diglycyl-*l*-proline, and diglycyl-*l*-hydroxyproline. There is no activation by Mn^{++} ions and in some cases a definite inhibition.

The presence of an enzyme which splits *l*-leucylglycylglycine is of special interest. This enzyme was found to act upon *l*-leucylglycylglycine at the peptide bond adjacent to the free amino group with the formation of leucine and glycylglycine. This enzyme cannot be identical with *l*-leucine-aminoexopeptidase. This conclusion is derived not only from the already mentioned fact that this enzyme is not activated by manganese, but also from

TABLE VII

Hydrolysis of Proline and Hydroxyproline Compounds by Extract of Intestinal Mucosa, Purified by Lead Acetate

All substrates were present in a concentration of 0.05 mM per cc. at 40°, buffered at pH 8.0 with 0.02 M veronal. The test solutions contained 0.001 M $MnSO_4$. Lead acetate-purified prolidase was employed as the enzyme.

Substrate	Enzyme concentration	Hydrolysis	
		1½ hrs.	24 hrs.
	mg. protein N per cc.	*per cent*	*per cent*
Glycyl-*l*-proline	0.00675	40	99
Glycyl-*l*-hydroxyproline	0.109	55	100
l-Prolylglycine	0.219	1	20
l-Hydroxyprolylglycine	0.219	0	12
Glycyl-*l*-proline diketopiperazine	0.219	0	0
Glycyl-*l*-hydroxyproline diketopiperazine	0.219	0	0
l-Prolinamide	0.107	1	3
l-Hydroxyprolinamide	0.107	−1	7
Carbobenzoxyglycyl-*l*-proline	0.320	3	22
Carbobenzoxyglycyl-*l*-hydroxyproline	0.320	1	9
Carbobenzoxyglycyl-*l*-prolinamide	0.320	5	27
Carbobenzoxyglycyl-*l*-hydroxyprolinamide	0.320	2	16
Carbobenzoxy-*l*-prolyl-*l*-proline	0.219	0	2
Glycyl-*l*-proline*	0.198	47	
Glycyl-*l*-hydroxyproline*	0.494	38	
l-Prolylglycine*	0.198	56	
l-Hydroxyprolylglycine*	0.494	59	

* These experiments were performed with a crude aqueous extract of intestinal mucosa as the enzyme; no Mn^{++} was added.

our observation that the preparation discussed here splits *l*-leucylglycylglycine about 80 times as fast as *l*-leucinamide and 500 times as fast as *l*-leucylglycine. If this enzyme were homospecific with the manganese-activated *l*-leucine-aminoexopeptidases, it should split the three substrates, *l*-leucylglycylglycine, *l*-leucylglycine, and *l*-leucinamide, with proteolytic coefficients of the same order of magnitude. The possibility must be considered that this enzyme has such a specificity type that it requires in

the back-bone of its substrate the groups underlined in the following formula of *l*-leucylglycylglycine.

(I) $$C_4H_9\cdot\underset{\underset{NH_2}{|}}{CH}\cdot CO\text{-}\underline{NH}\cdot CH_2\cdot\underline{CO}\text{—}\underline{NH}\cdot CH_2\cdot COOH$$

If this hypothesis is correct, the enzyme is an imidoendopeptidase or, in terms of an older terminology, an imidoproteinase. The existence of the specificity class of imidoproteinases has already been discussed (8). On the basis of experiments then available, chymotrypsin was assigned to this specificity class. However, it was found later that the enzymatic activity in question must be attributed to the aminoexopeptidase activity of chymotrypsin.

A closer investigation of the imidoendopeptidase which splits *l*-leucylglycylglycine is very desirable. Experiments performed in this laboratory by Dr. Joseph S. Fruton have shown that various animal tissues contain enzymes that hydrolyze *l*-leucylglycylglycine by a specific mechanism different from that of a leucine-aminoexopeptidase. The example discussed above of the imidoendopeptidase and the *l*-leucine-aminoexopeptidase represents a second case (12) of two proteolytic enzymes exhibiting different back-bone specificities but hydrolyzing the same substrate at identical linkages; *i.e.*, with the formation of the same split-products.

The hypothesis that a crude extract of intestinal mucosa contains, in addition to leucine-aminopeptidase, a second enzyme that splits *l*-leucylglycylglycine is in agreement with the data in Table I. The initial activities in the crude extract (Preparation A) indicate nearly twice the activity for *l*-leucylglycylglycine as for *l*-leucylglycine, whereas the purified enzyme (Preparation C) shows nearly equal activities towards the two substrates. Obviously, there has been removed during the purification an enzyme which acts upon *l*-leucylglycylglycine but very much less or not at all upon *l*-leucylglycine.

An enzyme of the specificity type characterized by Formula I would be expected also to hydrolyze other tripeptides containing a central glycine residue. It has already been mentioned that the partly purified enzyme preparation discussed in the present section acts indeed upon such tripeptides (Table VIII). However, our experimental material is by no means sufficient to prove that all these tripeptides are actually split by the same enzyme.

The hydrolysis of triglycine approximates a first order reaction more closely in the presence of Mn^{++}, because there is also contained in the enzyme solution an enzyme which slowly hydrolyzes glycylglycine and which is inhibited by Mn^{++}. This inhibition becomes noticeable in the 24 hour values (Table VIII).

The data for the hydrolysis of *l*-leucylglycylglycine closely resemble a zero order reaction, in the absence and in the presence of Mn^{++}. One possible explanation may be that we may have a low substrate concentration in relation to the enzyme concentration, a condition favoring zero order kinetics as recently emphasized by Van Slyke ((9) p. 33). The hydrolysis of *l*-alanylglycylglycine also follows zero order kinetics.

TABLE VIII

Action of Glycine-imidoendopeptidase Preparation on Various Substrates

Substrate concentration, 0.05 mM per cc.; temperature 40°; pH 7.9 to 8.0; 0.02 M veronal buffer.

Substrate	Enzyme concentration	Time	No Mn^{++} added			0.001 M Mn^{++} added		
			Hydrolysis	C	Average C	Hydrolysis	C	Average C
	mg. protein N per cc.	hrs.	per cent			per cent		
l-Leucylglycylglycine	0.00317	0.5	13			13		
		1.0	26			26		
		1.5	44			37		
		2.0	52			51		
		3.0	76			71		
		24.0	124			106		
Triglycine	0.00635	0.5	29			29	0.79	
		1.0	51			51	0.81	
		2.0	81			74	0.77	
		3.0	92			88	0.81	0.80
		24.0	127			102		
Diglycyl-*l*-proline	0.00635	0.5	20	0.51		20	(0.51)	
		1.25	40	0.47		46	(0.56)	
		2.0	55	0.46		65	(0.60)	
		3.0	78	0.50	0.49	98		
		24.0	184			197		
Diglycyl-*l*-hydroxyproline	0.00635	0.5	16	0.40		17	0.42	
		1.0	31	0.42		32	0.44	
		2.0	50	0.40		51	0.41	
		3.0	65	0.40	0.40	65	0.40	0.42
		24.0	99			160		

The hydrolysis of diglycyl-*l*-proline yields good first order velocity constants for the first 3 hours only in the absence of Mn^{++}. The calculated first order constants rise, in the presence of the metal, much earlier. This rise is the result of the concurrent rapid splitting of the dipeptide formed during the hydrolysis of diglycylproline. This dipeptide must be glycyl-*l*-proline and cannot be diglycine, since the hydrolysis of the former by prolidase is activated by Mn^{++}, while the hydrolysis of glycylglycine is

inhibited by the metal. Thus, it follows that the hydrolysis of diglycyl-*l*-proline takes place at the peptide bond adjacent to the free amino group and results in the formation of glycine and glycyl-*l*-proline as the first products. Our plan to test this conclusion by the isolation of the primary split-products could not be verified under present conditions.

For the enzymatic hydrolysis of diglycyl-*l*-hydroxyproline, good and almost identical first order velocity constants were obtained in the absence and in the presence of Mn^{++}. It is probable that here, as in the case of diglycylproline, the hydrolysis occurs between the two glycine residues. With the low enzyme concentration employed, the glycylhydroxyproline is hydrolyzed rather slowly even in the presence of activating manganese.

The data on the two stereoisomeric forms of alanylglycylglycine, also reported in Table IX, indicate that the *l* form is split about 90 times as rapidly as the *d* form. It is, at present, not known whether the same enzyme is responsible for the hydrolysis of the two stereoisomers.

Table IX provides some comparative data concerning the action of a similar and weaker enzyme preparation upon several substrates. The enzyme preparation contains in all probability other enzymes besides *l*-prolidase and glycine-imidoendopeptidase. One is justified, however, to conclude from Table IX that the imidoendopeptidase, which acts upon triglycine, is much less active towards glycylglycine, glycinamide, glycylglycinamide, and the tetrapeptides diglycyl-*l*-leucylglycine and diglycyl-*l*-glutamylglycine. The free carboxyl in the tripeptide appears to have a rather beneficial influence upon the action of the imidoendopeptidase.

Action of Kidney Cathepsin on Proline and Hydroxyproline Peptides

A highly active preparation of swine kidney cathepsin prepared according to Fruton, Irving, and Bergmann (8) was found to hydrolyze carbobenzoxyglycyl-*l*-proline to the extent to 20 per cent of one peptide bond within a period of 45 hours at pH 4.7 and 40°, with citrate buffer and cysteine as the activator. When no cysteine was added, only about 5 per cent splitting occurred. No hydrolysis was observed of the substrates carbobenzoxyglycyl-*l*-hydroxyproline, carbobenzoxyglycyl-*l*-prolinamide, and carbobenzoxyglycyl-*l*-hydroxyprolinamide. To our knowledge, this is the first reported observation of the hydrolysis of a proline peptide by a catheptic enzyme.

Instability of Dipeptides Containing Proline or Hydroxyproline

Freshly prepared solutions of glycyl-*l*-proline and glycyl-*l*-hydroxyproline are hydrolyzed to 100 per cent by solutions of prolidase. When such solutions are kept at room temperature before the enzyme is added, their carboxyl titer steadily decreases and simultaneously their sensitivity to-

wards prolidase diminishes. For the study of these peptides it is therefore necessary to use freshly prepared solutions.

TABLE IX

Hydrolysis of Various Peptides by Extract of Intestinal Mucosa, Purified by Lead Acetate

Enzyme concentration, 0.214 mg. of protein N per cc.; no Mn^{++} added; temperature 40°; pH 7.8 to 8.0.

Substrate	Time	Hydrolysis	C
	hrs.	*per cent*	
l-Alanylglycine	18	77	0.0028
	26	88	0.0028
	44	103	
Glycyl-*l*-alanine	26	17	0.00024
	44	25	0.00022
	72	35	0.00020
	95	46	0.00022
Glycylglycine	19	14	0.00026
	68	40	0.00026
Glycylglycinamide	19	35	0.00077
	68	85	0.00094
Glycinamide	19	7	0.00014
	68	26	0.00015
Diglycyl-*l*-leucylglycine	18	35	0.00081
	26	44	0.00076
	44	62	0.00075
	95	114	
Diglycyl-*l*-glutamylglycine	18	40	0.00097
	26	52	0.00096
	44	86	
	72	120	
	95	144	
l-Alanylglycylglycine	0.5	35	0.029
	1.25	77	0.040
	1.5	83	0.040
d-Alanylglycylglycine	19	22	0.0004‡
	68	51	0.00035
Triglycine	0.5	31	0.025
	1.25	73	0.035
	1.5	77	0.033

It is likely that the apparent decrease in peptide concentration is due to a slow transformation of the dipeptides into diketopiperazines in the slightly alkaline solution (pH, 8.0). It is noteworthy that the diketopiperazine of glycylproline has been repeatedly isolated from acid protein hydrolysates. Moreover, we have isolated diketopiperazines when carbo-

benzoxyglycyl-*l*-prolinamide and carbobenzoxyglycyl-*l*-hydroxyprolinamide were hydrogenated in acid solution. On removal of the carbobenzoxy group, ammonia is eliminated spontaneously and the diketopiperazine is formed under ring closure. Abderhalden and Nienburg (13) have found that carbobenzoxyprolylproline forms proline anhydride on hydrogenation. However, the hydrogenation of carbobenzoxyglycyl-*l*-proline and of carbobenzoxyglycyl-*l*-hydroxyproline yields the expected dipeptides.

TABLE X

Optical Rotation of Dipeptides Containing Glycine

The rotations of all peptides were determined in aqueous solution.

Peptide (1)	Bibliographic reference No. (2)	Specific rotation (3)	Molecular rotation (4)	Δ (a), (b) (5)
		degrees	*degrees*	*degrees*
Glycyl-*l*-leucine	16	−35.1	(*a*) −6,602	
l-Leucylglycine	17	+85.5	(*b*) +16,083	22,685
Glycyl-*l*-valine*	18	−19.17	(*a*) −3,430	
l-Valylglycine*	18	+93.5	(*b*) +16,278	19,718
Glycyl-*l*-alanine	19	−50.0	(*a*) −7,305	
l-Alanylglycine	20	+50.3	(*b*) +7,349	14,654
Glycyl-*l*-hydroxyproline	This paper	−128.4	(*a*) −24,152	
l-Hydroxyprolylglycine	" "	−22.4	(*b*) −4,213	19,939
Glycyl-*l*-proline	21	−113.8	(*a*) −19,585	
l-Prolylglycine†	22	−22.8	(*b*) −4,023	15,562

* These dipeptides, containing natural valine or natural alanine, were designated in the publication referred to in Column 2 as glycyl-*d*-valine, *d*-valylglycine, glycyl-*d*-alanine, and *d*-alanylglycine.

† This substance is described (22) as containing 1 molecule of water of crystallization.

Optical Rotation of Several Dipeptides Containing Glycine

The synthesis of glycyl-*l*-hydroxyproline by the carbobenzoxy method reported in the experimental section has yielded a preparation possessing an $[\alpha]_D^{26} = -128.4°$ (in water), while Abderhalden and Koppel (14) had reported for this peptide an $[\alpha]_D^{20} = -50.79°$ (in water). The difference may, at least in part, be attributed to the fact that Abderhalden and Koppel synthesized the dipeptide by means of the halogenacyl method. Fischer and Reif (15) have shown that the application of this method to halogenacyl derivatives of proline sometimes results in the formation of products which are isomeric with the expected peptides. Similar complications may be expected for the derivatives of hydroxyproline.

In order to obtain additional evidence, the difference between the rotation

of glycyl-*l*-hydroxyproline and *l*-hydroxyprolylglycine was calculated and the value thus obtained was compared with the values reported for other dipeptides containing an optically active aliphatic amino acid combined with glycine. It will be noted from the data reported in Table X that the peptides containing the glycine carbonyl combined with the nitrogen of an aliphatic monoaminomonocarboxylic acid have a specific rotation 90–120° more negative than the respective peptides with a reciprocal sequence of the two amino acid residues. The difference of the molecular rotations is 14,600–22,700°.

The difference of the molecular rotations for the reciprocal peptides containing proline or hydroxyproline respectively is very similar when the specific rotation of −128.4° is accepted for glycyl-*l*-proline. The value of −50.8°, on the other hand, as reported by Abderhalden and Koppel, is clearly out of line with the other data recorded in Table X.

The authors wish to thank Mr. Stephen M. Nagy, who performed the elementary analyses, for his valuable assistance.

EXPERIMENTAL

Enzymatic Studies

The activity and purity of different enzyme samples were determined by the methods defined by Irving, Fruton, and Bergmann (6). Wherever possible, the activity was determined by measuring the first order velocity constant K on the substrate S for the enzyme concentration E, where E is expressed as mg. of protein N per cc. of test solution. The proteolytic coefficient $C = K/E$. Although in some cases the reaction did not adhere strictly to a first order reaction, the initial constants were used in order to obtain comparative data. 1 unit of enzymatic activity is the quantity of enzyme which splits the substrate with a K of 0.0020 under the conditions used. The procedure was that followed in previous reports from this laboratory. Hydrolyses were carried out in 2.5 cc. volumetric flasks at a temperature of 40°. Hydrolysis was measured on 0.2 cc. samples by the titration method of Grassmann and Heyde (23). The substrate was present in a concentration of 0.05 M per cc. (with the sole exception of racemic substrates which were used in a concentration of 0.1 M per cc.). The solutions were buffered by the addition of 0.5 cc. of 0.1 M veronal buffer to the test solution.

Hydrolysis of l-Leucylglycylglycine by Lead Acetate-Treated Mucosa Extract

307 mg. of *l*-leucylglycylglycine were dissolved in water and brought to pH 8 by addition of NaOH. Enzyme was added equivalent to 0.317 mg. of protein N and the volume brought to 25 cc. After 35 minutes at 40°, the titration samples showed that 100 per cent splitting of one peptide bond had occurred. The reaction was stopped by addition of 4 cc. of M HCl and 20 cc. of absolute alcohol. The solution was concentrated *in vacuo*, and the residue dissolved in hot 95 per cent alcohol. The slight precipitate was filtered off and the solution again concentrated to dryness. The residue was dissolved in 5 cc. of hot water and 393 mg. of sodium 2-bromotoluene-5-sulfonate were added.[3] This is equivalent to 1.2 moles for the calculated amount of leucine; one-twenty-fifth of the total sample had been removed for estimation of the enzymatic splitting. After standing overnight at 0°, the solution was filtered and the mother liquor retained for the isolation of diglycine. Yield of dried leucine bromotoluene sulfonate, 420 mg., or 87 per cent of theory. The salt was recrystallized from hot water.

$C_{13}H_{20}NO_5SBr$.	Calculated.	C 40.8, H 5.3, N 3.7
382.3	Found.	" 40.6, " 5.5, " 3.7

To the mother liquor and washings (8 cc.) there were added 529 mg. of 5-nitronaphthalene-1-sulfonic acid. This sulfonic acid forms a sparingly soluble salt with glycylglycine which crystallizes as rhombohedral plates. Crystallization was completed overnight at 0°. The characteristic square plates of the glycine salt were absent. Yield, 337 mg., or 73 per cent of theory. The diglycine nitronaphthalene sulfonate was recrystallized from hot water.

$C_{14}H_{13}N_3O_8S$.	Calculated.	C 43.6, H 3.9, N 10.9
385.3	Found.	" 43.8, " 4.1, " 11.0

Glycyl-l-hydroxyproline

Carbobenzoxyglycyl-l-hydroxyproline—To a solution of 5 gm. of *l*-hydroxyproline in 25 cc. of 2 N NaOH there were added in portions with cooling and shaking an additional 20 cc. of 2 N NaOH and 10 gm. of carbobenzoxyglycyl chloride. On acidification with concentrated HCl, the compound precipitated as an oil. The oil was extracted with ethyl acetate. This solution was washed with dilute HCl and with water and then dried over Na_2SO_4. On concentration of this solution *in vacuo* to a thick syrup, sheaves of needles formed on standing at room temperature. The needles were filtered and washed with ether. Yield, 7.6 gm. On recrystallization from hot ethyl acetate, thin triangular plates were obtained. M.p. 124–124.5°.

$C_{15}H_{18}O_6N_2$.	Calculated.	C 55.9, H 5.6, N 8.7
322.3	Found.	" 56.0, " 5.7, " 8.5

[3] The leucine salt of 2-bromotoluene-5-sulfonic acid (21) is considerably less soluble than the glycine, leucylglycine, and diglycine salts of this sulfonic acid.

Glycyl-l-hydroxyproline—4 gm. of the carbobenzoxy compound were hydrogenated in the presence of palladium black in methyl alcohol containing 4 cc. of water and 4 cc. of glacial acetic acid. The oily precipitate was dissolved by the addition of water and the catalyst filtered off. The solution was evaporated *in vacuo* with the addition of methanol. The syrup thus obtained crystallized on addition of absolute ethanol. Yield, 2.3 gm.

$C_7H_{12}N_2O_4$.	Calculated.	C 44.7, H 6.4, N 14.9
188.1	Found.	" 44.4, " 6.5, " 14.8

$[\alpha]_D^{20}$ = −128.4° (1.8% in water)

Diglycyl-l-hydroxyproline

l-Hydroxyproline Benzyl Ester Hydrochloride—25 gm. of *l*-hydroxyproline were esterified twice with dry HCl in 350 cc. of benzyl alcohol, followed by removal of HCl and water *in vacuo* at 85°. The insoluble residue was filtered off and the ester hydrochloride was crystallized from the mother liquor by adding dry ether. Yield, 18.5 gm. of needles. They were dissolved in benzyl alcohol and crystallized by the addition of ether. M.p., 147–150°.

$C_{12}H_{16}NO_3Cl$.	Calculated.	C 55.9, H 6.3, N 5.4
257.7	Found.	" 56.0, " 6.4, " 5.4

Carbobenzoxydiglycyl-l-hydroxyproline Benzyl Ester—The carbobenzoxyglycylglycinazide used for this synthesis was prepared from the corresponding hydrazide according to Rinke (25). It was partially dried on a porous plate and then dissolved in ethyl acetate. This solution was successively washed with ice water, aqueous bicarbonate, and then with water. The solution was dried over Na_2SO_4 and then added to a dry ethyl acetate solution of hydroxyproline benzyl ester prepared from 6.4 gm. of the hydrochloride described in the preceding section. After standing at room temperature overnight, the solution was washed with water, dilute HCl, water, dilute bicarbonate, and again with water, dried over Na_2SO_4, and concentrated *in vacuo*. On addition of ether, the carbobenzoxy peptide ester crystallized in needles. It was recrystallized from ethyl acetate-petroleum ether. Yield, 4.7 gm. M.p., 123–127°.

$C_{24}H_{27}N_3O_7$.	Calculated.	C 61.4, H 5.8, N 9.0
469.5	Found.	" 61.4, " 5.9, " 8.9

Free Tripeptide—3 gm. of the carbobenzoxy tripeptide benzyl ester were hydrogenated in the usual manner in methyl alcohol containing 2 cc. of water and 2 cc. of glacial acetic acid. After concentration of the filtered

solution *in vacuo*, the substance crystallized on addition of absolute alcohol. Yield, 1.5 gm. It was recrystallized from water-alcohol.

$C_9H_{15}N_3O_4 \cdot H_2O$.	Calculated.	C 41.1, H 6.5, N 16.0
263.2	Found.	" 41.3, " 6.6, " 15.7

$[\alpha]_D^{20}$ = −97.7° (2.8% in water)

Diglycyl-l-proline

Carbobenzoxydiglycyl-l-proline Benzyl Ester—A dry ethyl acetate solution of *l*-proline benzyl ester[4] from 6 gm. of the hydrochloride was coupled in ethyl acetate solution with carbobenzoxylglycylglycinazide from 4.6 gm. of the corresponding hydrazide and the solution purified in a manner similar to that described above for the preparation of the corresponding carbobenzoxydiglycyl-*l*-hydroxyproline benzyl ester. After the ethyl acetate solution was concentrated *in vacuo*, anhydrous ether and petroleum ether were added to the residue. The carbobenzoxy benzyl ester crystallized as needles. Yield, 5.2 gm. After recrystallization of the ester from ethyl acetate-petroleum ether, its melting point was 87°.

$C_{24}H_{27}N_3O_6$.	Calculated.	C 63.5, H 6.0, N 9.3
453.5	Found.	" 63.5, " 6.3, " 9.3

Diglycyl-l-proline—4 gm. of the carbobenzoxy tripeptide benzyl ester were hydrogenated in the usual way in methyl alcohol containing 3 cc. of water and 3 cc. of glacial acetic acid. After evaporation of the filtered solution and the addition of absolute ethanol, the tripeptide crystallized as rectangular plates. Yield, 1.9 gm.

$C_9H_{15}N_3O_4 + \frac{1}{2}H_2O$.	Calculated.	C 45.4, H 6.8, N 17.6, H_2O 3.8
238.2	Found.	" 45.4, " 6.8, " 18.0, " 3.9

$[\alpha]_D^{20}$ = −101.5° (2.2% in water)

Carbobenzoxyglycyl-l-hydroxyprolinamide

l-Hydroxyproline Methyl Ester Hydrochloride—7 gm. of *l*-hydroxyproline were twice esterified with dry HCl in methanol, the solvent being removed by concentration *in vacuo* each time. Yield, 8.4 gm. of needles. The compound was twice recrystallized from methanol-ether. M.p., 162–164°, with evolution of gas.

$C_6H_{12}NO_3Cl$.	Calculated.	C 39.7, H 6.6, N 7.7
181.6	Found.	" 39.5, " 6.9, " 7.8

[4] The benzyl ester hydrochloride of *l*-proline was obtained by a procedure analogous to that described above for the benzyl ester hydrochloride of *l*-hydroxyproline. The proline ester, which crystallized readily from benzyl alcohol, could not be obtained analytically pure. The crude ester could, however, be used satisfactorily for syntheses.

Carbobenzoxyglycyl-l-hydroxyprolinamide—A dry solution in ethyl acetate of hydroxyproline methyl ester prepared from 5 gm. of the hydrochloride was coupled with 7 gm. of carbobenzoxyglycine chloride. The solution was washed with bicarbonate, water, and dilute HCl and dried over Na_2SO_4. The solvent was removed *in vacuo* and the resulting syrup dissolved in absolute methyl alcohol previously saturated with dry NH_3 gas at 0°. This solution was allowed to stand for 2 days in a pressure flask at room temperature. The solvent was then removed *in vacuo*, and the amide crystallized from methanol-ether. M.p., 208°.

$C_{15}H_{19}N_3O_5$.	Calculated.	C 56.0, H 6.0, N 13.1
321.2	Found.	" 55.9, " 5.9, " 12.9

Glycyl-l-hydroxyproline Diketopiperazine—3 gm. of the above carbobenzoxy dipeptide amide were hydrogenated in the usual manner in methanol containing 3 cc. of water and 3 cc. of glacial acetic acid. On concentration of the filtrate *in vacuo* with the repeated addition of absolute methanol, 2.1 gm. of large prisms were obtained. They were recrystallized from absolute methanol. This compound contained no amino nitrogen and gave the analytical values for the diketopiperazine.

$C_7H_{10}N_2O_3$.	Calculated.	C 49.4, H 5.9, N 16.5
170.2	Found.	" 49.4, " 6.0, " 16.5

$[\alpha]_D^{20} = -190.4°$ (1.9% in water)

Carbobenzoxyglycyl-l-prolinamide—A dry solution in ethyl acetate of *l*-proline benzyl ester from 6 gm. of the hydrochloride was coupled with 7 gm. of carbobenzoxyglycyl chloride. The solution was washed with bicarbonate, water, dilute HCl, and water, and then dried over Na_2SO_4. The solvent was then removed *in vacuo*, and the syrup dissolved in absolute methanol which had been previously saturated with dry NH_3 gas at 0°. After standing for 3 days at room temperature in a stoppered flask, the solution was repeatedly concentrated *in vacuo* with methanol until it was free of NH_3. Crystallization occurred on addition of ether and petroleum ether. Yield, 7.0 gm. of needles. They were recrystallized from methanol by the addition of ether and petroleum ether. M.p., 150–151°.

$C_{15}H_{19}N_3O_4$.	Calculated.	C 59.0, H 6.2, N 13.8
305.1	Found.	" 59.1, " 6.3, " 13.6

Glycyl-l-proline Diketopiperazine—3 gm. of the above amide were hydrogenated in the usual way in methanol containing 3 cc. of water and 3 cc. of glacial acetic acid. The filtrate was concentrated *in vacuo* with the repeated addition of methanol. On addition of absolute alcohol the diketopiperazine crystallized as regular octahedra. The substance contained no amino nitrogen. M.p., 213°.

$C_7H_{10}N_2O_2$.	Calculated.	C 54.5, H 6.5, N 18.2
154.2	Found.	" 54.4, " 6.7, " 18.2

$[\alpha]_D^{20} = -197.3°$ (8.1% in water)

Fischer and Reif (15) report a melting point of 213° and $[\alpha]_D^{20} = -217.4°$ for this compound. This compound has also been isolated from several different protein hydrolysates: from gelatin by Levene and Beatty (26) who give a melting point of 182–183°, from gliadin by Abderhalden (27) who reports a melting point of 209° and $[\alpha]_D^{20} = -206.5°$, and from edestin by Abderhalden and Komm (28) who give a melting point of 180–183° and $[\alpha]_D^{20} = -202°$.

l-Hydroxyprolylglycine

Carbobenzoxy-l-hydroxyproline Hydrazide—To 10 gm. of hydroxyproline methyl ester hydrochloride in 50 cc. of water with 100 cc. of chloroform there were added in portions with cooling and shaking 3 gm. of MgO and 15 gm. of carbobenzoxy chloride. 20 minutes after the last addition, 2 cc. of pyridine were added. 5 N HCl was then added until the aqueous layer reacted acid. The chloroform layer was washed with water, bicarbonate, and dilute HCl, and dried over Na_2SO_4. The solution was then concentrated *in vacuo* with the repeated addition of alcohol. The syrup thus obtained was dissolved in 50 cc. of absolute alcohol, and filtered. 3.8 gm. of hydrazine hydrate were added and the solution allowed to stand at room temperature overnight. The slight precipitate was filtered and the solution concentrated *in vacuo* repeatedly with ether. Yield, 5.6 gm. of needles. They were recrystallized from ethyl acetate-ether. M.p., 149–149.5°.

$C_{15}H_{19}N_3O_4$.	Calculated.	C 55.9, H 6.1, N 15.0
279.3	Found.	" 55.9, " 6.0, " 15.0

Carbobenzoxy-l-hydroxyprolylglycine Benzyl Ester—5 gm. of carbobenzoxy-*l*-hydroxyproline hydrazide were converted to the azide by dissolving in 50 cc. of water with 1 cc. of concentrated HCl and 3 cc. of glacial acetic acid. A solution of 1.5 gm. of sodium nitrite in 5 cc. of water was added with cooling and shaking. The oily precipitate was extracted into ethyl acetate and washed with cold water, bicarbonate, and again with water. After drying, the solution of the azide was added to a dry ethyl acetate solution of glycine benzyl ester prepared from 5.4 gm. of the hydrochloride, and the mixture allowed to stand overnight at room temperature. The solution was concentrated *in vacuo* to yield 4.7 gm. of needles. The substance was twice recrystallized from ethyl acetate. M.p., 153°.

$C_{22}H_{24}N_3O_6$.	Calculated.	C 64.1, H 5.9, N 6.8
412.4	Found.	" 64.3, " 6.1, " 6.9

l-Hydroxyprolylglycine—3 gm. of the above carbobenzoxy dipeptide benzyl ester were hydrogenated in the usual way in methanol containing 2 cc. of glacial acetic acid and 2 cc. of water. The peptide which crystallized during the hydrogenation was dissolved by the addition of a little water, and the solution was filtered and then repeatedly concentrated *in vacuo* with the addition of methanol. Yield, 1.3 gm. of needles. The dipeptide was recrystallized from water-methanol.

$C_7H_{12}N_2O_4$.	Calculated.	C 44.7, H 6.4, N 14.9
188.1	Found.	" 44.8, " 6.4, " 14.8

$[\alpha]_D^{20} = -22.42°$ (7.7% in water)

l-Prolinamide Hydrochloride—9 gm. of *l*-proline benzyl ester hydrochloride were converted to the free ester in ethyl acetate in the usual manner. After drying, the solvent was evaporated off with the three times repeated addition of methanol. The syrup was dissolved in 75 cc. of methanol, which was previously saturated with dry NH_3 gas at 0°. After standing at room temperature for 3 days in a stoppered flask, the solution was repeatedly concentrated with methanol. Yield, 3 gm. of needles. These were dissolved in methanol and an excess of dilute HCl was added. This solution was repeatedly concentrated *in vacuo* with methanol. The needles of the amide hydrochloride were recrystallized from methanol-ether. M.p., 173–175°.

$C_5H_{11}N_2OCl$.	Calculated.	C 39.9, H 7.4, N 18.6
150.6	Found.	" 40.0, " 7.4, " 18.5

l-Hydroxyprolinamide—*l*-Hydroxyproline methyl ester was prepared in dry chloroform solution from 4.6 gm. of the hydrochloride. The solution was concentrated *in vacuo*. Methanol was twice added and the solution again evaporated. The syrup was dissolved in 75 cc. of methanol, and the solution saturated with dry NH_3 and kept in a stoppered flask for 2 days at room temperature. The solution was repeatedly evaporated *in vacuo*, with the addition of methanol. Yield, 2.2 gm. of needles. They were recrystallized from ethyl acetate. M.p., 139°.

$C_5H_{10}N_2O_2$.	Calculated.	C 46.1, H 7.8, N 21.5
130.2	Found.	" 46.1, " 8.0, " 21.2

Carbobenzoxy-l-prolyl-l-proline—This was prepared as described by Abderhalden and Nienburg (13).

l-Alanylglycylglycine—This was prepared as described by Bergmann and Fruton (3). The carbobenzoxy-*l*-alanylglycine hydrazide used in the preparation of the tripeptide had, after recrystallization from hot water, a melting point of 157°. Bergmann and Fruton give for this compound a melting point of 145–147°.

Carbobenzoxyglycylglycine Hydrazide—This was prepared as described by Rinke (25).

Carbobenzoxyglycyl-l-proline and Glycyl-l-proline—These were prepared as described by Bergmann, Zervas, Schleich, and Leinert (21).

SUMMARY

1. The preparation of a *l*-leucine-aminoexopeptidase from aqueous extracts of swine intestinal mucosa is described. The purified enzyme protein is stable but becomes rather unstable when activated by Mn^{++}, thus frustrating attempts at establishing reliable proteolytic quotients.

2. Two procedures are described for the preparation of prolidase (substrates, glycyl-*l*-proline and glycyl-*l*-hydroxyproline) from intestinal mucosa. Prolidase is activated by Mn^{++}, but not by Mg^{++}, Co^{++}, Cu^{++}, or Zn^{++}. With the manganese-activated enzyme, satisfactory first order velocity constants were obtained.

3. Swine intestinal mucosa is found to contain one or several endopeptidases hydrolyzing carbobenzoxyglycyl-*l*-prolinamide and carbobenzoxyglycyl-*l*-hydroxyprolinamide.

4. Swine intestinal mucosa contains, in addition to *l*-leucine-aminoexopeptidase, a second enzyme that hydrolyzes *l*-leucylglycylglycine to yield leucine and glycylglycine. In contrast to the leucinepeptidase, the new enzyme is not activated by Mn^{++} and acts not at all or only very slowly upon *l*-leucylglycine. It is tentatively classified as a glycine-imidoendopeptidase.

5. Swine kidney contains an enzyme which, in the presence of added cysteine, hydrolyzes carbobenzoxyglycyl-*l*-proline.

6. Evidence is offered for the presence in swine intestinal mucosa of enzymes hydrolyzing glycylglycine and glycyl-*l*-leucine, glycyl-*l*-alanine, *l*-alanylglycine, *l*-prolylglycine and *l*-hydroxyprolylglycine, and *d*-leucylglycylglycine.

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J. Biol. Chem. 163(1): 29-38 (1946)

ZINC TOXICITY IN RATS

ANTAGONISTIC EFFECTS OF COPPER AND LIVER

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(Received for publication, December 28, 1945)

The evaluation of the nutritional quality of foods and feeds requires a more definite knowledge of the physiological rôle of the various micro nutrient inorganic elements in the animal body than is now available. Problems of immediate concern include the effects of one or more of these elements on the utilization of another, for example the effect of traces of arsenic in reducing the toxicity of selenium (Moxon and Rhian (8)) and of copper in reducing the toxicity of molybdenum (Ferguson *et al.* (3)). It is the purpose of this paper to summarize studies of an anemia and subnormal growth induced in rats by high zinc diets and the antagonistic effects of copper and liver supplements.

Ferguson *et al.* reported that certain "teart" pastures in England which were toxic for cattle contained a large amount of molybdenum and that this molybdenum toxicity could be prevented by drenching the cattle with a solution of copper sulfate. Such observations as well as those to be reported in this paper point to the possibility of deficiencies occurring in grazing animals, which are relative rather than absolute. It is usually concluded that if "sick" animals respond favorably to dietary supplement, such as copper for example, the animals and the feed they consumed were copper-deficient and copper therapy is advised. It may be that such therapy only partially corrects the condition, for, as pointed out in this study and in that of Ferguson *et al.*, copper deficiency may be relative to a high content of some other factor in the feed which renders a normally adequate copper content inadequate.

The present study was stimulated by the report of Sutton and Nelson (11) who observed that excess dietary zinc produced an anemia, subnormal growth, and reproductive failure in rats. These observations were fully confirmed by the authors. Further studies showed that feeding a mixture of iron, copper, and cobalt salts largely or completely prevented the anemia and that a supplement of liver extract partially prevented the subnormal growth. These preliminary observations led to more detailed experiments which are reported below.

EXPERIMENTAL

Methods

The basal diet used in these studies was a commercial calf meal which has proved over many years to be an adequate stock diet for rats (Maynard (7)). Zinc was thoroughly mixed into this calf meal as C.P. zinc carbonate and fed *ad libitum* to the rats. The supplements of liver extract[1] and iron, copper, and cobalt salts were mixed in a few ml. of milk and fed to the animals daily in Pyrex glass cups. When these supplements were fed, the daily amounts were 2 mg. of iron as ferric pyrophosphate, 0.2 mg. of copper as cupric sulfate, 0.2 mg. of cobalt as cobaltous chloride, and 1 gm. of liver extract. The rats were from our colony which originated from Sprague-Dawley stock. Young rats ranging from 4 to 6 weeks of age were started on each experiment. They were kept in individual wire screened cages during the studies which usually lasted from 4 to 6 weeks. Hemoglobin levels were determined periodically on blood samples secured by clipping the tails. At the end of the experiment approximately 0.5 ml. of blood was removed by heart puncture, mixed with heparin to prevent coagulation, and subjected to various determinations in duplicate. Hemoglobin was determined in a photoelectric colorimeter as oxyhemoglobin (Sheard and Sanford (9)). Red cells and leucocytes were counted in the usual manner. The red cell volume (centrifuge hematocrit) was determined with the modified centrifuge tubes described by Gruneberg (Smith (10)). Blood smears were prepared for microscopic study by staining with hematoxylin and eosin.

Results

In a series of preliminary experiments in which a level of 1 per cent of zinc in the diet was used, the following observations were made. Hemoglobin decreased to levels of severe anemia in 3 to 5 weeks. The rats eventually stopped growing and many of them died before the 6th week, particularly the youngest rats of the group. The hair was unkempt; the rats walked with a highly arched back and were very irritable when handled. If in addition to the high zinc diet a mixture of iron, copper, and cobalt salts was fed, the hemoglobin remained at or near to normal levels over the 4 to 6 week period, but body weight remained subnormal. If in addition to the 1 per cent zinc diet liver extract was fed, the hemoglobin dropped to levels comparable with those of rats fed the high zinc diet alone; however, growth was maintained near the normal level and the rats lived for longer periods of time. It thus appeared that the excess dietary zinc had at least

[1] Liver extract powder, Eli Lilly and Company.

two effects: (1) it produced an anemia, and (2) it produced a subnormal rate of growth that was not secondary to the anemia.

Two factorially designed experiments were then conducted. One was designed primarily to study the effects of iron, copper, cobalt, and liver extract on the course of the anemia, which for convenience will be referred to as "zinc anemia." The other experiment was designed to study the effect of liver extract on the subnormal growth induced by the feeding of

TABLE I

Mean Hemoglobin Values of Group on Each Treatment

Treatment	Mean Hb value* per 100 ml. blood
	gm.
None	10.64
Fe	13.02
Cu	14.04
Fe, Cu	13.84
Co	11.90
Fe, Co	11.06
Cu, "	14.24
Fe, Cu, Co	14.92
Liver	13.80
Fe, liver	13.10
Cu, "	14.46
Fe, Cu, liver	15.40
Co, liver	14.70
Fe, Co, liver	14.24
Cu, " "	14.50
Fe, Cu, Co, liver	15.62

* Mean of five rats.

excess zinc. Detailed studies were made of the blood picture of rats in the second experiment.

Zinc Anemia—The 1 per cent level of zinc used in the preliminary experiments was so toxic that many (up to 75 per cent) deaths resulted in 3 to 5 weeks. A pilot study was conducted testing the effect of various levels of zinc on the decrease in hemoglobin level. By feeding zinc at various levels ranging from 0.4 to 1.0 per cent of the diet it was found that a level of 0.7 per cent produced a marked anemia in 4 weeks and permitted the rats to live for relatively long periods of time. Accordingly, a level of 0.7 per cent zinc was chosen for the next experiment which involved the dietary supplements of iron, copper, cobalt, and liver extract, each at "high" and "low" levels in all possible combinations. The high level

indicates the addition of the supplement in amounts previously mentioned, and the low level indicates no addition of the supplement to the high zinc diet. This was a 2^4 factorial experiment with five replications. 80 rats of mixed sexes, ranging in age from 30 to 40 days and in weight from 36 to 71 gm., were divided at random (Fisher and Yates (4)) into sixteen groups of five animals each. Sixteen animals, one animal receiving each treatment, constituted a block. The rats in each block were arranged at random in a battery of cages, giving five batteries or blocks. The five

TABLE II

Analysis of Variance of Hemoglobin Levels of Rats Fed Various Supplements in Addition to High Zinc Diet

Source of variation	df	Mean square
Total	79	
Replications	4	8.34*
Fe	1	4.90
Cu	1	76.05*
Co	1	4.80
Liver	1	38.64*
Fe × Cu	1	0.39
" × Co	1	2.74
" × liver	1	0.00
Cu × Co	1	0.22
" × liver	1	8.58†
Co × "	1	2.38
Fe × Cu × Co	1	8.06†
" × " × liver	1	3.28
Cu × Co × "	1	7.20
Fe × " × "	1	0.92
" × Cu × Co × liver	1	3.04
Error	60	1.83

* Significant at the 1 per cent level.
† Significant at the 5 per cent level.

batteries were distributed in the air-conditioned animal room, and differences between batteries served as a measure of positional effects. The rats were weighed weekly during the experiment, which was terminated at the end of 6 weeks. At this time blood samples were taken by heart puncture, and the hemoglobin level determined. The mean hemoglobin values of the different groups are summarized in Table I. Table II presents a summary of the analysis of variance of the hemoglobin values.

The analysis of variance showed that the rats fed the high zinc diet plus copper or liver extract had higher hemoglobin levels than those rats fed the high zinc diet without added copper or liver extract. These differences

were highly significant. Also, there was a significant interaction of copper and liver extract due to the fact that copper supplements produced a greater response in the hemoglobin level in the absence of liver than it did in the presence of liver extract. There was a significant interaction of iron by copper by cobalt, which resulted in a higher hemoglobin level than would be expected if additive effects of the metals fed singly or in combinations of any two were involved. There was a highly significant difference between replications, indicating that some factor or factors associated with

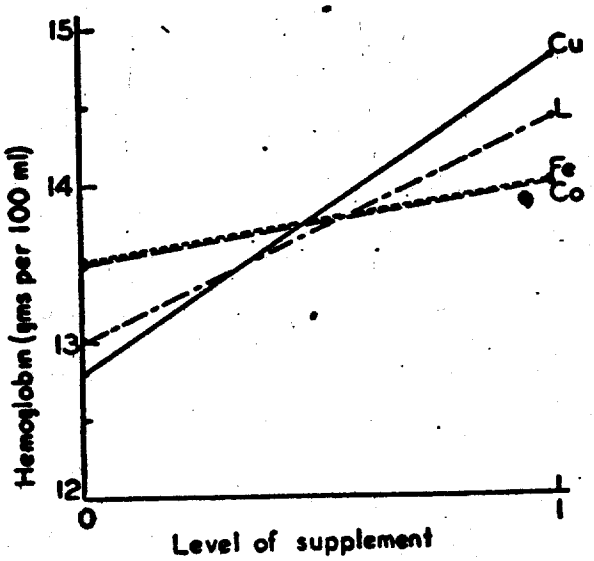

FIG. 1. The hemoglobin level of the rats fed the various supplements at 0 and 1 levels. The 0 level indicates that the supplement was not fed and the 1 level that it was fed. Each point is the average hemoglobin level of forty rats. The difference between the hemoglobin levels of the rats not fed additional copper salts and those fed copper salts in addition to the high zinc diet is highly significant. The same holds true for the difference between the rats not fed liver extract and those fed liver extract (*L*).

the position of the five replications introduced a difference in hemoglobin levels that was highly significant. Since the room was air-conditioned and maintained at a temperature of 24.4°, the factor probably is not temperature variation. All other sources of variation were non-significant.

The hemoglobin levels observed at high and low levels of iron, copper, cobalt, or liver extract are graphically presented in Fig. 1.

Growth—In the foregoing experiment the effects of the various treatments on growth were tested by an analysis of variance, and the addition of liver extract to the diet was associated with significantly better growth (odds 19:1) than was the case for those rats fed the basal diet plus 0.7 per cent

zinc. However, previous observations indicated that a much more marked effect could be obtained with liver extract when the higher levels of zinc were fed. Accordingly, a second factorial experiment was set up involving a basal diet containing 1 per cent of zinc and supplemented with liver extract and the mixture of iron, copper, and cobalt salts, each at a high and low level. The mixture of iron, copper, and cobalt salts was chosen, since it most effectively prevented a drop in hemoglobin level among the various combinations of the elements. This was a 2^2 factorial experiment with ten replications.

Forty rats, ranging in age from 26 to 29 days and in weight from 42 to 82 gm. and consisting of twenty males and twenty females, were allotted at random to the four groups treated differently, leaving five male and five female rats in each group. The rats were caged individually and distributed at random on five shelves of a cage battery, so that each shelf contained eight rats, one on each treatment, resulting in five replications. The rats were weighed weekly during the experiment which ran for 5 weeks. At the end of this time, blood samples were taken from all rats except the group which received all supplements; that is, iron, copper, cobalt, and liver extract. Red cell counts, leucocyte counts, hemoglobin, and hematocrit determinations were made. A group of ten comparable rats which received the calf meal alone (stock diet) was also studied to provide control values for comparison.

TABLE III

Mean Initial and Final Weights of Rats Fed High Zinc Diet and Various Combinations of Liver Extract and Iron, Copper, and Cobalt

Treatment	Mean initial weight*	Mean final weight*
	gm.	gm.
None	61.5	118.5
Liver	62.7	155.1
Fe, Cu, Co	64.5	120.8
Liver, Fe, Cu, Co	64.3	148.7
Stock diet	62.4	174.6

* Mean weights of ten rats (five males and five females).

The mean initial and final weights of the rats on each treatment are summarized in Table III. The analysis of variance of the growth data is summarized in Table IV.

It will be noted that the liver extract supplement to the high zinc diet induced a positive growth response which when compared to the growth of rats fed the high zinc diet alone gave a difference that was highly significant. Although the supplement of iron, copper, and cobalt maintained hemoglobin at levels significantly higher than in those rats fed the high

zinc diet alone (Table V), it had no effect in counteracting the subnormal growth induced by the excess dietary zinc. A significant (99:1) sex differ-

TABLE IV

Analysis of Variance of Growth Data of Rats Fed High Zinc Diet and Various Combinations of Liver Extract and Mixture of Iron, Copper, and Cobalt

Source of variation	*df*	Mean square
Total	39	
Replications	4	703
Sex	1	9,456*
Replications × sex	4	1,664
Liver	1	10,401*
Fe, Cu, Co	1	42
Sex × liver	1	2,512*
Liver × Fe, Cu, Co	1	189
Error 1	26	558
" 2†	28	615
" 3‡	32	746

* Significant at the 1 per cent level.

† Used to test the significance of liver and Fe, Cu, and Co.

‡ Used to test the significance of replications and sex.

TABLE V

Blood Pictures of Rats Fed the Zinc Diet Alone or Plus Liver Extract or Fe, Cu, and Co Supplements along with Those for Comparable Rats Fed a Stock Diet

Treatment	Mean Hb	Mean r.b.c.	Mean hematocrit	Mean cell volume	Mean cell Hb concentration	Mean cell Hb	Leucocyte count
	gm. per 100 ml.	*millions*	*per cent*	*cu. microns*	*per cent*	*micromicrograms*	*thousands*
None	8.36	9.64	35.5	37.1	23.4	8.6	29.0
	±0.622	±0.577	±1.72	±1.07	±0.52	±0.26	±2.68
Liver	8.77	9.27	35.5	38.6	24.5	9.5	40.3
	±0.604	±0.481	±1.39	±1.19	±0.69	±0.38	±4.90
Fe, Cu, Co	12.86	12.94	47.4	36.8	27.3	10.0	19.1
	±0.614	±0.611	±2.40	±1.49	±0.84	±0.33	±1.55
Stock	16.04	8.52	50.4	59.3	31.8	18.9	22.0
	±0.149	±0.194	±0.59	±1.15	±0.47	±0.24	±0.73

Each of the readings is the mean of ten (no treatment and liver) or eight rats with its standard error.

ence in growth was found due to the more rapid growth of the males, as would be expected. A highly significant sex by liver interaction was accounted for in the observation that male rats responded more than did female rats to the liver extract supplement.

Blood Picture—The results of the blood studies are summarized in Table

V. The significance of differences of treatment was determined by the *t* test.

In comparing the group that received no treatment, that is those rats which received the high zinc diet alone, with the stock rats, it will be observed that hemoglobin and hematocrit values are lower. These differences are highly significant. Although hemoglobin values are lower, the mean red cell count of the untreated group is slightly higher than that of the stock rats. This difference is not significant. The mean cell volume is significantly less (odds 99:1) than normal, which shows that the zinc anemia is microcytic. Also, the mean concentration of hemoglobin per red cell is significantly less (odds 99:1) than normal, and this shows the anemia to be hypochromic. The zinc anemia is, therefore, morphologically classified as microcytic and hypochromic. The leucocyte counts of the untreated group are significantly higher than normal. Apparently, the excess dietary zinc affects the leucogenic as well as the erythrogenic line of cells. Microscopic examinations of the blood smears of the untreated rats confirmed the presence of microcytosis and hypochromia. In addition there was a definite basophilia, as noted in the many red cells which took the hematoxylin stain. Poikilocytes were present, but not numerous.

The addition of liver extract to the high zinc diet did not significantly change the blood picture exhibited by the untreated group except to induce a further rise in the leucocyte counts. This difference was significant. In the previous experiment in which the dietary level of zinc was 0.7 per cent, liver extract maintained the hemoglobin at a level significantly higher than in those rats not fed the liver supplement. However, in this latter experiment, in which the zinc level was 1 per cent, liver extract had no significant effect on the hemoglobin level.

The addition of iron, copper, and cobalt salts to the high zinc diet produced a rise in hemoglobin level, red cell count, and hematocrit. These increases were all highly significant. The red cell counts of the iron-, copper-, and cobalt-supplemented rats were even higher than that of normal rats. This difference was highly significant.

DISCUSSION

The results of this study show that the addition of zinc to an otherwise adequate diet for rats induces at least two symptoms, (1) an anemia that can at least partially be prevented by an additional supplement of copper salts, and (2) subnormal growth that can be partially prevented by an additional supplement of liver extract.

In so far as the anemia is concerned, additional supplements of iron or cobalt salts had no effect, although the combination of iron, copper, and cobalt salts maintained hemoglobin at slightly higher levels than copper alone at the dietary levels of zinc and the supplements used in this study.

It is possible that the feeding of levels of copper higher than that used here would maintain hemoglobin at normal levels. Liver extract fed as a supplement to a 0.7 per cent zinc diet maintained a hemoglobin level higher than that found in rats fed the high zinc diet alone, but it had no significant effect on the level of hemoglobin when the level of zinc was 1 per cent. Liver extract may have affected the course of the anemia through its content of iron, copper, and cobalt. 1 gm. of liver extract furnished an average of 0.11 mg. of iron, 0.03 mg. of copper, and 0.0002 mg. of cobalt. These amounts were considerably less than those furnished by the salts.

A review of the literature revealed another suggestion of a dietary copper-zinc relationship. The condition of enzootic ataxia in lambs in Australia has been associated with a dietary deficiency of copper (Bennetts and Beck (1)). Swayback, a disease similar to the enzootic ataxia of Australia, is wide-spread in Great Britain, and copper supplementation has been found to be an effective preventive (Dunlop *et al.* (2)), but in contrast to the "ataxia" pastures of Australia the "swayback" pastures of Britain are well supplied with copper (Innes and Shearer (6)). These findings lead Shearer to suggest that some unknown factor in the "swayback" pastures affected the utilization of dietary copper. Such pastures were found to have a high calcium to phosphorus ratio and a high content of *zinc* and lead, and it was suggested that one of these may affect the absorption of copper.

Iron, copper, and cobalt salts had no effect on the subnormal growth induced by the high zinc diet, but liver extract supplements gave highly significant growth responses. Liver extract, then, furnished some factor or factors that the zinc additions rendered inadequate. In this connection Gross *et al.* (5) reported that 4 to 6 mg. of zinc chloride fed to rats in addition to a synthetic diet precipitated a deficiency syndrome that could be cured by the additional feeding of calcium pantothenate. Our rats fed the high zinc diets did not show any characteristic symptoms of pantothenic acid deficiency. In a pilot experiment, rats were fed the high zinc diet and in addition a supplement of 100 γ of calcium pantothenate per day. This supplement had no effect on the hemoglobin level or on the rate of growth. Apparently the deficiency induced by the zinc carbonate additions and corrected by the liver extract is not related to pantothenic acid. Further work is required to determine the active fraction in the liver extract.

The significant differences found among replications, *i.e.* positional effects, in the first study are worthy of further comment. The majority of nutritional experiments are conducted with the assumption, consciously or unconsciously, that positional effects are either not present or that they are of a magnitude that is insignificant. The results reported here showed that positional effects were significant as related to hemoglobin level even

in a small air-conditioned animal room. Even though positional effects may for the moment be uninterpretable or may not be considered to be of interest in a particular study, an estimation of their magnitudes would be justified in the interests of increased precision. In a properly designed experiment, the effect of position of the animals may be estimated and removed from the total source of variance, permitting greater precision in the estimation of the effects of the variables under study. Another reason for designing experiments in which positional effects can be estimated is to prevent confounding positional effects with treatment effects. Such confounding may lead the experimenter to conclusions that treatments were significantly different when the differences may have been due to the position of the animals.

SUMMARY

The feeding of excess zinc to young rats induced a microcytic and hypochromic anemia and subnormal growth. The additional feeding of copper maintained the hemoglobin at significantly higher levels and a mixture of iron, copper, and cobalt essentially maintained hemoglobin at normal levels, although iron or cobalt supplements alone had no effect. Iron, copper, and cobalt had no effect on the subnormal growth of zinc-fed rats, but supplements of a liver extract produced a highly significant growth response. The liver activity is apparently not due to pantothenic acid.

Studies such as this point to the possible occurrence of relative deficiencies in addition to absolute deficiencies in the field; that is, animals may be deficient in one mineral relative to an excess of some other.

The authors are grateful to Dr. H. L. Lucas for aid in the statistical analyses.

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Range-Finding Toxicity Data: List VII

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Acute toxicity and irritation data on about 200 compounds are presented, accumulated in a continuing program for screening potential commercial products.

DURING THE SEVEN years since the appearance of our last communication on the range-finding test,[1] many additional data have become available for presentation. Table I lists these data under names conforming to the nomenclature used in *Chemical Abstracts*. Table II lists only the oral LD_{50} values for a number of inorganic compounds. All materials included are either in commercial production or have been evaluated for commercial potential within the past few years.

As has been stated in earlier communications,[1-6] the range-finding test is relied upon only to allow predictions of the comparative hazards of handling new chemicals. Acute toxicity studies, no matter how precisely executed, yield no more than an indication of the degree of care necessary to protect exposed workmen or lead to an opinion that certain technically feasible applications of a chemical may or may not eventually be proved safe.

Our last paper[1] included data under the name of triisooctyl phosphine. The compound should have been entered as triisooctyl phosphite.

Experimental methods have remained unchanged. They are described in our 196[illegible] publication.[1]

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Received April 8, [illegible]

Table 1 Range-Finding Toxicity Data

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corneal Injury in Rabbits
Hydrocarbons								
2-(4-Cyclohexenyl)-bicyclo-(2.2.1)-hept-5-ene	4.29 (3.07 – 5.98)	3.54 (2.19 – 5.72)	8 hr.	–	–	–	5	2
Cyclopentene	2.14 (1.45 – 3.17)	1.59 (0.97 – 2.59)	3 m.	16,000	4	4/6	5	5
5-Ethylidene-2-norbornene	2.83	9.17 (1.35 – 6.33)	–	4,000	4	5/6	5	2
p-Menth-3-ene	20.75 (15.36 –28.03)	5.66 (2.59 –12.3)	30 m.	2,000	4	2/6	5	1
2-Norbornene	11.3 (7.0 – 18.3)*	> 5	5 m.	–	–	–	1	7
1,7-Octadiene	19.7 (15.0 – 25.8)	14.1 (4.66 –42.9)	15 m.	8,000	4	2/6	4	1
5a,4,7,7a-Tetrahydro-1H-indene	3.73 (2.68 – 5.21)	14.1 (8.75 –22.9)	4 hr.	–	–	–	3	2
Toluene	7.53 (6.73 – 8.43)*	14.1 (9.63 –20.77)	–	4,000	4	1/6	4	9
4-Vinyl-1-cyclohexene	3.08 (2.49 – 3.81)	20	15 m.	8,000	4	4/6	4	2
5-Vinyl-2-norbornene	5.19 (4.22 – 6.41)	15.9 (11.7 –21.5)	–	4,000	4	4/6	5	2
Hydroxyl Compounds								
p-tert. Butylphenol	3.25 (2.48 – 4.26)	2.52 (0.93 – 8.58)	8 hr.	–	–	–	6	9
Cyclohexenyl-4-phenol	2.83	3.18 (1.94 – 5.18)*	8 hr.	–	–	–	–	9
2-Cyclopenten-1-ol	0.47 (0.34 – 0.66)	0.18	1 hr.	500†	4	0/6	2	5
3,4-Dihydroxy-1,5-hexadiene	1.62 (1.24 – 2.13)	0.40 (0.29 – 0.54)	–	–	–	–	2	6
Eicosanol, mixed isomers	>64	>20	8 hr.	–	–	–	3	1
2-Ethylhexanol	2.46 (1.82 – 3.33)	2.38 (1.70 – 3.34)	8 hr.	–	–	–	3	5
Hydroxydihydrocyclopentadiene	3.25 (2.11 – 4.99)	3.15 (2.33 – 4.26)	8 hr.	–	–	–	5	9
3-Methyl-1-butanol	7.07 (4.82 – 10.4)	3.97 (2.93 – 5.37)	8 hr.	–	–	–	2	8
Methyl-3-cyclohexenyl-methanol	1.41	0.62 (0.42 – 0.92)	8 hr.	–	–	–	3	8
Nonylphenol, mixed isomers	1.62 (1.06 – 2.50)	2.14 (1.52 – 2.99)	4 hr.	–	–	–	6	10
2-Norbornanemethanol	1.62 (1.24 – 2.13)*	0.80	8 hr.	–	–	–	3	9
Tetradecanol, mixed isomers	32.5 (29.1 – 36.3)	7.13 (4.41 –11.52)	8 hr.	–	–	–	2	1
Ethers								
Bicyclo(2.2.1)hept-2,5-ylene bis allyl ether	3.73 (2.43 – 5.74)	5.95 (3.57 – 9.09)	8 hr.	–	–	–	3	1
2-Cyclopentyl ether	11.3	1.59 (1.03 – 2.59)	4 hr.	–	–	–	5	2
Ethyl propenyl ether	19.0 (13.4 –27.0)	>10	5 m.	8,000	4	0/6	2	2
Ethyl vinyl ether	8.16 (7.03 – 9.48)	>20	–	64,000	4	0/6	1	1
Methoxy triethylene glycol vinyl ether	11.3	10.0 (4.56 –21.9)	8 hr.	–	–	–	2	3
alpha-Methylbenzyl ether	9.80 (6.38 –15.06)*	–	8 hr.	–	–	–	4	[illegible]
2-Methoxypropene	1.87 (1.26 – 2.76)	>20	–	64,000	4	4/6	1	1
1,1,3,3-Tetramethoxyhexane	4.09 (3.52 – 4.76)*	–	8 hr.	–	–	–	2	2
Hydroxy Ethers								
1-Butoxyethoxy-2-propanol	4.00 (2.55 – 6.27)	2.83 (1.75 – 4.57)	8 hr.	–	–	–	3	5
3-Butoxy-1-propanol	5.95 (3.65 – 9.68)	1.59 (1.17 – 2.15)	8 hr.	–	–	–	2	7
Diethoxytetraethylene glycol	4.29 (2.90 – 6.34)	6.35 (3.89 –10.4)	8 hr.	–	–	–	2	[illegible]
3-Ethoxy-1-propanol	13.0 (8.46 –20.0)	2.83	8 hr.	–	–	–	2	5
2-Ethylbutoxypropanol, mixed isomers	3.56 (2.21 – 5.74)	6.0	8 hr.	–	–	–	2	9
2-Hexyloxy-2-ethoxyethyl ether	3.73 (2.68 – 5.21)	6.30 (4.66 – 8.52)	8 hr.	–	–	–	2	5
Isobutoxypropanol, mixed isomers	7.5	3.56 (1.95 – 6.52)	8 hr.	–	–	–	3	[illegible]

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corneal Injury in Rabbits
2-Isopropoxyethanol	5.66 (4.11 – 7.79)	1.60 (1.08 – 2.37)	2 hr.	4,000	4	4/6	3	5
3-Methoxy-1-propanol	13.0 (9.90 –17.0)	3.66	8 hr.	–	–	–	1	5
2-Propoxyethanol	4.89 (4.16 – 5.73)*	0.96 (0.93 – 0.99)	–	–	–	–	2	8
Propoxypropanol, mixed isomers	3.25 (2.48 – 4.26)	4.00 (2.70 – 5.92)	8 hr.	–	–	–	2	7
Aldehydes, Ketones								
Cyclohexanone	1.62 (1.20 – 2.20)	1.00 (0.63 – 1.63)	30 m.	2,000†	4	1/6	1	5
5-Ethyl-3-nonen-2-one	8.12 (7.27 – 9.06)*	8.48 (5.04 –14.24)	8 hr.	–	–	–	2	1
Hexanal, mixed isomers	9.51 (5.84 –15.5)	>10	30 m.	–	–	–	2	5
2-Methylcyclohexanone	2.14 (1.48 – 3.10)	1.77	–	2,800	4	3/6	3	5
2-Methyl-1,2,3,6-tetrahydrobenzaldehyde	5.66	3.15 (2.33 – 4.26)	8 hr.	–	–	–	3	5
b-Tetralone	0.81 (0.62 – 1.07)	>20	8 hr.	–	–	–	4	3
Valeraldehyde	5.66	6	15 m.	4,000	4	3/6	2	5
Acids, Anhydrides, Lactones								
3-Hydroxybutyric acid lactone	17.2 (12.3 –23.9)	>20	8 hr.	–	–	–	5	5
b-Methyl-delta-valerolactone	12.9 (8.4 –19.9)	>10	–	–	–	–	1	4
Methyltetrahydrophthalic anhydride	2.14 (1.48 – 3.10)	1.41	8 hr.	–	–	–	1	9
Tri-isobutenylsuccinic anhydride	0.94 (0.61 – 1.44)*	0.89 (0.55 – 1.44)*	–	–	–	–	4	7
Valeric acid, mixed isomers	2.00 (0.90 – 4.44)	0.31 (0.06 – 1.50)	8 hr.	–	–	–	6	9
Esters, Acetals								
Acetic acid, 2-methyl-2,4-pentanediol diester	3.36 (2.06 – 5.48)	16.0 (5.33 –76.8)	8 hr.	–	–	–	3	2
Acetic acid, 2-methyl-2-propene-1,1-diol diester	0.44 (0.28 – 0.68)	0.044(0.024– 0.082)	1 hr.	62.5	4	5/6	3	9
Acetic acid, phenyl ester	1.63 (1.03 – 2.74)	8.00 (3.65 –17.5)	8 hr.	–	–	–	2	2
Acetic acid, propyl ester	9.8 (7.5 –12.9)	>20	30 m.	8,000	4	4/6	1	2
Acetic acid, triethylene glycol diester	22.6	8.0 (1.91 –33.5)	8 hr.	–	–	–	1	1
Acrylic acid, 2-butoxyethoxy ester	6.50 (4.72 – 8.95)	0.64 (0.39 – 1.04)	8 hr.	–	–	–	3	2
Acrylic acid, decyl ester, mixed isomers	12.3 (9.98 –15.3)	3.54 (1.17 –10.7)	8 hr.	–	–	–	5	1
Acrylic acid, hexyl ester	26.0 (18.9 –35.8)	5.66 (3.50 – 9.14)	4 hr.	–	–	–	5	1
Acrylic acid, 2-hydroxypropyl ester	1.23 (0.89 – 1.70)	0.16 (0.10 – 0.26)	8 hr.	–	–	–	3	7
Acrylic acid, 2-methoxyethoxy ester	0.81 (0.59 – 1.21)	0.25 (0.17 – 0.37)	15 m.	500	4	3/6	4	5
Acrylic acid, 2-norbornyl ester	5.66	0.88 (0.54 – 1.43)	8 hr.	–	–	–	5	2
Azelic acid, dihexyl ester	16.0 (10.2 –25.0)	>20	–	–	–	–	2	1
Benzoic acid, 2-ethylhexanediol diester	29.2 (15.8 –53.9)	20	8 hr.	–	–	–	2	1
Fumaric acid, ethyl 2,3-epoxypropyl ester	1.62 (1.06 – 2.50)	0.35 (0.22 – 0.57)	–	–	–	–	5	8
Fumaric acid, dimethyl ester	2.24 (1.15 – 4.37)*	1.25 (0.84 – 1.85)*	8 hr.	–	–	–	5	9
Malonic acid, diethyl ester	14.9 (10.7 –20.8)	>16	8 hr.	–	–	–	2	5
Methacrylic acid, allyl ester	0.43 (0.31 – 0.60)*	0.50 (0.37 – 0.68)	8 m.	250†	4	0/6	5	2
Methacrylic acid, butyl ester	22.6	11.3 (5.19 –24.7)	8 hr.	–	–	–	5	1
Phthalic acid, butyl decyl ester	20.8 (16.8 –25.7)	15.9 (11.7 –21.5)	8 hr.	–	–	–	2	1
Phthalic acid, decyl hexyl ester	49.4 (39.9 –61.0)	>20	8 hr.	–	–	–	2	1

(Continued on Next Page)

Material Studied	Single Oral LD50 for Rats ml/Kg	Single Skin Penetration LD50 for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corne Injur in Rabbi
Phthalic acid, decyl octyl ester	45.2 (28.0 -73.2)	>20	8 hr.	–	–	–	2	1
Propionaldehyde dicrotyl acetal	2.14 (1.54 - 2.99)	3.54 (2.19 - 5.72)	30 m.	2,000	4	5/6	4	2
Succinic acid, di-2-[(2-ethylbutoxy)ethoxy] ester	3.3 *	7.11 (6.58 - 7.68)	–	–	–	–	3	2
Epoxy Compounds								
Bicyclopentadiene dioxide	2.14 (1.54 - 2.99)	1.77	8 hr.	–	–	–	3	5
Bis(3,4-epoxybutyl)ether	1.07 (0.77 - 1.50)	0.25 (0.17 - 0.37)	8 hr.	–	–	–	5	7
Bis(3,4-epoxy cyclohexylmethyl)adipate	4.39 (3.07 - 5.98)	>20	8 hr.	–	–	–	2	1
N,N-Bis(2,3-epoxypropyl)aniline	1.62 (1.18 - 2.24)	3.56 (1.68 - 7.57)	8 hr.	–	–	–	3	1
Butyl 2,3-epoxypropyl fumarate	0.71 (0.48 - 1.04)	1.26 (0.93 - 1.70)	8 hr.	–	–	–	5	8
4-Butylphenyl 2,3-epoxypropyl ether	3.73 (2.68 - 5.21)	5.66	8 hr.	–	–	–	5	2
1,2-Epoxy cyclohexane	1.09 (0.66 - 1.80)	0.63 (0.39 - 1.03)	30 m	2,000†	4	1/6	3	8
2,3-Epoxypropyl methacrylate	0.77 (0.57 - 1.04)	0.45 (0.21 - 0.95)	2 hr.	–	–	–	5	4
Propylene oxide	1.14 (1.05 - 1.23)	1.50 (1.07 - 21.0)	5 m. §	4,000	4	4/6	1	5
Heterocyclic Oxygen								
3-Aminomethyl-2,3-dihydro-4H-pyran	1.00 (0.64 - 1.57)*	0.18	4 hr.	–	–	–	8	9
2,5-Divinyltetrahydropyran	2.46 (1.79 - 3.39)	1.41 (0.65 - 3.08)	4 hr.	–	–	–	4	8
1,9-Divinyl-2,4,8,10-tetraoxaspiro (5,5) undecane	3.25 (2.48 - 4.26)*	7.92 (5.87 -10.73)*	8 hr.	–	–	–	2	2
6-Ethoxy-4-methyl-tetrahydropyran	5.16 (4.17 - 6.38)	> 5	4 hr.	–	–	–	2	9
Aliphatic and Alicyclic Amino Compounds								
1-Amino-3-aminomethyl-3,5,5-trimethyl cyclohexanol	1.30 (1.04 - 1.75)	–	8 hr.	–	–	–	7	9
2-Aminomethyltetrahydropyran	0.71 (0.44 - 1.13)	0.71 (0.44 - 1.14)	4 hr.	–	–	–	6	9
3-Aminopropoxy-2-ethoxy ethanol	6.50 (4.95 - 8.53)*	5.99 (4.27 - 8.42)	8 hr.	–	–	–	3	7
Amyl amine, mixed isomers	0.47 (0.33 - 0.65)*	1.12 (0.61 - 2.05)	30 m. §	2,000	4	4/6	6	9
Bis(2-dimethylaminoethoxy)ethane	2.83	1.2	–	–	–	–	7	8
Bis(2-dimethylaminoethyl) ether	1.23 (0.94 - 1.62)	0.28	8 hr.	–	–	–	6	9
Cyclohexylamine	0.71 (0.51 - 0.97)	0.32 (0.24 - 0.43)	2 hr.	4,000†	4	0/6	7	10
1,8-Diamino-p-menthane	0.77 (0.62 - 0.95)	0.63 (0.47 - 0.85)	4 hr.	–	–	–	7	9
2,4-Diamino-1-methylcyclohexane	1.41	0.50 (0.31 - 0.82)	8 hr.	–	–	–	6	9
Di(3-aminopropyl) ether of diethylene glycol	4.29 (3.07 - 5.98)	2.50 (1.14 - 5.48)	4 hr.	–	–	–	6	8
Di(3-aminopropoxy)ethane	2.83 (1.76 - 4.55)	1.25 (0.84 - 1.85)	8 hr.	–	–	–	6	8
N,N-Di(methyl-N-cyclohexylmethyl)amine	1.23 (0.94 - 1.62)*	0.21 (0.15 - 0.30)	8 hr.	–	–	–	7	9
N-(Methyl-N-cyclohexylmethyl)amine	1.41 *	0.63 (0.47 - 0.85)	4 hr.	–	–	–	6	9
N,N,N',N',N"-Pentamethyldiethylenetriamine	1.63 (1.24 - 2.13)	0.28	8 hr.	–	–	–	7	9
N,N,N',N'-Tetramethyl-1,3-butanediamine	0.93 (0.67 - 1.30)	0.40 (0.29 - 0.54)	8 hr.	–	–	–	6	9
N,N,N',N'-Tetramethyl-1,3-propanediamine	0.41 (0.31 - 0.53)	0.30 (0.21 - 0.42)	15 m.	1,000	4	2/6	5	9
Tetramethyl-2-tetrazene	1.78 (1.12 - 2.83)	—	4 hr.	–	–	–	2	4
Tripropylamine	0.096(0.071- 0.13)*	0.57 (0.26 - 1.23)	8 hr.	250	4	3/6	4	1
Aromatic Nitro and Amino Compounds								
3-Aminopyrene	1.07 (0.77 - 1.50)*	> 1	8 hr.	–	–	–	–	8
N-Methyl-1-naphthylamine	1.41 (1.03 - 1.95)		8 hr.	–	–	–	2	1

Material Studied	Single Oral LD50 for Rats ml/Kg	Single Skin Penetration LD50 for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corne Injur in Rabbi
Amides								
N(2-Acetoxyethyl)-N-ethylacetamide	20.8 (16.8 -25.7)	>10	8 hr.	–	–	–	2	4
ε-Caprolactam	2.14 (1.54 - 2.99)*	1.41	4 hr.	–	–	–	–	7
N-Ethyl-N-vinylacetamide	2.46 (1.88 - 3.23)	1.25 (0.84 - 1.85)	4 hr.	2,000	4	3/6	2	5
Propyl-N,N-diethylsuccinamate	12.93 (8.42 -19.87)	>20	8 hr.	–	–	–	4	5
Heterocyclic Compounds								
3,5-Dimethyl-1-(trichloromethylmercapto)-pyrazole	0.57 *	0.20 (0.15 - 0.27)*	4 hr.	–	–	–	6	10
Isopropylmorpholine	0.71	0.10 (0.07 - 0.15)	15 m.	500	4	5/6	6	7
Isopropylpiperazine	2.83	0.64 (0.47 - 0.86)	8 hr.	–	–	–	6	9
3,4-Lutidine	0.71 (0.40 - 1.24)	0.14	1 hr.	250†	4	0/6	6	8
N-Methyl-ε-caprolactam	1.62 (1.18 - 2.24)	1.41	8 hr.	–	–	–	3	5
3-Methyl-2-oxazolidone	7.13 (3.52 -15.30)	10.8 (6.17 -16.5)	8 hr.	–	–	–	1	4
2-Methyl-5-vinylpyridine	1.30 (0.71 - 2.37)	0.80 (0.54 - 1.18)	4 hr.	4,000	8	4/6	5	8
1-(Trichloromethyl mercapto) pyrazole	0.35 *	0.063(0.047- 0.085)	–	200	1	6/6	7	9
1-(Trichloromethyl-mercapto)-4-methylpyrazole	1.04 (0.81 - 1.28)	0.079(0.059- 0.107)	2 m. §	–	–	–	7	8
Cyano Compounds								
Bis(2-isocyanatoethyl)carbonate	1.83 (1.21 - 2.79)	>12	8 hr.	–	–	–	3	9
Bis(2-isocyanatoethyl)-4-cyclohexene-1,2-dicarboxylate	10.4 (5.55 -19.4)	> 8	8 hr.	–	–	–	5	5
Bis(2-isocyanatoethyl)-5-norbornene-2,3-dicarboxylate	22.6 (13.4 -38.2)	16.1 (4.5 -57.2)	8 hr.	–	–	–	5	5
3-Butenenitrile	0.115(0.097- 0.138)*	1.41	5 m.	250†	4	0/6	2	3
N,N-Dibutyl-3-aminopropionitrile	3.25 (2.48 - 4.26)	5.04 (3.73 - 6.82)	8 hr.	–	–	–	2	2
1,6-Hexamethylene diisocyanate	0.71	0.57 (0.35 - 0.91)	8 hr.	–	–	–	8	9
Lactonitrile	0.087(0.060- 0.125)*	0.029(0.015- 0.027)	–	62.5†	4	0/6	2	–‡
3-Methoxypropionitrile	4.39 (3.07 - 5.98)*	>10	8 hr.	–	–	–	2	5
Methyl isocyanate	0.071(0.027- 0.18)*	0.22 (0.12 - 0.41)	–	31.25†	4	0/6	6	10
Tridecanitrile, mixed isomers	3.73 (2.68 - 5.21)	3.18 (1.43 - 7.06)	8 hr.	–	–	–	4	5
Halogen Compounds								
1-Bromooctane	4.49 (2.09 - 9.64)	8.00 (4.93 -13.0)	–	–	–	–	5	1
2-Chlorobutane	20.0 (12.8 -31.3)	20	5 m. §	8,000	4	3/6	1	2
Chloromethyl ether	0.21 (0.12 - 0.37)	0.28 (0.13 - 0.62)	–	8†	4	1/6	6	10
Dibromobicycloheptane, mixed isomers	0.21 (0.15 - 0.30)*	0.25 (0.19 - 0.34)	1 hr.	–	–	–	6	–
Dichlorobenzyl alcohol, mixed isomers	0.81 (0.50 - 1.31)	0.40 (0.24 - 0.65)	8 hr.	–	–	–	5	9
1,2-Dichloropropane(propylene dichloride)	1.9 (1.7 - 2.1)	8.75 (8.31 - 9.20)	10 m.	2,000	8	3/6	1	2
trans-2,3-Dichloro-1,4-dioxane	1.41	0.44 (0.27 - 0.71)	4 hr.	–	–	–	5	7
1,2-Dichloroethane(ethylene dichloride)	0.77 (0.67 - 0.89)*	3.89 (3.40 - 4.46)	–	–	–	–	2	3
Di(2-chloroethyl)acetal	0.31 (0.24 - 0.40)*	0.20 (0.14 - 0.30)	1 hr.	–	–	–	3	2
Fumaroyl chloride	0.81 (0.51 - 1.30)	1.41	–	500	4	3/6	6	10
1,1,1,3,3,3-Hexachloro-2,2-difluoropropane	0.54 (0.30 - 1.01)	4.33 (2.80 - 7.32)	–	–	–	–	3	2
2-Hydroxyethanesulfonyl chloride	2.46 (1.79 - 3.39)	0.79 (0.49 - 1.30)	8 hr.	–	–	–	3	9

(Continued on Next Page)

Range-Finding Toxicity Data (Continued)

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Cornea Injury in Rabbits
Phenyl chloroformate	1.41 (0.83 - 2.40)	3.97 (2.93 - 5.37)	15 m.	44	4	3/6	6	10
Propylene chlorohydrin	0.22 (0.19 - 0.27)	0.48 (0.34 - 0.67)	15 m.	500	4	1/6	1	8
Tetrachloroethane	0.20 (0.16 - 0.27)	3.99 (3.10 - 5.13)	-	1,000	4	3/6	6	-
1,1,2,3-Tetrachloropropene	0.35 *	0.40 (0.27 - 0.59)	30 m.	250	4	5/6	5	9
1,1,2-Trichloroethane	0.58 (0.47 - 0.71)	3.73 (3.30 - 4.21)	-	500	8	4/6	2	2
Trichloroethylene	4.92 (3.75 - 6.46)	>20	-	8,000	4	4/6	5	4
Phosphorous Compounds								
Bis(2-chloroethyl) phosphite	0.26 (0.19 - 0.35)*	0.141 (0.065- 0.308)	8 hr.	-	-	-	2	3
Diethyl phosphite	5.19 (2.81 - 9.58)	2.02 (1.24 - 3.29)	4 hr.	-	-	-	1	5
Trimethyl phosphate	2.83 (1.75 - 4.57)	2.83	8 hr.	-	-	-	4	2
Tris(dipropylene glycol) phosphine	19.7 (15.0 -25.8)	>10	8 hr.	-	-	-	2	1
Tris(dipropylene glycol) phosphonate	29.9 (21.4 -41.6)	15.9 (11.7 -21.5)	8 hr.	-	-	-	3	1
Sulphur Compounds								
Bis(2-acetoxyethyl) sulfone	14.1	11.3	8 hr.	-	-	-	4	2
Bis(1,2-dichloroethyl) sulfone	0.25 (0.19 - 0.32)*	1.00 (0.40 - 2.51)	8 hr.	-	-	-	7	9
2,3-Epithiopropyl methoxy ether	2.14 (1.54 - 2.99)	> 5	1 hr.	-	-	-	3	2
n-Hexyl vinyl sulfone	0.57	0.84 (0.50 - 1.40)	-	-	-	-	7	5
Isopropyl sulfate	1.09 (0.64 - 1.87)	1.41 (0.88 - 2.29)	1 hr.	-	-	-	6	5
2(Methoxyethylthio) ethanol	13.00 (7.70 -21.93)	5.04 (3.09 - 8.23)	8 hr.	-	-	-	2	5
Methylvinyloxyethyl sulfide	3.25 (2.48 - 4.26)	> 8	-	3,200	8	4/6	2	2
Tetrahydrothiophene-1,1-dioxide (sulfolane)	1.54 (1.25 - 1.91)	3.18 (2.35 - 4.29)	8 hr.	-	-	-	2	4
2,2'-Thiodiethanol diacetate	9.25 (7.51 -12.9)	>16	8 hr.	-	-	-	3	1
Trichloromethyl allyl perthioxanthate	2.18 (1.39 - 3.42)*	0.6	-	-	-	-	6	1
Trichloromethyl methyl perthioxanthate	1.54 (0.89 - 2.68)*	1.3	-	-	-	-	6	2
Silicon Compounds								
Amyl trimethoxy silane	4.92 (3.75 - 6.46)	10.00 (4.56 -21.9)	4 hr.	-	-	-	4	1
2(3,4-Epoxycyclohexyl) ethyl trimethoxy silane	12.3 (9.98 -15.3)	6.30 (4.66 - 8.52)	8 hr.	-	-	-	3	1
3(2,3-Epoxypropoxy) propyl trimethoxy silane	22.6	3.97 (2.93 - 5.37)	8 hr.	-	-	-	3	2
2-Ethylbutyl silicate	19.7 (15.0 -25.8)	>10	8 hr.	-	-	-	3	1
2-Mercaptoethyl trimethoxy silane	2.46 (1.88 - 3.23)	-	2 hr.	-	-	-	3	2
3-Mercaptopropyl trimethoxy silane	2.83 (1.61 - 4.98)	5.66	-	-	-	-	3	2
Methyl trimethoxy silane	12.5 (9.98 -15.3)	>10	1 hr.	-	-	-	3	2
Trimethoxy silane	9.33 (6.69 -13.0)	6.30 (4.66 - 8.52)	15 m.	62.5	4	5/6	3	5
Vinyl trimethoxy silane	11.3	3.54 (2.03 - 6.16)	2 hr.	4,000	4	1/6	2	1

* as gm/kg in a suitable vehicle

§ inhalation time shown killed all six rats

† twice concentration shown killed all six rats

‡ caused death when introduced into the rabbit eye

Table II

Acute Oral Toxicity of Some Inorganic Compounds for Rats

Material*		Formula	Single Oral LD_{50} for Rats gm/Kg	Concentration Intubated gm/ml**	
Aluminum isopropylate	T	$Al[OCH(CH_3)CH_3]_3$	11.3 (7.0 -18.3)	0.200	A
Aluminum nitrate		$Al(NO_3)_3 \cdot 9H_2O$	4.28 (3.86 - 4.76)	0.100	
Ammonium metavanadate	T	NH_4VO_3	0.16 (0.12 - 0.21)	0.0025	B
Ammonium paratungstate	T	$(NH_4O)_6W_7O_{24} \cdot 6H_2O$	11.3	0.400	A
Ammonium persulfate		$(NH_4)_2S_2O_8$	0.82 (0.53 - 1.26)	0.200	
Borax		$Na_2B_4O_7 \cdot 10H_2O$	5.66	0.010	
Boric acid		H_3BO_3	5.14 (4.75 - 5.58)	0.200	
Calcium acetate		$Ca(OOCCH_3)_2 \cdot H_2O$	4.28 (3.86 - 4.76)	0.100	
Calcium acrylate	T	$Ca(OOCCH{=}CH_2)_2 \cdot H_2O$	4.92 (3.75 - 6.46)	0.200	A
Calcium cyanide		$Ca(CN)_2$	0.039(0.030- 0.051)	0.001	
Calcium hydroxide		$Ca(OH)_2$	7.34 (4.83 -11.14)	0.100	
Calcium propionate	T	$Ca(OOCCH_2CH_3)_2 \cdot H_2O$	5.16 (4.17 - 6.38)	0.100	
Chromium acetate	T	$Cr(OOCCH_3)_2 \cdot H_2O$	11.26	0.200	
Chromium chloride (ous)	T	$CrCl_2$	1.87 (1.21 - 2.87)	0.050	
Chromium nitrate (ic)		$Cr(NO_3)_3 \cdot 9H_2O$	3.25 (2.11 - 4.99)	0.200	
Cobalt oxide	T	CoO	1.70 (1.07 - 2.82)	0.100	A
Copper acetate		$Cu(OOCCH_3)_2 \cdot H_2O$	0.71 (0.60 - 0.83)	0.010	
Copper nitrate		$Cu(NO_3)_2 \cdot 3H_2O$	0.94 (0.61 - 1.43)	0.200	
Copper sulfate		$CuSO_4 \cdot 5H_2O$	0.96 (0.71 - 1.30)	0.050	
Cuprous oxide	T	Cu_2O	0.47 (0.34 - 0.66)	0.100	A
Diammonium decaborane	T	$(NH_4)_2B_{10}H_{10}$	3.56 (1.85 - 6.86)	0.050	
Dicumene chromium	T	$(C_9H_{12})_2Cr$	0.81 (0.62 - 1.07)	0.100	M
Ferric nitrate		$Fe(NO_3)_3 \cdot 9H_2O$	3.25 (2.56 - 4.26)	0.200	
Ferrous ammonium sulfate		$(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$	3.25 (2.48 - 4.26)	0.300	
Lithium carbonate		Li_2CO_3	0.71	0.200	
Magnesium chloride		$MgCl_2 \cdot 6H_2O$	8.1 (7.3 - 9.1)	0.100	
Manganous acetate		$Mn(OOCCH_3)_2 \cdot 4H_2O$	3.73 (2.68 - 5.21)	0.200	
Nickel nitrate		$Ni(NO_3)_2 \cdot 6H_2O$	1.62 (1.06 - 2.50)	0.200	
Potassium acetate		$KOOCCH_3$	3.25 (2.48 - 4.26)	0.100	
Potassium carbonate		K_2CO_3	1.87 (1.34 - 2.60)	0.200	
Potassium hydroxide		KOH	1.23 (0.80 - 1.89)	0.100	
Potassium permanganate		$KMnO_4$	1.09 (0.70 - 1.71)	0.020	
Silver oxide	T	Ag_2O	2.82	0.200	A
Sodium arsenite		$NaAsO_2$	0.041(0.031- 0.053)	0.010	
Sodium cyanide		$NaCN$	0.015(0.011- 0.021)	0.0004	
Sodium fluoride		NaF	0.18 (0.12 - 0.26)	0.005	
Sodium nitrite		$NaNO_2$	0.18	0.010	
Sodium phosphate, diabasic		$Na_2HPO_4 \cdot 7H_2O$	12.93 (9.85 -16.97)	0.200	
Sodium phosphate, tribasic		$Na_3PO_4 \cdot 12H_2O$	7.40 (6.81 - 8.03)	0.200	
Sodium tripolyphosphate	T	$Na_5P_3O_{10}$	6.50 (4.03 -10.48)	0.200	
Sulfuric acid		H_2SO_4	2.14 (1.54 - 2.99)	0.250	
Vanadium dichloride	T	VCl_2	0.54 (0.38 - 0.75)	0.010	A
Vanadium oxytrichloride	T	$VOCl_3$	0.14 (0.09 - 0.22)	0.050	M
Vanadium tetrachloride	T	VCl_4	0.16	0.090	M
Vanadium trichloride	T	VCl_3	0.35 (0.22 - 0.57)	0.100	
Zinc acetate		$Zn(OOCCH_3)_2 \cdot 2H_2O$	2.46 (1.60 - 3.78)	0.200	
Zinc naphthenate	T		4.92 (3.75 - 6.46)	0.200	L

* T Preceding formula designates technical grade, or development sample, otherwise reagent grade

** Intubation concentrations are in water except as noted by following symbols:

M In mineral oil

L In lard

A Suspended in 0.25% agar

B Suspended in 5% bentonite

Effect of Zinc Toxicity on Calcium, Phosphorus and Magnesium Metabolism of Young Rats[1,2]

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ABSTRACT The effects of high levels of dietary zinc on bone mineralization, on growth and on the metabolism of calcium, phosphorus and magnesium of young male rats in the presence and absence of several dietary supplements were studied in a series of experiments. Zinc toxicity resulted in marked decreases in bone calcium and phosphorus levels and marked increases in bone zinc levels at the end of the first week animals were on experiment. Significant decreases in weight gain associated with zinc toxicity did not occur until the second week. Bone magnesium levels were not affected by zinc toxicity. Supplements of calcium and phosphorus alleviated the adverse effects of zinc on weight gain and on the deposition of calcium and phosphorus in the bone. Calcium and phosphorus supplements prevented the marked accumulation of zinc in the bones of young rats fed high levels of zinc. Zinc toxicity resulted in highly significant decreases in the retentions of calcium, phosphorus and magnesium by young rats which occurred at the end of the first week the animals were on experiment.

Although Sadasivan (1) reported more than a decade ago that high levels of dietary zinc (0.5 and 1.0%) prevented the normal deposition of calcium and phosphorus in the bones of young rats, the exact nature of this interference of zinc with bone mineralization has not been determined. Other reports indicate that high levels of dietary zinc are associated with decreases in the retention of phosphorus in rats (2, 3) and decreases in the retentions of both calcium and phosphorus in lambs (4). Whiting and Bezeau (5), however, reported that dietary zinc increased the retention of calcium in pigs, but had no influence on phosphorus retention.

Abelson and Aldous (6) reported that excessive zinc interferred with the metabolism of magnesium in several species of bacteria. Adiga et al. (7) reported that zinc toxicity caused a conditioned magnesium deficiency in *Aspergillus niger* which could be alleviated by supplementing the culture media with magnesium. Recently, Sastry et al. (8) have found that the toxicity of several metals, including zinc, on the growth of *Neurospora crassa* can be alleviated by magnesium supplementation.

The specific objectives of the present study were to determine the effect of zinc toxicity on calcium, phosphorus and magnesium levels of the bones of young rats in the presence and absence of dietary supplements of calcium, phosphorus and magnesium and to investigate the effect of a high level of zinc on the absorption, utilization and retention of calcium, phosphorus and magnesium. Changes in weight gains and bone zinc levels of the zinc-fed rats under the various dietary conditions were observed also.

MATERIALS AND METHODS

Weanling male Sprague-Dawley rats were used in all experiments. The animals were housed in individual wire-bottom cages in an air conditioned room maintained at 24.5°C. Food and water were offered ad libitum. All animals used in a particular experiment were randomized into replications on the basis of initial body weight.

The composition of the basal diet was: (in per cent) casein, 19;[4] starch, 63;[5] vege-

Received for publication May 31, 1963.

[1] Contribution from the Home Economics Department, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as Paper no. 1631 of the Journal Series. The data were taken from a thesis submitted by the senior author in partial fulfillment of the requirement for the Master of Science degree.

[2] Supported in part with a grant from The University of North Carolina at Greensboro Research Council.

[3] Present address: 211 Towne House Apartments, Chapel Hill, North Carolina.

[4] Vitamin Test Casein, Nutritional Biochemicals Corporation, Cleveland.

[5] Globe Easy-flow Corn Starch 3366, Corn Products Sales Company, Greensboro, North Carolina.

table oil, 10;[6] mineral mix, 4;[7] vitamin mix, 2;[8] cellulose, 2;[9] and oleum percomorphum.[10] Chemical analyses of samples of the basal diet taken periodically throughout the study revealed that it contained an average of 0.52% of calcium, 0.55% of phosphorus, 0.04% of magnesium and 10 ppm of zinc. Supplements were incorporated into the basal diet at the expense of equal amounts of starch. Zinc was fed as the carbonate; supplements of calcium, phosphorus and magnesium were in the carbonate, potassium dihydrogen phosphate and sulfate forms, respectively.

Bone mineralization and growth experiments

With the exception of experiment 2, rats from randomly selected replications were killed at the end of each experiment, and the femurs of each animal were removed. The bones were cleaned of muscle and fat, dried in an oven and weighed on an analytical balance. The bone samples were wet-ashed with nitric and perchloric acids on a hot plate. The ash of each pair of bones was dissolved in 3 ml of 0.6 N HCl and brought to a volume of 100 ml with distilled water. Five milliliters of the first dilution were rediluted to 100 ml, and appropriate aliquots of the second dilution were taken for the calcium and phosphorus determinations. Appropriate aliquots of the first dilution of each bone sample were used for the magnesium and zinc determinations.

Calcium, phosphorus, magnesium and zinc determinations were made on the bone samples by the methods of Weybrew et al. (9), Simonsen et al. (10, 11) and McCall et al. (12), respectively.

Experiments 1 and 2. The objective of these experiments was to characterize the effect of zinc on bone mineralization with respect to the dietary level of zinc required to produce a change in mineral levels and to the time of onset of the symptoms. Levels of zinc tested in these experiments were 0.0, 0.25, 0.5 and 0.75%. In experiment 1 six animals were maintained with each of the 4 dietary regimens for 4 weeks. Forty-eight animals were divided equally into 4 groups in experiment 2. Each group received one of the dietary regimens previously mentioned. At weekly intervals 4 animals from each group were killed, and the femurs of each animal were removed for subsequent analyses.

Experiments 3 and 4. The primary purpose of these experiments was to determine the effects of calcium, phosphorus and magnesium supplements on bone mineralization of zinc-fed rats. In experiment 3 supplements of 0.4% of calcium or phosphorus and 0.08% of magnesium were added to diets containing 0.75% of zinc (table 1). Supplements of 0.4, 0.8 and 1.2% of calcium or phosphorus were added to diets containing 0.75% of zinc in experiment 4 (table 2).

Mineral balance study

Eight animals, averaging 53 g in weight initially, were divided into 2 groups. One group received the basal diet; the other group received the basal diet containing 0.75% of zinc. The basal diet used in the balance study, by chemical analyses, contained 0.45% of calcium, 0.59% of phosphorus and 0.03% of magnesium; and the diet with added zinc contained 0.46% of calcium, 0.58% of phosphorus and 0.03% of magnesium. Each animal was placed in a wire-bottom metabolism cage which was fixed in such a manner so that feces and urine could be collected separately. Fresh feed was provided daily, and distilled water was available to the animals at all times.

All animals received the basal diet during a 4-day preliminary period prior to the experimental period. Four consecutive 7-day collection periods were used during the experimental period. During a collection period the respective excreta from an individual animal were collected daily,

[6] Crisco, Procter and Gamble Company, Cincinnati.

[7] Salt Mixture W, Nutritional Biochemicals Corporation, Cleveland. The composition of this salt mixture is listed as: (in per cent) $CaCO_3$, 21.000; $CuSO_4 \cdot 5H_2O$, 0.039; $FePO_4 \cdot 2H_2O$, 1.470; $MnSO_4$, 0.020; $MgSO_4$, 9.000; $KAl(SO_4)_2 \cdot 12H_2O$, 0.009; KCl, 12.000; KH_2PO_4, 31.000; KI, 0.003; NaCl, 10.500; NaF, 0.057 and $Ca_3(PO_4)_2$, 14.900.

[8] Each 100 g of vitamin mix contained: (in milligrams) 0.1% vitamin B_{12} (with mannitol), 0.1; biotin, 1; folic acid, 5; thiamine·HCl, 25; pyridoxine·HCl, 23; 2-methyl-naphthoquinone, 50; riboflavin, 50; nicotinic acid, 50; Ca pantothenate, 150; p-aminobenzoic acid, 500; (in grams) inositol, 5; choline chloride, 7.5; DL-methionine, 30; cornstarch, 56.6. All vitamins and methionine were purchased from Nutritional Biochemicals Corporation, Cleveland.

[9] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[10] Each kilogram of diet contained 24 drops of oleum percomorphum, Mead Johnson and Company, Evansville, Indiana.

TABLE 1

Responses of zinc-fed rats [1] to supplements of calcium, phosphorus and magnesium [2]

Diet	Wt gain at 4 weeks [3]	Bone constituent [4]			
		Ca	P	Mg	Zn
	g	mg/g dry weight			
Basal	182±8	162.7 ± 8.7	87.0±4.0	2.98±0.13	0.11±0.01
+0.75% Zn	97±8	110.9 ± 5.2	77.0±6.4	2.99±0.13	2.69±0.26
+0.75% Zn+0.4% Ca	126±9	133.9 ± 8.4	80.9±1.4	2.92±0.07	1.72±0.29
+0.75% Zn+0.4% P	106±9	109.4 ± 8.4	74.8±3.3	2.77±0.09	1.95±0.24
+0.75% Zn+0.08% Mg	113±7	100.2 ± 6.1	73.3±0.6	3.20±0.18	2.29±0.27
+0.75% Zn+0.4% Ca +0.4% P	154±5	158.2 ± 8.2	87.5±4.7	3.00±0.14	1.06±0.04
+0.75% Zn+0.4% Ca +0.08% Mg	129±7	145.3 ±14.8	85.0±2.4	3.48±0.09	1.43±0.12
+0.75% Zn+0.4% P +0.08% Mg	128±13	117.2 ± 8.0	85.6±5.7	3.11±0.24	1.54±0.16
+0.75% Zn+0.4% Ca +0.4% P+0.08% Mg	139±11	125.9 ± 4.8	83.0±6.0	3.18±0.15	0.84±0.10

[1] Sprague-Dawley rats averaging 49 g in weight initially.
[2] Each figure represents mean ± se.
[3] Each figure is the mean of 6 animals.
[4] Each figure is the mean of 4 animals.

TABLE 2

Effects of calcium and phosphorus supplements on rats [1] fed high levels of zinc [2]

Diet	Wt gain at 4 weeks	Bone constituent			
		Ca	P	Mg	Zn
	g	mg/g dry weight			
Basal	148±5	163.4 ±4.8	83.4±1.8	2.41±0.25	0.10±0.01
+0.75% Zn	104±9	116.4 ±5.9	73.2±1.4	2.32±0.20	1.29±0.16
+0.75% Zn+0.4% Ca	124±4	132.3 ±5.4	79.0±2.9	2.18±0.20	1.03±0.20
+0.75% Zn+0.4% P	113±3	118.3 ±4.4	73.5±1.5	2.28±0.09	0.99±0.15
+0.75% Zn+0.8% Ca	131±2	149.1 ±3.9	79.9±1.1	2.18±0.18	0.99±0.09
+0.75% Zn+0.8% P	116±5	124.6 ±3.2	69.9±3.5	2.27±0.10	0.68±0.10
+0.75% Zn+1.2% Ca	120±4	146.7 ±3.0	78.3±1.3	2.27±0.19	1.13±0.17
+0.75% Zn+1.2% P	119±3	126.0 ±4.5	72.6±1.9	1.91±0.11	0.40±0.05
+0.75% Zn+0.4% Ca +0.4% P	131±3	146.0 ±6.6	74.9±1.7	2.42±0.18	0.76±0.15
+0.75% Zn+0.8% Ca +0.8% P	132±6	146.6 ±6.6	80.4±0.9	1.79±0.19	0.28±0.06 [3]
+0.75% Zn+1.2% Ca +1.2% P	115±4	156.7 ±3.8	80.5±1.9	1.41±0.20 [3]	0.26±0.06 [3]

[1] Sprague-Dawley rats averaging 47 g in weight initially.
[2] Each figure represents mean of 6 animals with se unless otherwise indicated.
[3] Mean of 5 animals

pooled and kept in sealed containers in a refrigerator. Samples of the pooled fecal and urine collections from each animal for each 7-day period were prepared for mineral analyses by the wet-ashing procedure previously mentioned. The ash of each sample was dissolved in 3 ml of 6 N HCl and brought to a volume of 100 ml with distilled water. Representative samples of the 2 diets were prepared for mineral analyses by the same procedures. Calcium, phosphorus and magnesium determinations were made on appropriate aliquots of these samples by the methods previously mentioned.

Statistical analyses

All data were subjected to an analysis of variance. Statements of significance are based on odds of at least 19 to 1 ($P \leq 0.05$).

RESULTS

Bone mineralization and growth experiments

Experiment 1. The results of this experiment are shown in table 3. Increasing levels of dietary zinc resulted in significant linear decreases ($P \leq 0.05$) in bone calcium and phosphorus deposition, highly significant linear increases ($P \leq 0.01$) in bone zinc levels and highly significant decreases ($P \leq 0.01$) in weight gains. Dietary zinc, at the levels used in this experiment, had no apparent effect on the deposition of magnesium in the bones of young rats.

Experiment 2. Results of this experiment (table 4) indicated that high levels of dietary zinc resulted in marked and highly significant changes ($P \leq 0.01$) in

TABLE 3

Effect of zinc on growth and bone mineralization of young rats [1,2]

Level of dietary zinc	Wt gain at 4 weeks	Bone constituent			
		Ca	P	Mg	Zn
%	g	mg/g dry weight			
None	188 ± 8	146.3 ± 8.2	71.6 ± 2.4	3.45 ± 0.05	0.09 ± 0.01
0.25	197 ± 4	143.8 ± 5.5	69.5 ± 1.5	3.37 ± 0.09	0.58 ± 0.04
0.50	161 ± 6	131.4 ± 10.1	65.5 ± 0.6	3.44 ± 0.08	1.34 ± 0.14
0.75	113 ± 9	107.4 ± 8.8	61.3 ± 2.8	3.44 ± 0.13	2.34 ± 0.15

[1] Sprague-Dawley rats averaging 53 g in weight initially.
[2] Each figure represents mean of 6 animals with SE.

TABLE 4

Mean weight gain and bone mineral values of young rats [1] *fed diets containing high levels of zinc* [2]

Weeks on experiment	Level of zinc	Wt gain [3]	Bone constituent [4]			
			Ca	P	Mg	Zn
	%	g	mg/g dry weight			
1	None	39 ± 1	140.8 ± 4.3	91.5 ± 1.3	2.57 ± 0.14	0.13 ± 0.02
	0.25	33 ± 1	131.8 ± 2.4	87.1 ± 1.5	2.72 ± 0.09	0.40 ± 0.04
	0.50	31 ± 1	113.2 ± 3.6	85.0 ± 3.7	2.56 ± 0.10	0.61 ± 0.09
	0.75	27 ± 1	116.1 ± 4.5	84.4 ± 2.2	2.50 ± 0.08	1.01 ± 0.15
2	None	87 ± 2	150.6 ± 1.4	92.5 ± 0.6	2.59 ± 0.15	0.40 ± 0.00
	0.25	76 ± 4	143.4 ± 5.2	89.8 ± 1.3	2.54 ± 0.17	0.54 ± 0.05
	0.50	67 ± 2	122.5 ± 8.3	84.4 ± 1.7	3.21 ± 0.35	0.90 ± 0.10
	0.75	61 ± 2	114.2 ± 3.4	81.4 ± 1.9	2.54 ± 0.02	1.46 ± 0.12
3	None	134 ± 3	156.0 ± 5.6	93.7 ± 0.9	2.81 ± 0.17	0.13 ± 0.01
	0.25	121 ± 4	144.6 ± 5.8	89.4 ± 0.8	2.75 ± 0.05	0.77 ± 0.09
	0.50	107 ± 2	142.3 ± 7.3	88.7 ± 1.2	2.71 ± 0.07	1.77 ± 0.06
	0.75	88 ± 4	125.6 ± 7.0	84.4 ± 1.0	2.83 ± 0.08	2.39 ± 0.12

[1] Sprague-Dawley rats averaging 49 g in weight initially.
[2] Each figure represents mean ± SE.
[3] Mean of 16, 12 and 8 animals for weeks 1, 2 and 3, respectively.
[4] Each figure is the mean of 4 animals.

the levels of calcium and zinc and a significant change ($P \leq 0.05$) in the level of phosphorus in the bones at the end of the first week. Bone magnesium values varied from week to week and from diet to diet, but these differences were not found to be statistically significant. Analysis of the data indicated that weight gains of animals fed 0.75% of zinc were significantly lower ($P \leq 0.01$) than the weight gains of the controls at the end of the second week.

Experiment 3. Analysis of the results (table 1) revealed that supplementing the high zinc diet with calcium resulted in bone calcium levels which were significantly higher ($P \leq 0.05$) than those of rats fed only zinc. Although supplements of phosphorus or magnesium alone did not prevent the marked decreases in bone calcium, the combination of either of these minerals with calcium resulted in increases in bone calcium which were greater than those observed in the zinc-fed rats receiving only calcium. The addition of either phosphorus or magnesium, but not calcium, to the high zinc diet was associated with increases in the accumulation of phosphorus in the bones. Various combinations of these supplements resulted in bone phosphorus values in zinc-fed rats which were essentially the same as those observed in the control animals. The deposition of magnesium in the bones of animals receiving zinc diets supplemented with magnesium was significantly greater ($P \leq 0.05$) than the level of magnesium that accumulated in the bones of animals fed diets not supplemented with magnesium.

The degree to which zinc accumulated in the bones was closely related to the type of supplementation. The addition of either calcium or phosphorus to the high zinc diet was associated with a highly significant reduction ($P \leq 0.01$) in the amount of zinc deposited in the bone, whereas the addition of magnesium had no apparent effect on bone zinc. A combination of all 3 mineral supplements, however, resulted in the least amount of zinc accumulation in the bones of animals receiving added zinc in their diets.

The results indicated that supplements of calcium, but not phosphorus or magnesium, partially alleviated the marked reduction in weight gain associated with zinc toxicity. Greatest improvement in weight gain of zinc-fed rats occurred when a supplement of calcium and phosphorus was included in the diet. A combination of phosphorus plus magnesium or one of calcium, phosphorus and magnesium also resulted in substantial increases in weight gains of rats fed high levels of zinc.

Experiment 4. As noted in the previous experiment, supplements of calcium resulted in a significant improvement in bone calcium levels of zinc-fed rats, whereas the addition of phosphorus to the diet resulted in little improvement in bone calcium levels (table 2). Increasing the calcium supplement from 0.4 to 0.8% resulted in an additional increase in bone calcium levels of zinc-fed rats. A supplement of 1.2% of calcium resulted in the same increase in bone calcium as did the 0.8% supplemental level. The mean bone calcium values of the zinc-fed rats supplemented with either 0.4% of calcium and phosphorus or 0.8% of calcium and phosphorus were no different from the mean bone calcium values of the rats supplemented with either 0.8 or 1.2% of calcium. A supplemental level of 1.2% of calcium and phosphorus completely alleviated the marked decrease in the calcium level of the bones of the zinc-fed animals since the mean calcium value of these rats was not significantly different from that of the control animals.

In this experiment the addition of calcium supplements partially alleviated the decrease in bone phosphorus, whereas phosphorus supplementation had no beneficial effect on bone phosphorus levels. A combination of either 0.8% of calcium and phosphorus or 1.2% of calcium and phosphorus supplemented in the diet resulted in bone phosphorus levels that approached those of the controls.

No significant reduction in the magnesium content of the bone occurred with an intake of 0.75% of zinc, and no significant increases in the levels of bone magnesium resulted with supplementation.

As observed in experiment 3, supplements of calcium or phosphorus effectively prevented the accumulation of excessive amounts of zinc in the bone. In general a combination of 2 supplements was more

effective than either supplement used alone.

Analysis of the weight gain data (table 2) indicated a highly significant increase ($P \leq 0.01$) in weight gain when calcium was supplemented at the 0.8% level or when calcium and phosphorus were given in combination at either the 0.4% or the 0.8% levels. Supplements of only phosphorus at all levels tested resulted in slight increases in weight gains of the zinc-fed rats.

Mineral balance study

Calcium. A summary of the calcium balance data is given in table 5. A highly significant decrease ($P \leq 0.01$) in the retention of calcium by rats fed the high zinc diet was noted at the end of one week. The data also indicated that calcium retention decreased in the zinc-fed rats as the length of time the animals were kept on the dietary regimen increased. With the basal diet the amount of calcium retained by the animals was approximately the same each week of the experimental period.

Although the feeding of zinc was associated, initially, with a highly significant increase ($P \leq 0.01$) in urinary calcium, the amounts of calcium excreted *via* the urine by both groups of animals were essentially the same by the end of the fourth week. The feeding of zinc resulted in a highly significant increase ($P \leq 0.01$) in fecal calcium during the first week of the experimental period. During the first 2 weeks, approximately twice as much calcium was excreted in the feces of the zinc-fed rats as was excreted by the controls. The amount of fecal calcium of the zinc-fed rats was approximately 1.5 times that excreted by the controls during the third and fourth weeks.

Phosphorus. Table 6 contains the results of the phosphorus balance. Analysis

TABLE 5

Calcium balance summary[1]

Weeks on experiment	Diet	Ca intake	Fecal Ca	Urinary Ca	Ca retention
		mg/day	*mg/day*	*mg/day*	*mg/day*
1	Basal	48.46	16.52	0.08	31.86
	+0.75% Zn	45.69	31.11	0.28	14.30
2	Basal	56.24	21.07	0.07	35.10
	+0.75% Zn	51.26	45.00	0.23	6.03
3	Basal	58.49	27.43	0.16	30.90
	+0.75% Zn	54.20	40.30	0.30	13.60
4	Basal	67.40	34.13	0.26	33.01
	+0.75% Zn	57.89	48.49	0.28	9.12

[1] Each figure is the mean of 4 animals.

TABLE 6

Phosphorus balance summary[1]

Weeks on experiment	Diet	P intake	Fecal P	Urinary P	P retention
		mg/day	*mg/day*	*mg/day*	*mg/day*
1	Basal	63.53	8.02	14.80	40.72
	+0.75% Zn	57.99	21.05	7.48	29.46
2	Basal	73.74	8.60	20.40	44.73
	+0.75% Zn	65.06	28.64	14.82	21.59
3	Basal	76.68	11.72	23.56	41.40
	+0.75% Zn	68.80	21.61	14.10	33.09
4	Basal	88.36	14.90	26.71	46.74
	+0.75% Zn	73.48	26.33	14.40	32.74

[1] Each figure is the mean of 4 animals.

TABLE 7

Magnesium balance summary[1]

Weeks on experiment	Diet	Mg intake	Fecal Mg	Urinary Mg	Mg retention
		mg/day	*mg/day*	*mg/day*	*mg/day*
1	Basal	3.35	0.97	0.10	2.28
	+0.75% Zn	2.70	1.16	0.12	1.42
2	Basal	3.88	1.15	0.02	2.71
	+0.75% Zn	3.03	1.44	0.25	1.34
3	Basal	4.04	1.24	0.16	2.64
	+0.75% Zn	3.20	1.55	0.13	1.52
4	Basal	4.65	1.57	0.09	2.99
	+0.75% Zn	3.42	1.47	0.16	1.79

[1] Each figure is the mean of 4 animals.

of the data showed that a dietary level of 0.75% of zinc resulted in a highly significant decrease ($P \leq 0.01$) in phosphorus retention as early as the first week of the experimental period. In contrast with the results obtained on the calcium balance, the amount of phosphorus retained by either the control animals or the zinc-fed animals was essentially the same for the respective groups during the entire experimental period.

Zinc toxicity resulted in a highly significant decrease ($P \leq 0.01$) in urinary phosphorus and an increase in the amount of phosphorus in the feces which was significant at the 1% level of probability. In general, the urinary and fecal excretion of phosphorus remained fairly constant in the zinc-fed rats throughout the entire experimental period. There were slight increases in urinary and fecal excretion of phosphorus in the control animals as the number of weeks increased.

Magnesium. Analysis of the results of the magnesium balance (table 7) indicated that a highly significant decrease ($P \leq$ [illegible]1) in magnesium retention occurred in animals fed the high zinc diet. The significant decrease in magnesium retention in zinc-fed rats occurred during the first week of the experimental period. The amount of magnesium retained by either the control animals or the zinc-fed animals increased slightly as the number of weeks increased.

In general, the feeding of zinc to young rats was associated with increases in the fecal and urinary excretion of magnesium. The fecal excretion of magnesium of both groups of animals increased as the number of weeks increased.

Covariance analyses. The effects of zinc toxicity on the excretion and the retention of calcium, phosphorus and magnesium were still significant after adjustments were made for food intake by covariance. The results of the covariance analyses indicated that the significant reduction in the retention and the marked increase in the excretion of these minerals were due to dietary zinc and not to food intake.

DISCUSSION

The overall results of this study indicate that zinc has an antagonistic effect on the normal deposition of calcium and phosphorus in the bones of young rats which can be alleviated with calcium and phosphorus supplements. Since the feeding of zinc was associated with an increase in the excretion of calcium and phosphorus, there is a possibility that additional calcium and phosphorus supplements to a high zinc diet supply sufficient amounts of these minerals to replace the amounts lost by the adverse action of zinc. Added dietary supplements of calcium and phosphorus may, on the other hand, facilitate the removal of excess zinc from the animal body so that near normal conditions exist with respect to zinc absorption and utilization. That calcium and phosphorus supplementation prevented the marked increase in the accumulation of zinc in the bone tends to support this possibility. Lewis et al. (13) reported that increases in the level of dietary calcium resulted in decreases in the zinc levels of several or-

gans of swine and proposed that excessive amounts of calcium probably reduced the zinc content of the organs by hindering zinc absorption from the gastrointestinal tract.

Results obtained in this study showing that calcium and phosphorus supplements alleviated the adverse effect of zinc on growth are consistent with the idea that these supplements facilitate the removal of excess zinc from the animal body. Supplementing a high zinc diet with calcium and phosphorus resulted in a marked increase in weight gain. A smaller, but significant, increase in weight gain was evident with calcium alone. Although phosphorus alone was largely ineffective in promoting increases in weight gains of zinc-fed rats, it appears to be essential for establishing and maintaining the proper calcium-to-phosphorus ratio which is conducive to normal growth.

The thesis that the beneficial effect of supplemental calcium and phosphorus is to remove excess zinc is further supported by the results of the mineral balance study. The overall results of this study indicate that the primary effect of zinc toxicity on calcium and phosphorus is to interfere with the normal absorption and to increase the fecal excretion of these minerals in the young rat. Approximately, 42% of the total daily calcium intake and 14% of the total daily phosphorus intake were excreted in the feces by animals receiving the basal diet. In the zinc-fed rats, these figures were 78 and 37%, respectively. Presumably, calcium and phosphorus are utilized to aid in the elimination of excess zinc, and smaller amounts of these minerals are available to the animal for normal absorption. When excessive amounts of calcium and phosphorus are added to the high zinc diet, sufficient amounts of these minerals are then available to remove the excess zinc and to allow for adequate retention of these minerals by the animal body. Results of Newland et al. (14) indicating that increasing the level of calcium in diets containing added zinc resulted in increases in the fecal excretion of zinc support this possibility. In connection with parakeratosis, Hoekstra et al. (15) suggested that calcium might accentuate the disease by either hindering zinc absorption or by promoting the excretion of zinc. Their data (15), however, indicated that calcium was not altering the pH of the intestinal tract and thereby affecting the absorption of zinc.

Since the calcium-to-phosphorus ratios of some of the diets used in this study are fairly extreme, there is a possibility that some of these ratios could have accounted for some of the depressing effects on calcium and phosphorus levels. Wasserman (16), however, has pointed out that although the calcium-to-phosphorus ratio should be considered in assessing dietary adequacy, the absolute levels of these minerals in the diet should be recognized as important factors also. Presumably, the calcium-to-phosphorus ratio of the diet is an important factor when either element is present in deficient amounts. Under some conditions of severe changes of calcium-to-phosphorus ratio, there may be depression of the availability of the ion present in the least amount, but this relationship may not necessarily hold under other conditions (16). The calcium and phosphorus supplied by the basal diet used in this study do not appear to be present in deficient amounts. In most cases those combinations of calcium and phosphorus supplements that did not alleviate the effect of zinc toxicity in this study did not result in weight gains and bone calcium and phosphorus levels which were lower than those observed when zinc alone was added to the basal. Thus, the depressing effect of excessive dietary calcium and phosphorus does not appear to be a major factor under the conditions of this study.

Zinc toxicity did not significantly decrease weight gain until the second week of the experimental period. Since significant changes in bone mineralization occurred in zinc-fed rats during the first week, there is the possibility that the interference of zinc with bone formation precedes the interference with growth.

The data indicate that high levels of zinc have an adverse effect on both the absorption and the utilization of magnesium. In rats receiving the basal diet, an average of about 67% of the total daily magnesium intake was retained, whereas in the zinc-fed rats, the average value was approximately 49%. In some instances supple-

ments of calcium and phosphorus to the high zinc diet resulted in a further reduction in bone magnesium levels. There is a possibility that the excessive calcium and phosphorus supplied by these diets had some depressing effect on magnesium as it has been shown that the magnesium requirement of the rat is increased when the calcium and phosphorus content of the diet is increased (17–19).

The data of this investigation indicate that calcium is affected to a greater extent by high levels of zinc than is phosphorus or magnesium. The effects of zinc on the movements of phosphorus and magnesium appear to be similar. Some of the data also suggest the possibility of a complex interrelationship in the animal involving calcium, phosphorus, magnesium and zinc.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. Gennard Matrone at North Carolina State of the University of North Carolina, Raleigh, for his active interest in this study and for his help in preparing this manuscript.

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Poult. Sci. 40: 340-4 (1961)

The Availability of Zinc in Various Compounds to Broad Breasted Bronze Poults[1]

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(Received for publication May 17, 1960)

A CONSIDERABLE number of recent investigations have concerned the zinc requirement of chickens and turkeys. This work was stimulated by the results of studies conducted by O'Dell and Savage (1957) and Supplee *et al.* (1958). Compounds which have been used as zinc sources in the various investigations include zinc carbonate, zinc chloride, zinc oxide, zinc sulfate and certain zinc proteinates. A few comparisons have been made, but only one detailed study has been conducted to determine the biological availability of zinc from various sources to chicks and poults.

Roberson and Schaible (1958) reported that the zinc in zinc sulfate and zinc chloride was equally available to starting chicks. One-hundred parts per million (ppm.) of zinc from each compound was added to a zinc-deficient, purified diet. This level of supplementary zinc, however, was more than three times the amount required by chicks. No definite conclusions regarding availability can be drawn from these data. Pensack *et al.* (1958) studied the relative growth-stimulating effect of several organic and inorganic zinc salts for chicks. Levels of 6, 20 and 40 ppm. of zinc from each source were added to a cerelose-casein-gelatin diet. Zinc was equally available from all sources (zinc carbonate, zinc oxide, zinc chloride and zinc proteinates) tested. Scott (1959) reported that zinc chloride, zinc oxide and possibly zinc carbonate could be used effectively in poultry rations. The results of Kienholz *et al.* (1959) indicated that the zinc in un-autoclaved field peas was not available to chicks, or that the peas contained a heat-labile factor which increased the requirement for zinc. Edwards (1959) has conducted a detailed study of the availability to chicks of zinc in various compounds and ores. Two levels, 10 and 20 ppm. of zinc from each material, were fed in a purified diet of low zinc content. The growth data obtained indicated that the zinc in zinc sulfate, willemite, zinc carbonate, zinc metal, zinc oxide (technical grade), smithsonite, hemimorphite, zinc oxide (A.R. grade) and zincite was relatively available to the young growing chicken. The zinc in sterling black and brown crude ore was of lower availability and that from sphalerite and franklinite was relatively unavailable.

The present investigation was undertaken to determine the relative availability of zinc from various compounds to Broad Breasted Bronze (BBB) poults.

[1] Published with the approval of the Director as Paper No. 1046, Journal Series, Nebraska Agricultural Experiment Station.

EXPERIMENTAL PROCEDURE

Straight-run BBB poults were used in all three of the experiments. The basal diet, composed largely of isolated soybean protein and sucrose, was identical, and the experimental procedure followed was essentially the same as that described in an earlier publication (Sullivan, 1960). The name, formula and percentage of zinc in each of the compounds tested are shown in Table 1. The dietary treatments consisted of the basal diet plus either 20, 30 or 40 ppm., depending on the experiment involved, of zinc from each compound. The unsupplemented basal diet, which contained 14.2 ppm. of zinc by analysis, served as the negative control in each experiment. Each dietary treatment was fed to two replicate groups of 10 poults each. Precautions were taken to prevent the ingestion of unknown quantities of environmental zinc; the feed troughs were coated with a plastic-type (phenolic modified polyester) finish. Distilled water, which was dispensed in glass founts, and the experimental diets were supplied *ad libitum*.

The experiments were of 28 days duration. Individual body weights were taken at weekly intervals and the total diet consumption of the 20 birds assigned to each dietary treatment was determined at the termination of each experiment. When experiments 2 and 3 were terminated, the incidence of distinct zinc-deficiency symptoms in the legs and feathers of surviving birds was determined. When experiment 3 was terminated, three birds of average weight from each experimental group were sacrificed to obtain bone samples. A femur, a tibiotarsus and a tarsometatarsus were taken from each. After the adhering tissue was removed, the length of each bone was measured in centimeters.

The growth data from all three experiments and the bone measurements from experiment 3 were subjected to analysis of variance (Snedecor, 1956). Duncan's (1953) New Multiple Range Test for significance was used to compare the treatment means when significant differences were detected by the analysis of variance. In every instance the level of significance is 5 percent, except where the 1 percent level is specifically indicated.

TABLE 1.—*Zinc compounds tested for availability of zinc to poults, 0–4 weeks of age*

Name and description	Formula	Percent zinc
Zinc carbonate (technical grade)		54.0
Zinc chloride (A.R. grade)	$ZnCl_2$	45.6
Zinc oxide (technical grade)	ZnO	71.6
Zinc sulfate (technical grade)	$ZnSO_4 \cdot H_2O$	36.4
Zinc sulfate (U.S.P. grade)	$ZnSO_4 \cdot 7H_2O$	22.7

[1] Samples and zinc analysis data furnished by the Calbium Carbonate Company, 520 South Fourth Street, Quincy, Illinois.

RESULTS AND DISCUSSION

The effect of supplementary zinc from various compounds on the growth of poults, 0–4 weeks of age, is shown by the data presented in Table 2. In experiment 1, where 40 ppm. of zinc from each compound was added to the basal diet, there were no significant differences between the gains of poults fed the various sources of supplementary zinc. The gains of those birds fed the unsupplemented basal diet were significantly less ($P < .01$) than the

TABLE 2.—*Effect of supplementary zinc from various compounds on the growth of poults, 0–4 weeks of age.*

	Average gain in 4 weeks, grams[1]						
	Source of supplementary zinc[2]						
	None	$ZnCl_2 \cdot 4H_2O$	ZnO	$ZnCl_2$	$ZnSO_4 \cdot H_2O$	Zinc carbonate	
Experiment 1	210	469	[illegible]	444	[illegible]	448	
Experiment 2	146	462	462	477	423	429	
Experiment 3	146	259	[illegible]	316	345	327	

[1] Any two means in a column followed by the same line are significantly different at the 5 percent level.

[2] Each value was computed from the gains of 20, 18 and 19 surviving birds per treatment, respectively, in experiments 1, 2 and 3.

[3] The level of supplementary zinc added to the basal diet in each experiment was [illegible] ppm. in experiment 1, 30 ppm. in experiment 2, and 20 ppm. in experiment 3. The basal diet contained 14.2 ppm. of zinc by analysis.

TABLE 3.—*Effect of supplementary zinc from various compounds on feed efficiency and survival of poults, 0–4 weeks of age*

	Source of supplementary zinc[1]					
	None	$ZnSO_4 \cdot H_2O$	ZnO	$ZnCl_2$	$ZnSO_4 \cdot 7H_2O$	Zinc carbonate
Experiment 1						
Feed gain	1.00	1.00	1.60	1.71	1.63	1.63
Survival	9/20	19/20	18/20	20/20	19/20	19/20
Experiment 2						
Feed gain	2.15	1.65	1.49	1.38	1.48	1.50
Survival	10/20	16/20	19/20	20/20	19/20	19/20
Experiment 3						
Feed gain	1.67	1.58	1.94	1.72	1.61	1.66
Survival	8/20	17/20	19/20	20/20	20/20	18/20

[1] The level of supplementary zinc added to the basal diet in each experiment was as follows: 60 ppm., experiment 1; 30 ppm., experiment 2; and 20 ppm., experiment 3. The basal diet contained 14 ppm. of zinc by analysis.

gains of birds fed any of the supplemented diets, this was the case in each of the three experiments. In experiment 2 the compounds were compared by adding 30 ppm. of zinc from each to the basal diet. Differences in availability were then evidenced by differences in body weight gains; the gains of poults fed $ZnSO_4 \cdot 7H_2O$ and zinc carbonate were significantly greater than the gains of poults fed zinc oxide and $ZnSO_4 \cdot H_2O$. The gains of poults fed zinc chloride were significantly greater than those of poults fed $ZnSO_4 \cdot H_2O$. The difference in growth response to zinc oxide and $ZnSO_4 \cdot H_2O$ was not significant. In experiment 3, 20 ppm. of zinc from each compound was added to the basal diet. The gains of poults fed zinc carbonate, $ZnSO_4 \cdot 7H_2O$, and zinc chloride were significantly greater than the gains of poults fed zinc oxide and $ZnSO_4 \cdot H_2O$. The response to zinc carbonate was significantly greater than the response to zinc chloride, but not significantly greater than the response to $ZnSO_4 \cdot 7H_2O$. The difference in the response to zinc oxide and $ZnSO_4 \cdot H_2O$ was not significant.

The effect of supplementary zinc from various compounds on feed efficiency and survival is shown by the data presented in Table 3. The feed efficiency of birds fed the unsupplemented basal diet was much poorer than the feed efficiency of poults receiving any of the supplemented diets. In experiment 1 the various zinc supplements affected feed efficiency similarly; however, in experiment 2 the poults fed $ZnSO_4 \cdot H_2O$ showed poorer feed efficiency than did poults receiving the other zinc compounds. In experiment 3 poults fed zinc carbonate and $ZnSO_4 \cdot 7H_2O$ showed the best feed efficiency. These data are in general agreement with the growth data. The survival data indicate that mortality was not excessive in any of the groups fed supplementary zinc, while fewer than 50 percent of the control birds survived the four weeks experimental period.

Linear bone growth was used as a criterion for determining zinc availability, because in previous studies both chicks and poults fed diets limiting in zinc exhibited a shortening and thickening of the long bones. The bone length data, presented in Table 4, were taken from experiment 3 and are in close agreement with the growth data obtained in that test. Femur and tarsometatarsus samples from poults fed zinc carbonate and $ZnSO_4 \cdot 7H_2O$ were of significantly greater length than similar samples from poults fed the other compounds. Tibiotarsus samples from poults fed zinc carbonate and $ZnSO_4 \cdot 7H_2O$

TABLE 4.—*Effect of supplementary zinc from various compounds on bone development in poults four weeks of age*

	Average[1] bone length in centimeters					
	Source of supplementary zinc[2]					
	None	$ZnSO_4 \cdot H_2O$	ZnO	$ZnCl_2$	Zinc carbonate	$ZnSO_4 \cdot 7H_2O$
Femur	3.73	4.15	4.23	4.42	4.77	4.97
Tibiotarsus	5.22	5.85	5.88	6.28	6.57	6.95
Tarsometatarsus	3.87	4.35	4.53	4.60	5.25	5.08

[1] Each value represents the average of six individual bone measurements, three from each replicate group per treatment in experiment 3. See footnote 1 of Table 2.
[2] Twenty ppm. of zinc from each compound was added to the basal diet which contained 14 ppm. of zinc by analysis.

TABLE 5.—*Effect of supplementary zinc from various compounds on the incidence[1] of zinc deficiency symptoms in poults four weeks of age*

	Source of supplementary zinc[2]					
	None	$ZnSO_4 \cdot H_2O$	ZnO	$ZnCl_2$	$ZnSO_4 \cdot 7H_2O$	Zinc carbonate
Experiment 2						
Leg symptoms	10/10	9/18	14/19	2/20	2/19	5/19
Feather symptoms	10/10	5/18	8/19	1/20	1/19	2/19
Experiment 3						
Leg symptoms	8/8	17/17	19/19	19/20	16/20	16/18
Feather symptoms	8/8	16/17	15/19	11/20	7/20	9/18

[1] Incidence of deficiency symptoms in surviving birds at the termination of the experiment.
[2] The level of supplementary zinc added to the basal diet was 30 ppm. in experiment 2 and 20 ppm. in experiment 3. The basal diet contained 14 ppm. of zinc by analysis.

were of significantly greater length than samples from poults fed zinc oxide and $ZnSO_4 \cdot H_2O$. There were no significant differences in the length of bone samples from poults fed zinc chloride, zinc oxide and $ZnSO_4 \cdot H_2O$.

The effect of supplementary zinc from the various compounds on the incidence of zinc deficiency symptoms is shown by the data presented in Table 5. Zinc deficiency symptoms as evidenced by shortened legs, enlarged hocks and retarded feathering were exhibited by all of the surviving birds in the control groups. Poults fed zinc oxide and $ZnSO_4 \cdot H_2O$ showed a greater incidence of deficiency symptoms than did poults fed the other compounds. Practically all of the poults fed zinc oxide and $ZnSO_4 \cdot H_2O$ in experiment 3 showed abnormal bone and feather development, while poults fed the other compounds showed these conditions to a lesser degree. Abnormal leg development was definitely more pronounced than abnormal feather development in all of the groups.

The data obtained in these experiments clearly indicate that the zinc in zinc carbonate and $ZnSO_4 \cdot 7H_2O$ is readily available to starting poults. The zinc in zinc chloride was, perhaps, slightly less available than the zinc in zinc carbonate and $ZnSO_4 \cdot 7H_2O$. The deliquescent or hygroscopic nature of zinc chloride may have caused this to occur and would limit the use of this compound in poultry rations. Zinc oxide and $ZnSO_4 \cdot H_2O$ were definitely inferior sources of zinc for starting turkey rations. These results are not in complete agreement with previously reported data. Edwards (1959) reported that the zinc in zinc oxide was equally or slightly more available than the zinc in $ZnSO_4 \cdot 7H_2O$. The zinc oxide and $ZnSO_4 \cdot H_2O$ as well as the zinc carbonate used in the current study were technical grade compounds containing various impurities. It is possible, but quite unlikely, due to the amounts involved, that certain impurities could have been responsible for the relatively poor response to zinc oxide and $ZnSO_4 \cdot H_2O$.

In view of recent reports (Norris and Ziegler, 1959; Davis and Norris, 1960) there appears to be two distinct factors which influence the biological availability of minerals. These factors are: (1) the binding of metals by protein or some other moiety or additive in the diet such as free amino acids or ethylenediaminetetraacetic acid (EDTA) and (2) the particular solubility or some other property of the chem-

ical form being used. In this study "availability" as determined by the latter has been investigated. The availability of zinc in the various compounds, as determined in this study, might be different in the presence of different proteins, other free amino acids or chelating agents such as EDTA.

SUMMARY

The effect of supplementary zinc from various compounds on growth, survival, feed efficiency, bone development and the incidence of deficiency symptoms was studied in three experiments with Broad Breasted Bronze poults, 0–4 weeks of age. The results obtained indicate that the zinc in zinc carbonate and $ZnSO_4 \cdot 7H_2O$ is readily available. The zinc in zinc chloride was, perhaps, slightly less available than the zinc in zinc carbonate and $ZnSO_4 \cdot 7H_2O$. Zinc oxide and $ZnSO_4 \cdot H_2O$ were relatively inferior sources of dietary zinc for poults.

ACKNOWLEDGMENT

The author is grateful to the Central Soya Company, Inc., Chemurgy Division, 1825 Laramie Ave., Chicago 39, Illinois, for supplying the isolated soybean protein (Promine); to Merck and Company, Inc., Animal Science Research, Rahway, New Jersey, for supplying the vitamin B_{12}, d-biotin, menadione, pyridoxine HCl, pteroylglutamic acid, riboflavin, thiamine HCl, calcium pantothenate and penicillin; to the Dow Chemical Company, Abbott Road Buildings, Midland, Michigan, for supplying the DL-methionine and glycine and to the Calcium Carbonate Company, 520 South Fourth Street, Quincy, Illinois for supplying the calcium carbonate, zinc carbonate, zinc oxide and zinc sulfate ($ZnSO_4 \cdot H_2O$) used in this study. A grant-in-aid which partially supported the work was also furnished by the Calcium Carbonate Company.

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J. Biol. Chem. 180:1279 (1949)

ACTIVATION OF PHOSPHOGLUCOMUTASE BY METAL-BINDING AGENTS

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(Received for publication, June 27, 1949)

Cysteine, glutathione, and other reducing agents have been used to activate phosphoglucomutase (1, 2), especially crystalline mutase, which was found to be essentially inactive without the addition of cysteine (2). It has been found that cysteine can be replaced by a number of metal-binding agents which approach or equal cysteine in effectiveness. This activation by metal-binding agents has been demonstrated with mutase from skeletal muscle, heart muscle, and yeast and is described in this paper. A summary of these findings has been reported previously (3).

Methods and Preparations

The conversion of glucose-1-phosphate (G-1-P) to glucose-6-phosphate (G-6-P) was measured by the disappearance of labile phosphate; that is, organic phosphate hydrolyzed by normal sulfuric acid during a 5 minute period in a boiling water bath. The inorganic phosphate liberated was then determined by the method of Fiske and Subbarow (4). In some experiments formation of G-6-P was also measured by the appearance of material able to reduce alkaline copper reagents such as the Nelson reagent (5). The usual reaction mixture contained 6×10^{-3} M G-1-P and 1.5×10^{-3} M magnesium sulfate. When cysteine was used, its final molarity was 1.2×10^{-2}. The enzyme preparation was added to a mixture of sufficient volume to permit subsequent withdrawal of aliquots over a period of time. The temperature of incubation was either 30° or 37°.

Crude mutase was prepared by heating water extracts of skeletal or heart muscle 20 minutes at 52° and then removing the denatured protein by filtration. Crystalline mutase was prepared from rabbit muscle as described by Najjar (2). The first crystals were recrystallized once or twice before use. Yeast mutase was found to be enriched in a protein fraction from yeast which represented an intermediate step in the preparation of *Zwischenferment* as described by Negelein and Gerischer (6). Washed and dried brewers' yeast was extracted with 3 volumes of water for 5 hours at 37°, centrifuged, and filtered. Two isoelectric precipitates at pH 4.6 and 8.9 were discarded. The protein fraction precipitating between 0.4 and 0.6 saturated ammonium sulfate was collected and this

fractionation was repeated a second time. The precipitate was dissolved, made 0.4 saturated with ammonium sulfate, and heated for 1 hour at 40°. Denatured protein was discarded and the solution was dialyzed for 19 hours against distilled water.[1] This preparation is very similar to one which has been used for the preparation of the enzyme from aqueous extracts of muscle.

Glucose-1-phosphate was prepared chemically by the method of Cori, Colowick, and Cori (7), or enzymatically with starch, inorganic phosphate, and phosphorylase from potatoes or from rabbit muscle.[2]

EXPERIMENTAL

When amorphous, zinc-free, or well dialyzed crystalline zinc insulin was added to muscle mutase, either crude or purified, the rate of the enzymatic reaction was increased. This stimulation occurred in the absence of cysteine, required relatively large amounts of insulin, and was non-specific in the sense that other proteins, *e.g.* crystalline albumin, could be substituted for insulin. By contrast it was found that non-dialyzed zinc insulin inhibited mutase. This inhibition is probably caused by zinc ions, to which the enzyme is quite sensitive (Table I). It suggested moreover that the activation of mutase by proteins and by cysteine represented a metal-binding effect. A number of heavy metal-binding agents were therefore investigated; they differ from cysteine, which is also a heavy metal binder, in having no reducing action.

Histidine was found to be more effective than insulin (Fig. 1), and in larger concentrations (0.04 M) approached cysteine in the degree of activation of mutase. Histamine was less effective than histidine, while carnosine had no activating effect. (Fig. 1).

Diphenylthiocarbazone (dithizone) is a metal-binding agent which is almost insoluble in water, but can be used in carbon tetrachloride solution. When the complete reaction mixture was shaken with carbon tetrachloride containing 0.01 per cent dithizone, a rapid enzymatic reaction took place, while the control, which was shaken with the same amount of carbon tetrachloride containing no dithizone, showed no reaction at all (Fig. 2). 8-Hydroxyquinoline, being more soluble in water, could be added directly to the reaction mixture and approached optimal amounts of cysteine in effectiveness (Fig. 2). Activation by 8-hydroxyquinoline or cysteine could also be demonstrated with mutase from heart muscle and from yeast (Fig. 3). In contrast to the mutase preparations of mammalian tissues that of yeast showed considerable activity in the absence of metal-binding

[1] Dr. M. Slein kindly provided a sample at this stage.

[2] The preparations used contained glucose-1,6-diphosphate in amounts sufficient to give maximal activation with respect to this coenzyme.

TABLE I

Effect of Metal Ions on Activity of Yeast Mutase

The reaction mixture consisted of 6×10^{-3} M glucose-1-phosphate and 1.5×10^{-3} M magnesium ions. No metal-binding agents were added.

Metal	Charge	Concentration of metal, final molarity	Inhibition
			per cent
Cobalt	++	5×10^{-5}	0
Iron	++	5×10^{-5}	0
Zinc	++	1×10^{-4}	91
"	++	5×10^{-5}	53
Lead	++	1×10^{-4}	85
Copper	++	1×10^{-4}	100
"	++	5×10^{-5}	33
Mercury	++	5×10^{-5}	97
Silver	+	5×10^{-5}	96
"	+	5×10^{-5}	32

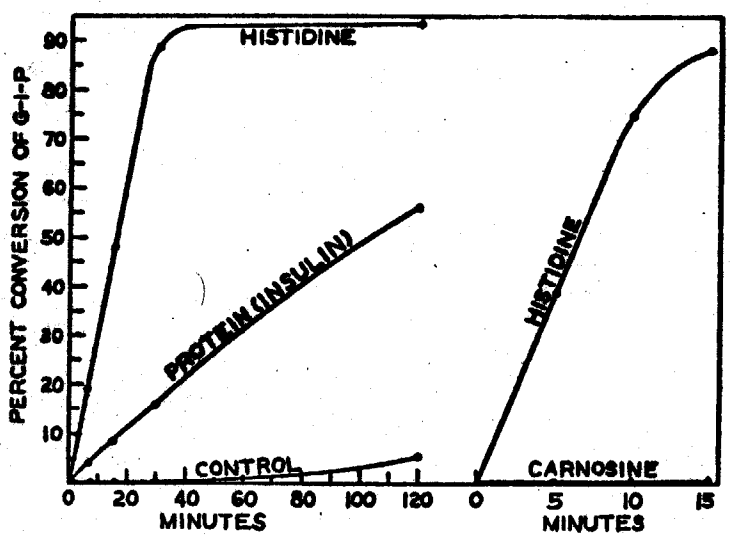

FIG. 1. Effect of histidine, insulin, and carnosine on crystalline phosphoglucomutase. The reaction mixtures contained enzyme, 6×10^{-3} M glucose-1-phosphate, and 1.5×10^{-3} M magnesium ions. The final concentration was 0.024 M for histidine in the first experiment and 0.032 M for histidine and carnosine in the second experiment. Crystalline zinc insulin was dialyzed 18 hours against acidified distilled water, and was then added to give a final concentration of 1.3 mg. per ml.

agents. The inhibition caused by the addition of small amounts of zinc ions was completely overcome by the addition of the metal-binding agents (Fig. 3).

In several experiments with yeast mutase varying amounts of metal ions were added to the reaction mixture, which included 1.5×10^{-3} M magnesium ion but no metal-binding agent. Metal ions which inhibited the yeast enzyme in low concentrations were zinc, lead, copper, mercury, and silver (Table I). Copper and silver ions partially inhibited the spontaneous activity of the yeast mutase at 5×10^{-5} M concentration, but, since other proteins were present as impurities, the enzyme might actually be

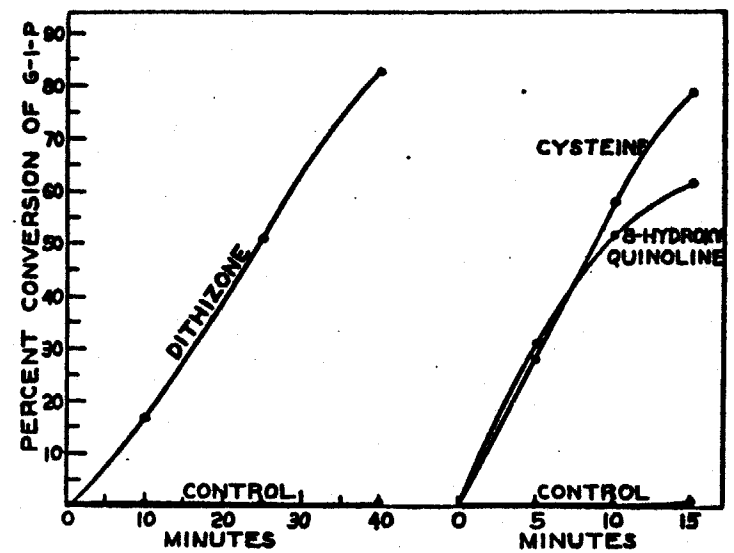

FIG. 2. Effect of dithizone, cysteine, and 8-hydroxyquinoline on crystalline phosphoglucomutase. The reaction mixtures contained enzyme, 6×10^{-3} M glucose-1-phosphate, and 1.5×10^{-3} M magnesium ions. In the first experiment the reaction mixture was shaken with carbon tetrachloride alone in the control and with carbon tetrachloride containing 0.01 per cent dithizone in the other. In the second experiment the final molarity of cysteine was 0.025 and that of the 8-hydroxyquinoline 0.002.

considerably more sensitive to these metals. Crystalline mutase could not be used in such experiments because it was inactive without the addition of metal-binding agents. This is presumably due to the presence of traces of heavy metal. The inhibition of yeast mutase by metal ions was reversible under the conditions tested; *i.e.*, after complete inhibition of the mutase reaction was obtained with metal ions, addition of metal-binding agents restored the rate to that of the original uninhibited reaction.

Since the marked sensitivity to metals suggested involvement of sulfhydryl groups, the effect of iodoacetate was investigated. When 1×10^{-3}

M iodoacetate was added directly to the reaction mixture, no inhibition was noted. However, when the yeast enzyme was preincubated with 2×10^{-3} M iodoacetate for 10 minutes at room temperature before addition to the reaction mixture, an inhibition was observed which amounted to about 50 per cent.

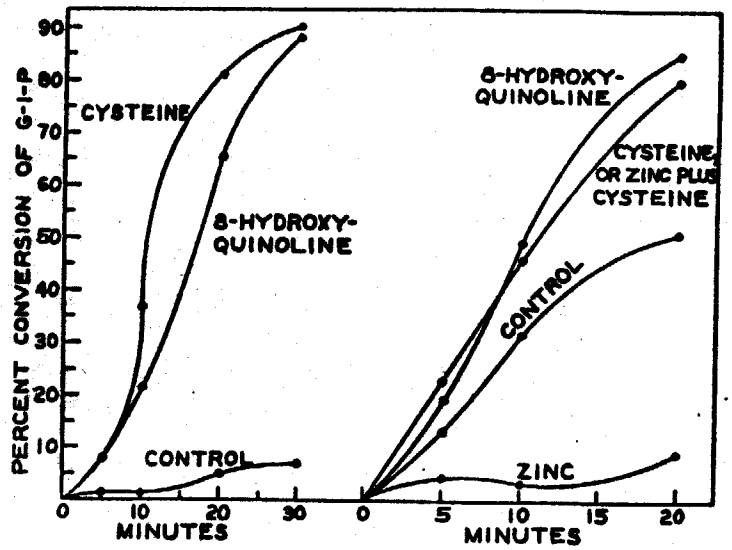

FIG. 3. Effect of metal-binding agents on phosphoglucomutase from heart (first experiment) or yeast (second experiment). The reaction mixtures contained enzyme, 6×10^{-3} M glucose-1-phosphate, and 1.5×10^{-3} M magnesium ions. In both experiments the final molarity of cysteine was 0.02 and that of 8-hydroxyquinoline 0.003. When added, zinc ions were present in a final concentration of 5×10^{-5} M.

DISCUSSION

Apart from metal-binding agents, phosphoglucomutase requires glucose-1,6-diphosphate for its activity (see the following paper (8)). The experiments reported in this paper were carried out in the presence of sufficient glucose-1,6-diphosphate to saturate the enzyme with respect to this cofactor. In the absence of metal-binding agents, 1,6-diphosphate, even in a final concentration of 2×10^{-3} M, failed to activate crystalline mutase.

Cysteine, which is a reducing agent as well as a metal-binding agent, was found to be slightly more effective than other metal-binding agents. It is known from work with other enzymes (phosphorylase, glyceraldehyde phosphate dehydrogenase) that the "reducing action" of cysteine as measured by the increase in enzyme activity is not instantaneous but requires 5 or more minutes for completion. By contrast phosphoglucomutase shows a rapid decrease in activity when it is exposed to the reducing action of cysteine. In order to obtain maximal activation of mutase with cysteine, the latter must not be in contact with the enzyme prior to the start of the reaction (2). It therefore seems probable that cysteine activates mutase merely in its capacity as a metal binder.

Since proteins are metal-binding agents, crude mutase preparations in general show less need of metal-binding agents than do purified preparations. With a relatively crude yeast fraction, addition of metal-binding agents increased the activity by about 50 per cent. Addition of small amounts of heavy metal ions magnified the effect of metal-binding agents. Much stronger activation by metal-binding agents was observed with crude mutase preparations from heart or skeletal muscle. Such differences may in part be accounted for by the capacity of the contaminating proteins to bind metal ions. That muscle extract contains a protective protein fraction has been observed previously (9). The crystalline enzyme from muscle is apparently extremely sensitive to traces of heavy metal, since it is completely inactive in the absence of metal-binding agents.

SUMMARY

Phosphoglucomutase from skeletal muscle, heart, or yeast can be activated by metal-binding agents. Various proteins (zinc-free insulin, serum albumin) exert such an effect which is not, however, maximal. Zinc insulin causes inhibition, since the enzyme is quite sensitive to zinc ions (as well as to copper, mercury, and silver ions). Histidine, diphenylthiocarbazone, and 8-hydroxyquinoline were found to activate the enzyme nearly as effectively as cysteine. The activation by these metal-binding agents is distinct from the activation by glucose-1,6-diphosphate.

The author wishes to thank Dr. Carl F. Cori and Dr. Gerty T. Cori for advice and encouragement during the course of this investigation.

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Studies on Zinc.

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Young rats weighing between 40 and 50 gm. were placed in groups of 5, three females and 2 males, in screen bottom cages. Food and water were supplied *ad libitum*. C. P. zinc carbonate was incorporated in the growing ration at 3 levels, corresponding to 0.10, 0.50, and 1.00% of zinc. The growing ration is a mixture of naturally occurring foods, and has served this laboratory well, during many years, for the growth and reproduction of rats. The zinc salt contained as a maximum the following impurities: arsenic 0.000%, chloride 0.002%, iron 0.01%, lead 0.002%, nitrate 0.000%, and sulphate 0.01%. Hemoglobin was determined by the Newcomer method.

Growth was normal on the rations containing 0.10 and 0.50% of zinc. The animals were vigorous when the experiment terminated, and there was practically no difference in the weight of the rats receiving zinc and those on the stock diet. The limit of tolerance to zinc carbonate lies between 0.50 and 1.0% of zinc in the diet. The animals on the 1% zinc diet failed to grow in the majority of cases, although some individuals weighed 140 to 160 gm. after 10 to 12 weeks. Some of the animals began dying within 4 weeks after being placed on this diet; some were partly eaten.

Growth and reproduction on the lowest level of zinc were normal. Several generations of these rats on the same zinc diet have shown no abnormalities. Reproduction was markedly affected, when the animals received 0.50% of zinc. The females on this diet became pregnant between the fourteenth and seventeenth weeks. One female had 3 young and lost 30 gm. at parturition. Another female had 3 young (one born dead) but lost 40 gm. at parturition. Another female when observed had 5 dead young weighing 24 gm. The young appeared white and bloodless. The females with the live young mothered them satisfactorily. The young averaged 30 gm.

at 21 days of age. The original females again mated when returned to their cages. The young were dead in every case and no live young were born after the first pregnancy. A total of 23 still born rats were counted and others were undoubtedly consumed by the mothers. The females on 0.50% zinc ceased to become pregnant after 5 months on the ration, although their weights and outward appearances were normal. No reproduction occurred on the 1% zinc level.

TABLE I.
Effect of Zinc Carbonate on Hemoglobin and Red Cells of the Rat.

Lot No.	Zinc Intake %	Initial Hemoglobin gm. per 100 cc.	Final Hemoglobin gm. per 100 cc.	Initial R.B.C. millions per cu.mm.	Final R.B.C. millions per cu. mm.	Weeks on Ration
1	0.10	14.5	14.7	8.4	9.1	39
2	0.50	13.9	10.2	8.6	8.5	39
3	1.00	14.2	6.1	8.5	6.6	12-16
4	1.00	13.6	6.5	7.8	6.5	10-16
5	1.00	13.7	6.8	8.0	6.8	12-16
6	1.00	13.5	7.8	8.1	5.8	10
7	0.00	14.1	14.1	7.8	8.9	34
8	0.00	14.6	15.1	—	—	30

Table I shows that normal hemoglobin values were found in the group of rats receiving 0.10% of zinc after 39 weeks on this diet. That there was something organically wrong with the animals on the 0.50% zinc level was first made evident from observations on their reproductive behavior. At the age of initial reproduction of these females the hemoglobin values were only slightly lower than those of the control group. They were still within the limits given by some investigators as normal, but when the rats were kept longer on the ration they became anemic. The average hemoglobin value at 30 weeks was 10.2 gm. No change in the number of R.B.C. was observed. Both hemoglobin and red blood corpuscles were diminished on the 1% zinc diet. Hemoglobin values in this lot usually started to decrease after 3 to 5 weeks on the ration. Low values of 4 gm. % were sometimes observed. Rats often lived for 5 weeks with hemoglobin values below 6 gm. %, while others succumbed with the hemoglobin only slightly below normal. The blood cell picture of the animals on 1% of zinc was interesting. Although the red cells became irregular in size and shape within 5 weeks after being placed on this diet, the number was not appreciably diminished until after 8 to 10 weeks on this regime. There was apparently an excess of white and immature red blood corpuscles in every case.

Normal females gave average blood hemoglobin values at 7 days

and one day before parturition of 14.6 and 12.6 gm. per 100 cc. respectively. At parturition and one week after, the corresponding values were 12.26 and 14.2. We believe that this normal anemic condition is so increased by zinc, when ingested at a 0.50% level, that normal reproduction is impossible, although growth is not impaired. Hemoglobin and reproduction became normal in the animals on 0.50% zinc, when the salt was removed from the ration. The same is true of the hemoglobin of rats on 1% zinc; whether reproduction is permanently impaired on the highest level of zinc is not known.

BLOOD SUGAR CHANGES IN THE RAT PRODUCED BY SALTS OF BERYLLIUM, MAGNESIUM, AND ZINC WITH SOME OBSERVATIONS ON HEMOGLOBIN AND RED BLOOD CORPUSCLES

W. R. Sutton and Victor E. Nelson

This report is the direct result of research in this laboratory upon the physiological role of several metallic salts. The influence of magnesium salts upon the blood sugar content of rabbits was studied by Underhill (1) who found that hyperglycemia subsequent to magnesium injection was most evident during the period of anesthesia. Meltzer and Auer (2) had shown that an animal anesthetized by an injection of magnesium sulfate could be quickly restored to normal by an intravenous injection of calcium chloride. Underhill (1) showed that the hyperglycemia produced in rabbits anesthetized with magnesium salt was reduced to normal in two or three hours time by calcium chloride. Salant and Wise (3) observed the production of glycosuria in rabbits by zinc salts. They gave results which show that an intravenous injection of zinc malate caused hyperglycemia and glycosuria. These workers report that intravenous injection of calcium chloride did not cause a decrease in the glycosuria resulting from the zinc malate injection. They did not report the effect of calcium chloride upon the blood sugar.

Methods

Young rats weighing between 130 and 160 grams were used in these experiments. The animals had been reared on our growing ration, which is composed of natural foods and has been used in this laboratory for the growth of rats for many years. Blood samples for glucose, hemoglobin, and erythrocyte counts were obtained, with few exceptions, from the external saphenous vein. In a few cases the blood pressure dropped to such a low level in two to three hours after administration of the metallic salts that it was impossible to obtain samples from the saphenous vein. In these cases blood samples were obtained directly from the heart. The initial blood sugar values were obtained from the unanesthetized animals after a twenty hour fast. All animals at the time they received the test solutions were anesthetized with an intraperitoneal injection

Table I — Blood Sugar Changes Produced by Zinc Chloride, Zinc Chloride and Glucose, and Glucose Administered by Stomach Tube to the Fasting White Rat under Nembutal Anesthesia.

Rat No.	Substance Administered	Volume of solution and mgs. of dissolved substance fed or injected	Glucose mg./100cc. after 20 hr. fast. (Animals not anesthetized)	Glucose mg./100cc. 1 hr. after dose. (Animals anesthetized)	Glucose mg./100cc. 2 hrs. after dose. (Animals anesthetized)	Glucose mg./100cc. 3 hrs. after dose. (Animals anesthetized)
1	$ZnCl_2$	2cc. (30 mg. Zn)	94	125	180	167
2	$ZnCl_2$	2cc. (30 mg. Zn)	80	114	171	190
3	$ZnCl_2$	1cc. (50 mg. Zn)	90	130	133	210
4	$ZnCl_2$ Glucose	2cc. (30 mg. Zn-250 mg. Glucose)	102	140	227	263
5	$ZnCl_2$ Glucose	2cc. (30 mg. Zn-250 mg. Glucose)	109	137	208	N.D.
6	$ZnCl_2$ Glucose	1cc. (50 mg. Zn-250 mg. Glucose)	87.5	125	200	250
7	$ZnCl_2$ Glucose	1cc. (50 mg. Zn-250 mg. Glucose)	95	137	Died	N.D.
8	Glucose	2cc. (250 mg. Glucose)	94	113	104	96
9	Glucose	1cc. (250 mg. Glucose)	82.5	95	116	140
10	Nembutal	1.5cc. (4.5 mg. Nembutal) injected	96.5	90	83	78
11	Nembutal	1.5cc. (4.5 mg. Nembutal) injected	102	90	88	N.D.
12	Nembutal	1.5cc. (4.5 mg. Nembutal) injected	97	83	75	N.D.

N.D. — No determination.

Table II — Changes in Blood Sugar in Various Intervals of Time after the Administration of Beryllium Sulfate by Stomach Tube to the Fasting White Rat under Nembutal Anesthesia

Rat No.	Substance Administered	Volume of solution and mgs. of dissolved substance fed	Glucose mg./100cc. after 20 hr. fast (Animals not anesthetized)	Glucose mg./100cc. ½ hour after dose (Animals anesthetized)	Glucose mg./100cc. 1 hour after dose (Animals anesthetized)	Glucose mg./100cc. 2 hours after dose (Animals anesthetized)	Glucose mg./100cc. 3 hours after dose (Animals anesthetized)
1	$BeSO_4$	2cc. (9 mg. Be)	94	94	.....	.....	.....
2	$BeSO_4$	1cc. (9 mg. Be)	90	85	.....	.....	.....
3	$BeSO_4$	1cc. (9 mg. Be)	83	83	113	.....	.....
4	$BeSO_4$	1cc. (9 mg. Be)	92	90	98	.....	.....
5	$BeSO_4$	1cc. (9 mg. Be)	109	.....	.....	136	.....
6	$BeSO_4$	1cc. (9 mg. Be)	89	.....	.....	141	.....
7	$BeSO_4$	1cc. (9 mg. Be)	95	.....	.....	144	.....
8	$BeSO_4$	1cc. (9 mg. Be)	83	.....	113	150	.....
9	$BeSO_4$	1cc. (9 mg. Be)	82	.....	121	160	121
10	$BeSO_4$	1cc. (9 mg. Be)	83	.....	131	145	160
11	$BeSO_4$	1cc. (6 mg. Be)	90	.....	100	84	.....
12	$BeSO_4$	1cc. (6 mg. Be)	100	.....	106	110	100
13	$BeSO_4$	1cc. (6 mg. Be)	92	.....	100	89	.....

Table III — The Effect of Calcium Chloride upon Blood Sugar, Hemoglobin, and Red Blood Corpuscle Changes Produced by Administration of Zinc Sulfate by Stomach Tube to the Fasting White Rat under Nembutal Anesthesia

Rat No.	Substance Administered	Volume of solution and mgs. of dissolved substance fed	Volume of solution and mgs. dissolved substance injected subcutaneously	Glucose mg./100cc. after 2 hrs. (Animals anesthetized)	Hemoglobin g./100cc. after 2 hrs. (Animals anesthetized)	RBC million/cmm after 2 hrs. (Animals anesthetized)
1	$ZnSO_4$	1cc. (65mg. Zn)	0	126	21	13.64
2	$ZnSO_4$	1cc. (65mg. Zn)	0	125	23	13.82
3	$ZnSO_4$	1cc. (65mg. Zn)	0	129	—	—
4	$ZnSO_4$	1cc. (65mg. Zn)	1.5cc. (7.5mg. $CaCl_2$)	75	23	13.60
5	$ZnSO_4$	1cc. (65mg. Zn)	1.5cc. (7.5mg. $CaCl_2$)	70	21	11.50
6	$ZnSO_4$	1cc. (65mg. Zn)	1cc. (5mg. $CaCl_2$)	98	23	13.60
7	$ZnSO_4$	1cc. (65mg. Zn)	1cc. (5mg. $CaCl_2$)	103	—	—
8	Glucose	1cc. (250 mg. Glucose)	0	113	15.5	9.65
9	Glucose	1cc. (250 mg. Glucose)	1cc. (5mg. $CaCl_2$)	139	14.6	8.80
10	Na_2SO_4	1cc. (96mgs. SO_4 = 142mgs. Na_2SO_4)	0	84	14.4	8.55

$CaCl_2$ was injected simultaneously with the zinc salfate.

of nembutal at the rate of 30 mg. per kilogram body weight. Hemoglobin was determined by the Newcomer method, and glucose was determined by the method of Folin and Malmros (4). The salt solutions were administered by stomach tube to rats fasted for 20 hours. For this purpose insulating tubing used in radio work and called "spaghetti" was employed. The concentration of the salts is given in the tables.

Discussion

Table I shows the rise in blood sugar caused by zinc in the form of the chloride; in addition, Table I also shows that the rise in blood sugar which accompanies the administration of glucose is increased by zinc chloride. Nembutal anesthesia causes a lowering of the blood sugar. Table II shows the effect of beryllium sulfate upon the blood sugar. Experiments tend to show that, when compared on a weight basis, beryllium as the sulfate is more toxic than zinc as the sulfate. Table III shows that subcutaneous injection of calcium chloride inhibits the increase of blood sugar caused by zinc sulfate, without diminishing the accompanying rise in hemoglobin and red blood corpuscles. Calcium chloride does not inhibit the blood sugar rise caused by administration of glucose. Calcium chloride injected subcutaneously one hour before or one hour after administration of $ZnSO_4$ did not lower the blood sugar, but when the two salts were administered simultaneously, the hyperglycemia was markedly reduced. Salant and Wise (3), however, did observe that calcium chloride did not affect glycosuria produced by zinc malate in rabbits. The absorption of zinc sulfate is slower than the absorption of zinc chloride as evidenced by the rate of blood sugar increase. The data given in Table IV for the rat agree with the work of Underhill (1) on the effect of $CaCl_2$ on hyperglycemia produced by magnesium sulfate in the rabbit. In addition it was found that

Table IV — The Inhibiting Effect of Calcium Chloride and Nembutal upon the Hyperglycemia Produced by Anesthetic Doses of Magnesium Sulfate upon the Fasting Rat

Rat No.	Volume of solution and mgs. of Mg as $MgSO_4$ dissolved and injected subcutaneously	Treatment of Animals	Volumes and mgs. of $CaCl_2$ dissolved. Sol. injected subcutaneously	Glucose mg./100cc 1.5 hours after dose
1	3/5cc. (14.4mg. Mg)	unanesthetized	none	135
2	3/5cc. (14.4mg. Mg)	anesthetized	none	87
3	3/5cc. (14.4mg. Mg)	unanesthetized	1cc. (5mg. $CaCl_2$)	88
4	3/5cc. (14.4mg. Mg)	anesthetized	1cc. (5mg. $CaCl_2$)	86
5	3/5cc. (14.4mg. Mg)	anesthetized	1cc. (5mg. $CaCl_2$)	87

The calcium chloride and magnesium sulfate were injected simultaneously.

nembutal anesthesia also inhibits the rise in blood sugar caused by subcutaneous injections of magnesium sulfate. Sulfuric acid also causes hyperglycemia and an increase in hemoglobin and red blood corpuscles when administered by stomach tube to rats fasted for 20 hours (Tables V and VI).

Table V — The Effect of Sulfuric Acid Administered by Stomach Tube on the Blood Sugar of the Fasting White Rat under Nembutal Anesthesia

Rat No.	Volume of solution and conc. of acid given	Initial blood sugar (mg./100cc.) after 20 hr. fast (Animals not anesthetized)	Blood sugar (mg./100cc.) 1 hr. after giving acid (Animals anesthetized)	Blood Sugar (mg./100cc.) 2 hrs. after giving acid (Animals anesthetized)
1	1cc. 2N H_2SO_4	102	102	126
2	1cc. 2N H_2SO_4	96	111	121
3	1cc. 2N H_2SO_4	83	105	125
4	1cc. 2N H_2SO_4	89	117	125
5	1cc. 2N H_2SO_4	82	88	143
6	1cc. 2N H_2SO_4	102	123	125

Table VI — The Effect of Sulfuric Acid Administered by Stomach Tube on the Hemoglobin, and Number of Red Blood Corpuscles of the Fasting White Rat under Nembutal Anesthesia

Rat No.	Volume of solution and conc. of acid given	Hemoglobin (gm./100cc.)		Red blood corpuscles $\frac{\text{million}}{\text{cmm.}}$	
		Initial after 20 hr. fast (Animals not anesthetized)	Final 2 hrs. later (Animals anesthetized)	Initial after 20 hr. fast (Animals not anesthetized)	Final 2 hrs. later (Animals anesthetized)
1	1cc.2NH_2SO_4	17.0	24+	10.48	14.54
2	1cc.2NH_2SO_4	16.4	22.5	10.86	11.48
3	1cc.2NH_2SO_4	17.5	24+	9.88	12.94
4	1cc.2NH_2SO_4	14.8	24+	9.74	15.28

Summary

Blood sugar may be increased 100-130 per cent above the normal fasting level by administration of certain zinc salts. When the same amount of zinc is given with glucose, the blood sugar may be increased 150-190 per cent above the normal fasting level in three hours. The absorption of glucose from the gastro-intestinal tract is delayed during nembutal anesthesia as evidenced by the blood sugar values when comparison is made with the data of Keil and Nelson (5). Nembutal anesthesia causes a lowering of the blood sugar of fasting rats.

Beryllium sulfate causes an increase in the blood sugar of fasting rats. The rise is not as rapid nor as marked as with a correspondingly toxic dose of zinc sulfate.

Calcium chloride may entirely inhibit the increase of blood sugar caused by zinc sulfate. In depressing the rise in blood sugar caused by zinc sulfate, calcium chloride has no effect upon the rise in hemoglobin and red blood corpuscles caused by zinc sulfate. Calcium chloride alone does not influence the number of red blood cells or hemoglobin values, nor does it have any effect upon blood sugar during the normal rise accompanying glucose absorption. The sulfate ion as sodium sulfate has no effect upon the blood sugar, red blood cell, or hemoglobin values.

The time of injection of calcium chloride relative to the administration of zinc sulfate is important to the inhibitory action of the former upon the blood sugar rise.

Magnesium sulfate causes no effect upon blood sugar when the salt is given by mouth. When subcutaneously injected, magnesium sulfate in proper concentration produces anesthesia. In this condition the blood sugar value rises. The rise in blood sugar produced by anesthetic doses of magnesium sulfate may be inhibited by either calcium chloride or nembutal.

Sulfuric acid also causes hyperglycemia and an increase in hemoglobin and red blood corpuscles when administered by stomach tube to rats fasted for 20 hours.

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Zinc deficiency causes growth retardation in chicks, particularly in the feathers and the tibia. It has been confirmed that 45-55 ug/g of zinc is required for the normal growth of a chick [1,2].

The upper limit of zinc intake has also been under investigation, and 1,500 ug/g or more of zinc added to the feed in the forms of zinc sulfate and zinc carbonate was found to decrease gain in body weight and food intake [3,4]. It has been reported [5] that 2,320 ug/g of zinc sulfate in drinking water stops the production of eggs by all experimental chickens on the third day.

An experiment on the effect of excessive intake of zinc on the growth of chicks was conducted to reconfirm the findings by Edward [6] and the results of an experiment previously conducted at this laboratory (unpublished), using three zinc compounds, zinc sulfate, zinc carbonate, and zinc oxide, which are often used as the source of zinc in chicken feed.

EXPERIMENTAL MATERIALS AND PROCEDURE

A total of 120 male Rock Horn chicks which hatched on May 20, 1961 were divided into 8 groups, each consisting of 15, and were subjected to this experiment for 25 days from the 5th day to the 30th day after hatching. In order to prevent mixture of zinc from other sources, they were raised in a plastic-covered battery bottomed with metal net, and feed and water were placed in synthetic paint-coated containers. Distilled water was given to the chicks.

Water and feed were given upon demand. The amount of water taken was measured daily and the amount of feed at five-day intervals by subtracting the remainder from the amount initially left in the container.

One-liter mess cylinders were used for measuring the amount of water taken, and caution was exercised to avoid errors at all times. Body weight was also measured along with feed intake, and the feed demand rate (intake in grams/ gain in body weight in grams) was obtained. A complete preparation with minerals and vitamins was used as the basal feed. The zinc content in the basal feed was 8.37%. General ingredients were measured by normal method [7].

Colorimetric determination [8] was performed with dithizone for the measurement of zinc.

TABLE 1. COMPOSITION OF BASAL FEED

Keys: a, raw material; b, content; c, yellow corn; d, wheat; e, wheat gluten; f, fat-free rice bran; g, fish waste; h, soy bean waste; i, alfalfa meal; j, table salt; k, powder cod liver oil; l, Orofac 10*; m, vitamin B_2; n, cholin-chloride; o, folic acid; p, biotin; q, *antibiotic additive containing 22 g of chlortetracyclin per kilogram; r, ingredient composition; s, water; t, crude protein; u, crude fat; v, crude fiber; w, crude ash content; x, soluble nitrogen-free substance; y, zinc.

TABLE 2. EXPERIMENTAL MATERIALS

Keys: a, experimental group; b, rate of zinc; c, source of zinc; d, additives other than zinc

The first group in Table 2 is the control, and three types of zinc compound were added to the basal feed for other groups at 2,000 and 3,000 ug/g. The amounts of zinc compounds added were decided according to the results of other experiments described in the literatures [3-5] and an experiment previously conducted at this laboratory (unpublished).

While expecting to see growth retardation due to the high levels of zinc, we conducted this experiment as a preliminary step toward the future study on the upper limit of zinc without adverse effects on growth, in which the amount

of zinc will be further subdivided.

Group 8 was given 3 mg% $CuSO_4 \cdot 5H_2O$ mixed in water in addition to zinc, following the experiment described in literatures 9 and 10.

RESULTS AND DISCUSSION

1. Effects on Growth

The table showing the changes in growth in the experimental groups (Table 3) clearly indicates differences according to the rate and the type of zinc added. With the exception of 2,000 ug/g ZnO group (group 2), the growth of the chicks was retarded as compared to the control group, the retardation particularly notable in 3,000 ug/g groups.

Of the three types of compound, $ZnSO_4 \cdot 7H_2O$ caused most notable growth retardation, followed in rank by $ZnCO_3$. The effect of ZnO was least notable. According to Roberson and Schaible [3] who investigated the effects of these three zinc compounds on chicks for 35 days, $ZnCO_3$ exhibited the strongest growth-inhibitive effect, followed in rank by $ZnSO_4$. ZnO showed the least effect. $ZnSO_4 \cdot 7H_2O$ at 3,000 ug/g (group 6), $ZnCO_3$ at 3,000 ug/g (group 7), and 3,000 ug/g of $ZnSO_4 \cdot 7H_2O$ + 3 mg% of $CuSO_4 \cdot 5H_2O$ (group 8) produced significant differences from the control within 1%, and $ZnSO_4 \cdot 7H_2O$ at 2,000 ug/g (group 3), within 5%. In the computation of the rate of demand for feed (feed intake in grams/gain in body weight in grams), decreases were noted in both factors as a result of high levels of zinc. With the exception of ZnO groups, all 3,000 ug/g groups showed higher rates of demand than the control.

TABLE 3. GROWTH PATTERN

Keys: a, rate of zinc added; b, source of zinc; c, gain in body weight, 5th to 30th day after hatching; d, index; e, feed demand rate (1), 5th to 30th day after hatching; f, (1) feed demand rate (feed intake in g/ gain in body weight in grams). (2) standard deviation. (3) *the difference

from the control significant at $P < 0.05$. ** the difference from the control significant at $P < 0.01$.

Figure 1. Growth Rate.

Keys: a, 5 days old; b, 10 days old; c, 15 days old; d, 20 days old; e, 25 days old; f, 30 days old; g, control group (group 1).

Growth rate curves (Figure 1) were obtained by plotting weight gains measured at 5-day intervals and expressed against the control point set at 100.

With the exception of 2,000 ug/g ZnO group (group 2), the growth retardation attributable to zinc became notable from the 5th to 10th day of the experiment.

TABLE 4. FEED AND WATER INTAKES (15 chicks per group)

Keys: a, total intake; b, index; c, total water intake; d, index; e,

Note: The total feed and water intakes refer to the intakes during the period of experiment (25 days). (1) standard deviation. (2) *the difference from the control group significant at $P < 0.05$.

As compared to the control, all groups indicated retardation in growth. Both feed and water intakes decreased gradually with time (Table 4). The correlation coefficient (r) of the two was +0.845, a considerably high value.

In the groups whose feed intake was less than that of the control, the intake of water was also lower, particularly in 3,000 ug/g groups.

2. Effects on the Tibia.

Zinc deficiency increases the width of the tibia, inhibits its lengthwise growth [3], widens the heals in chicks, and cause parakeratosis of the epidermis cuticle of the legs as commonly observed in pigs with zinc deficiency [11].

In this experiment, 5 chicks were selected at random from each group, and

the width, length, and weight of their tibial bones were measured. The tibial width in the central part of the bone and the length from the convex at the terminal of the bone on the femoral side to the concave at the terminal on the metatarsal side were measured with M-type 15 cm scaled slide calipers.

According to O'Dell et al. [1], the tibial width divided by the body weight then multiplied by 1000, the length subtracted by the width, and the weight of the tibia are indicated.

The results of measurement (Table 5) showed differences between the groups. With the exception of ZnO group, 3000 ug/g groups showed marked differences from the control group. As O'Dell et al. [1] and Roberson et al. [3] observed in their zinc deficiency experiments, excessive intake of zinc salts also resulted wider and shorter tibia.

It is yet to be clarified what physiological action causes such changes when zinc accumulates in the tibia.

3. Effects on the Blood.

A high level of zinc in mice is known to cause anemia [9]. The hematocrit values (blood corpuscle volume) of zinc deficiency chicks have been published [1]. However, no report has yet been made on hematological findings of chicks fed on excessive amount of zinc. In this experiment, hematocrit values and erythrocyte sedimentation speed were studied.

Hematocrit value was measured by the hematocrit method using Wintrobe's hematocrit tube and erythrocyte sedimentation speed, by Westergren's method [13]. Five chicks from each group were subjected to this experiment, and immediately after the test, they were phlebotomized by cardiocentesis. A 3.8% sodium citrate solution was used to prevent blood coagulation.

As shown in Table 5, the erythrocyte sedimentation speed increased but hematocrit value decreased in all 3,000 ug/g groups except for the ZnO group.

The results seem to indicate that the excessive intake of zinc caused

anemia as in chemical intoxication. Effects of zinc at high levels were also noted in the comb and the conjuctiva even in this experiment.

O'Dell et al. [1] observed a decrease in hematocrit value in their deficiency experiment. In this experiment, the value was also lower, and the correlation with erythrocyte sedimentation speed was witnessed by Sturkie et al. [14] was also noted.

4. Effects of internal zinc accumulation.

Five chicks from each group were subjected to this analysis.

TABLE 5. EFFECTS ON THE GROWTH OF THE TIBIA AND HEMATOLOGICAL IMAGE (5 to 50 days in age)

Keys: a, rate of zinc added; b, source of zinc, c, the tibia; d, (tibial width/body weight) x 10^3 (1): e, length/width (2); f, weight of the tibia; h, 6 hours; g, change in erythrocyte sedimentation speed (3); i, 24 hours; j, hematocrit value (4); k, (1) body weight at completion of experiment (g) :tibial width (cm) ratio multiplied by 10^3. (2) tibial length : tibial width ratio. (3) measured value at a specific time. (4) hematocrit value : erythrocyte volume %, measured by the hematocrit method. The measured values are the mean values of five chicks.

After the liver, the tibia and the pectoral muscle were carcinated, colorimetric determination of zinc was performed with dithizone. The liver was also subjected to the colorimetric determination of iron with o-pherantroline [15].

As shown in Table 6, internally accumulated zinc exerted similar effects on the gain in body weight and growth. In the $ZnSO_4$ at 3,000 ug/g plus $ZnCO_3$ group, the accumulation of zinc was considerable.

Comparing the accumulation of zinc per gram of the liver, the tibia and the pectoral muscle, the liver and the tibia gave approximately the same value with a high correlation coefficient of +0.940 at a risk of less than 1%.

Significant differences within 1 to 5% was noted in the zinc accumulation in the liver and the tibia between each of the experimental groups and the control group, but the amount of zinc stored in the pectoral muscle was about the same for both the control and the experimental groups, the amount being smaller than that in the liver or the tibia.

The amount of iron in the liver was approximately the same in all groups. This disagrees from the results of Cox's experiment [9] in which a decrease in the amount of iron in the liver was related to high level of zinc given to the animals.

In this experiment, a similar growth pattern was shown by 3,000 ug/g $ZnSO_4 \cdot 5H_2O$ group (group 8), but the latter exhibited an increase in the erythrocyte sedimentation speed and an increase in the zinc accumulation per gram of the liver, as compared to the former.

The experiment failed to confirm the observation by Maynard and Loosli [10] that the anemia in mice due to excessive intake of zinc is cured by the addition of copper.

It is hoped that the investigation be expanded to include the effect of the difference in the type of copper salt and the amount of copper added.

Copper is essential to the formation of hemoblobin (Hb) by iron, and is believed to possess a catalytic action. The observation by Cox et al.[9] that iron in the liver decreases in anemia caused by the deficiency or excessive intake of zinc is yet to be confirmed. Reviewing the levels of iron in the liver in this experiment, it is unlikely that excessive amount of zinc lowers the iron level in the liver to a notable degree. Even if Hb was decomposed in

the liver by excessive zinc, iron is retained in the liver cells and remains as brown hemosiderin pigment. Thus, a number of problems are yet to be studied in the future, such as the passage of iron in the blood, the binding of iron with copper and other inorganic substances, etc.

The sites of internal physiological mechanism where zinc is retained at relatively high levels were investigated. As a result, the liver and the tibia were found to retain a considerable amount of zinc. In our experiment (unpublished), the amount of zinc in the feathers was less than that in the tibia. In regard to internal distribution of zinc, Rahman et al. [16] demonstrated that the distribution changed with elapsed time. They also observed a relatively high level of zinc in the liver, the observation being in agreement with the results of this experiment.

The correlation between gain in body weight and zinc accumulation is illustrated in Figure 2, where x axis represents the logarithm of zinc accumulation (ug) in the liver, the tibia and the pectoral muscle and y axis, gain in body weight (g). High negative correlation values, -0.798 and -0.9266 were obtained for the liver and the tibia, respectively, and a positive correlation value, +0.777, for the pectoral muscle.

The regression equation was $Y = -122\ x + 570$ for the liver and $y = -136x + 621$ for the tibia within the risk of 1 - 5%, and $y = 323\ x - 541$ for the pectoral muscle. In other words, zinc accumulation in the liver and the tibia was high in the groups with marked growth retardation due to excessive intake of zinc, whereas the level of zinc in the pectoral muscle was lower in such groups.

The accumulation of zinc was highest in the 3,000 ug/g $ZnSO_4 \cdot 7H_2O$ group. The group to which 3 mg% of $CuSO_4 \cdot 5H_2O$ was also given for the anticipation that the addition may prevent the growth retardation due to zinc and the accumulation of zinc, failed to confirm such effects of copper.

TABLE 6. ZINC ACCUMULATION

Keys: a, experimental group; b, rate of zinc added; c, source of zinc; d, mean analytical value (1); e, the liver; f, zinc; g, iron; h, zinc in the tibia; i, zinc in the pectoral muscle; j, (1) The measured values are the mean values of 5, indicating the amount accumulated (ug) in 1 g of the organ. (2) Standard deviation. (3) *The difference from the control group is significant at $P < 0.05$. ** The difference from the control is significant at $P < 0.01$. (4) Correlation between the amount of zinc per gram of the liver and that per gram of the tibia. Correlation coefficient (r) = +0.940 was obtained at a risk less than 1%. Reliability limit of the correlation coefficient (p) $+0.508 < P < +0.994$.

FIGURE 2. CORRELATION BETWEEN GAIN IN BODY WEIGHT AND AMOUNT OF INTERNALLY ACCUMULATED ZINC.

Keys: a, gain in body weight; b, the liver; c, the tibia; d, the pectoral muscle; e, regression equation; f, correlation coefficient; g, amount of zinc accumulated.

The zinc compounds showed different internal distribution patterns. The difference may be attributed to the pattern in which they are decomposed in the intestine, or the presence of other inorganic substances in the excretory process. There are still many unknown facts on the internal physiological action of zinc, which must be investigated in the future.

Zinc sulfate among the three compounds tested showed the most notable growth inhibitive effect. Roberson et al. [3] who tested the same zinc compounds at 2,000 - 3,000 ug/g reported that zinc carbonate showed the strongest effect on the growth.

In the measurement of zinc in the liver, zinc sulfate was accumulated in the largest quantity among the three compounds. To what degree SO_4 is related

to the accumulation and excretion of zinc is not yet confirmed.

It has been confirmed by many investigators [17-19] that the amount of Ca, P, Mn, Fe, and Cu added has a close relationship to the accumulation and excretion of zinc.

SUMMARY

1. Three types of zinc compound (zinc oxide, zinc sulfate, and zinc carbonate) were given to chicks to investigate the effects of zinc at high level. As a result, growth retardation, considered to be the direct effect of a large intake of zinc, was most notable in zinc sulfate groups, followed in rank by zinc carbonate groups. The effect was least significant in zinc oxide groups.

2. The effects of zinc were clearly indicated by 3,000 ug/g sinz sulfate and zinc carbonate groups, and less notable in 2,000 ug/g groups and 3,000 ug/g zinc oxide group.

3. A large intake of zinc caused growth retardation, changes in the tibial bones, poor feathers and anemia.

4. Approximately the same amount of zinc was accumulated in the liver and the tibia, with correlation coefficient (r) = +0.940, a significantly high value. The accumulation of zinc was highest in the groups where the growth retardation due to excessive intake of zinc wasfound to be more notable.

REFERENCES

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EXPLANATION OF THE PHOTOGRAPHS

Photograph 1. The growth of feathers in the experimental groups.

No. 1. Control. Normal growth.

No. 2. 2,000 ug/g ZnO group and (No. 4) 2,000 ug/g $ZnCO_5$ group. No marked

difference from the control group.

No. 3. 2,000 ug/g $ZnSO_4 \cdot 7H_2O$ group. Hypoplasia of the dorsum and the alary muscle.

No. 5. 3,000 ug/g ZnO group, No. 7. 3,000 ug/g $ZnCO_3$ group. Hypoplasia of the dorsum, the alary muscle, and the tail.

No. 6. 3,000 ug/g $ZnSO_4 \cdot 7H_2O$ group. Overall hypoplasia.

Photograph 2. Comparison between the control group and No. 6 with severe hypoplasia of the feathers.

No. 1. Control group. Normal growth.

No. 6. 3,000 ug/g $ZnSO_4 \cdot 7H_2O$. Overall hypoplasia.

Photograph 3. Comparison between the leg of the control group and that of No. 6 with most severe hypoplasia of the feathers.

No. 1. Control group. Normal.

No. 6. 3,000 ug/g $ZnSO_4 \cdot 7H_2O$. Slightly pale (anemic symptom)

AMER. J. PHYSIOL. 80: 65-74. (1927).

THE EFFECT OF ZINC ADMINISTRATION UPON REPRODUCTION AND GROWTH IN THE ALBINO RAT, TOGETHER WITH A DEMONSTRATION OF THE CONSTANT CONCENTRATION OF ZINC IN A GIVEN SPECIES, REGARDLESS OF AGE

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Received for publication September 17, 1926

THE EFFECT OF ZINC FEEDING UPON REPRODUCTION AND UPON THE GROWTH OF YOUNG RATS. A few years ago Bertrand and Vladesco (1920, 1921c) made the observation that the normal zinc concentration of various male reproductive organs and fluids in the sheep, bull, pig, horse, herring and man was often noticeably high compared with the concentration in the entire body. In the herring, a high concentration in the testes seemed to be correlated with the period of physiologic functioning. The results obtained in this laboratory during the past two years on rats, cats and dogs have indicated that the normal zinc concentration of both male and female reproductive organs is often very high. In the male cat and dog, the testes only were analyzed and were found to be rather low in zinc. In the rat, however, the seminal vesicles were included in the analysis and the zinc concentration was uniformly high compared with the concentration in the total animal. These findings gave added weight to the suggestion advanced by Bertrand and Vladesco that zinc may normally play a very important rôle in the phenomenon of reproduction. In this connection, we felt it of interest to determine whether or not zinc feeding would produce any variation from the normal in the content of the reproductive organs or in their physiological functioning.

Experimental procedures. The albino rat was chosen as being the most suitable animal in which to study growth and reproduction without unduly prolonging the experimental period. One year of a rat's life, as worked out by Donaldson (1924, p. 3) is approximately comparable to thirty years in the human cycle. White rats reproduce freely and often, the gestation period being only three weeks, and the number of young in a litter averaging about seven.

Our experimental parent rats were, for the most part, adult males and females which had received previously for twenty-nine weeks from the time they were six weeks old, in the form of organic zinc salt solutions

(malate, acetate and citrate), daily doses of zinc ranging from 4.4 to 12.4 mgm. A few of the parent males, however, were white rats of different stock which had been on heavy suspensions of zinc oxide in gum acacia —34.4 mgm. of zinc daily—for twenty-nine weeks (from the time they were twenty-six weeks old).

Under the régime of zinc feeding, the organic zinc salt rats had grown in a normal manner and appeared perfectly healthy. The zinc oxide males, however, though their growth had been normal, had, unfortunately, been in contact with other rats which had so-called rat pneumonia. As it developed soon after the experiment was begun that the zinc oxide males were infected with this disease, they were eliminated from the experiment after they had served as sires in three matings, one of which proved sterile, and were replaced with one of the zinc malate sires and with another rat which had been on zinc acetate solution (2 mgm. of zinc daily) for forty-six weeks (from the time he was twelve weeks old), but which had been in isolated confined surroundings and on a slightly different diet. This zinc acetate rat was in good health, but was, like the zinc oxide sires, nearly twice as old as the female with which he was mated.

For controls, we mated rats which were of the same age and which had been maintained in the same surroundings for the same length of time and on the same diet as the zinc salt rats, but with water to drink instead of zinc salt solutions.

Throughout the experiment, the daily diet was as follows:

Stock mixture:	
Rolled oats	5 parts
Hominy	3 parts
Beef scraps	2½ parts
Milk powder	1½ parts
Salt	trace
Molasses horse feed (alfalfa, corn, oats and molasses)	
Dog biscuit	
Lettuce twice weekly	

Liquid, which was always measured in order to have a record of fluid consumption, was given either in the form of distilled water, a zinc salt solution, or a suspension of zinc oxide in 3 per cent gum acacia, according to the group to which the mated pair belonged. In the case of the zinc salt rats, the zinc dose was calculated from the fluid consumption. For the zinc oxide rats, a solution was made up weekly containing a known analyzed amount of zinc oxide and from this stock the cage burettes were filled as needed. At the end of the week all that was left of the stock solution was analyzed for zinc and subtraction of the two analyses gave the figure for the amount of zinc the pair of rats had ingested. This procedure was necessary, owing to the tendency of the zinc oxide to settle

TABLE 1

Results of fertility experiments with zinced rats and their controls

SERIES	PARENTS*			OFFSPRING		REMARKS
	Average daily dose of each pair of rats after mating (calculated as zinc)	Average daily dose of each rat before mating (calculated as zinc)†		September 28, 1925–January 2, 1926		
		Male	Female	Total number per female in number of litters	Deaths	
	mgm.	*mgm.*	*mgm.*			
D	Zinc acetate, 6.3	Zinc malate, 12.4	Zinc acetate, 4.4	6 in 2 litters	0	
E	Zinc malate, 16.5	Zinc malate, 12.4	Zinc acetate, 4.4	18 in 2 litters	0	8 embryos found at autopsy (1 litter)
F	Zinc malate, 11.1	Zinc malate, 12.4	Zinc citrate, 9.7	11 in 2 litters	1	
H	Zinc citrate, 10.2	Zinc oxide, 34.4	Zinc citrate, 9.7	15 in 3 litters	3	
L	Zinc acetate, 1.75	Zinc acetate, 2.0				
I	Zinc oxide, 6.5	Zinc oxide A, 34.4	Zinc citrate, 9.7	20 in 2 litters	19	Series I was sterile
J	Zinc oxide, 22.2	Zinc oxide B, 34.4				
M	Zinc oxide, 38.0	Zinc malate, 12.4				
Totals for five zinced females				70 in 11 litters	23	
Average number per litter				6.4		
Controls						
A	Water	Water	Water	14 in 3 litters	14	
B	Water	Water	Water	23 in 3 litters	12	
C	Water	Water	Water	19 in 2 litters	2	4 embryos found at autopsy (1 litter)
Totals for three control females				56 in 8 litters	28	
Average number per litter				7.0		

* All rats approximately 250 days old when mated, except the males in series H, L, I and J, which were approximately 410 days old.

† All rats were on zinc for 29 weeks previous to mating, except the male in series L (46 weeks).

out and thus to render inaccurate, dose figures obtained from fluid consumption.

The males were kept with the females throughout the experiment, so that second and third matings were dated from the time the first and second litters were born.

At the birth of a litter, two of the young were weighed and chloroformed for zinc analysis. The rest of the litter, regardless of number, was left with the mother for twenty-three days. The young rats were then weaned and distributed in other cages according to whatever zinc dose was allotted to them. In this manner, each pair of parent rats had two litters, each of which was allowed to grow to at least sixty days of age, in order to determine the effect of zinc on their early growth curves. In a few cases, three litters were born.

As the experiment carried to this point had not demonstrated any significant variation from the normal that could be attributed to zinc, we did not proceed with enlarging the numbers in the second generation or with breeding the second and third generations of rats, as had been our original plan, the work entailed far outweighing the value of the results to be anticipated.

Experimental results. Table 1 shows how the rats were mated; the periods of time during which they had been zinced previous to mating: their zinc doses during this experiment; the number of litters; the total number of offspring: and the number of young which died during the period they were with their parents. The figure for deaths among the young includes deaths from all causes—still-born births, young killed and eaten by the parents (a very common occurrence among normal rats (Evans and Bishop, 1923)), etc.—and is not at all an index of the health of the young at birth. No young rats died after weaning.

To the five females in the zinced group, seventy young were born (also eight unborn embryos found in one female at autopsy) during a 96-day period. There were eleven litters (the twelfth was embryonic) from fifteen matings. This is 15.6 offspring (including embryos) per mother, or 5.2 per mating. Thirty-two and nine-tenths per cent of the young born died or were killed by the parents.

In the control group, to the three mothers fifty-six young were born (also four embryos found) during the same period of time. There were eight litters (the ninth was embryonic) resulting from nine matings. This is 20.0 offspring, including embryos, per mother, or 6.6 per mating. Fifty per cent of the young born died or were killed by the parents.

The good health subsequent to weaning of the young in the various litters—zinced rats and water controls alike—as indicated in figures 1 and 2, which present the average growth curves of the various litters as compared with Donaldson's (1924, p. 178) average normal growth curves

for male and female albino rats. At the time of weaning, each litter was put on the same régime as the parents, i.e., litters from water parents were put on water, litters from zinc malate parents were put on zinc malate, etc. There was one exception to this procedure. Owing to lack of zinc oxide offspring, after weaning we put one of the second litters of zinc malate parents on zinc oxide.

The average curves given in figures 1 and 2 are made up of the following number of individual curves:

	Males	*Females*
Water rats	9	12
Zinc malate	13	4
Zinc citrate	3	5
Zinc acetate	4	1
Zinc oxide	3	3
Donaldson's normal rats	50	50

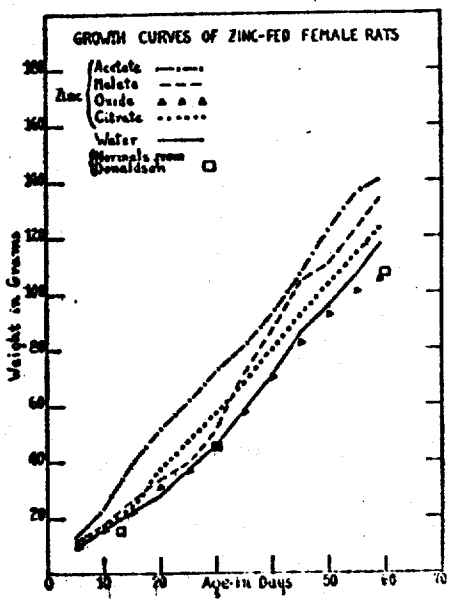

Fig. 1

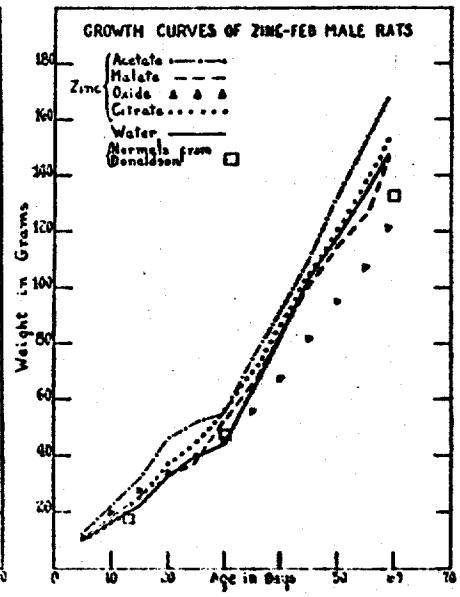

Fig. 2

Fig. 1, Growth curves of zinc-fed female rats plotted against the average growth curve of normal female rats, as determined by Donaldson.

Fig. 2. Growth curves of zinc-fed male rats plotted against the average growth curve of normal male rats, as determined by Donaldson.

Several of the second litters of offspring were transferred, at the end of the 60-day period, to another laboratory, where they were later used for breeding purposes. They reproduced in a perfectly normal manner, having large litters with no history of the death of any considerable number of the offspring.

At autopsy, the reproductive organs of the mated zinced male rats showed no difference in zinc concentration from that of unmated zinced males, and the reproductive organs of the zinc-fed rats showed no increase in zinc over those of the normals. Rat ovaries were not analyzed, since they are too small to give reliable analytical results.

Summarizing, we may say that the feeding of organic zinc salts or of zinc oxide in doses of from 2 to 38 mgm. of zinc daily to albino rats, not only for many weeks previous to mating, but during pregnancy and lactation as well, has no significant effect upon the health of the parents, upon fertility, nor upon the health and the early growth of the offspring. The offspring of zinced parents, weaned at twenty-three days of age and themselves put upon daily doses of zinc, grow at a normal rate and to a normal adult size.

The experiment has not thrown any light upon the cause of the high zinc concentration often found in reproductive organs, nor upon the possible rôle which zinc may play in the phenomenon of reproduction.

THE EFFECT OF ZINC FEEDING UPON THE TOTAL ZINC CONTENT OF RATS OF DIFFERENT AGES, TOGETHER WITH DATA UPON THE NORMAL ZINC CONTENT OF NEW-BORN, 60-DAY OLD AND ADULT RATS. In 1921, Bertrand and Vladesco (1921a) reported figures upon the zinc content of rabbits, guinea pigs, hens, mice and fish, raised under controlled conditions and ranging in age from three days to seven years. On the basis of figures obtained on the mouse, the hen, and the fish, *Idus Orfus*, these authors concluded that in general zinc is at a maximum in young life, being in all probability highest at birth; that it then diminishes gradually to a minimum level; and that it may rise again in old age. A second experiment by these same investigators (1921b) on rabbits a few hours to six weeks old, raised under controlled conditions but in a different laboratory and on a different diet from those in the first experiment, led to the conclusion that the total zinc content of the rabbit is at a maximum at birth, declines during lactation, and increases rapidly again when the animals are put on a regular mixed diet after being weaned. In our opinion, many more corroborative figures are needed in order to rule out normal variations which may possibly occur at any given age, even on a constant diet.

Giaya (1929) from analyses of viscera obtained at fourteen human autopsies on individuals who had given no history of having ingested zinc in excess of the normal amount in food, concluded that zinc content increases with age. The figures which Giaya presented support his assumption, but, in our opinion, the surroundings and diet of his subjects were too uncontrolled to warrant his results serving as a sound basis from which to draw such conclusions.

Lutz (1926) analyzing ten normal rats living on a constant diet, found approximately uniform concentrations of zinc (0.022 to 0.035 mgm. per

TABLE 2

Effect of zinc-feeding on zinc concentration of new-born, 60-day old and adult rats

AGE OF RAT	ZINC CONCENTRATION, MILLIGRAM OF ZINC PER GRAM OF TISSUE						REMARKS
	From water parents	From zinc malate parents	From zinc acetate parents	From zinc citrate parents	From zinc oxide parents		
New-born	0.030 0.030 0.030 0.032 0.032 0.040 0.040 0.040 0.041 0.050 0.051	0.038 0.039 0.040 0.042 0.048 0.051 0.052	0.033	0.033 0.042	0.034 0.043 0.052 0.054 0.042		
Average.....	0.038	0.044	0.033	0.039	0.045		
	On water	On zinc malate: 8.5 mgm. zinc daily	On zinc acetate: 5.0 mgm. zinc daily	On zinc citrate 16 mgm. zinc daily			
60 days	0.035 0.040 0.043	0.031 0.042 0.047 0.060	0.038 0.042	0.037 0.042 0.043			On zinc doses from the age of 3 weeks
Average.....	0.039	0.046	0.040	0.041			
	On water or 3 per cent gum acacia	On zinc malate: 13.6 mgm. zinc daily	On zinc acetate: 6.3 mgm. zinc daily	On zinc citrate: 10.2 mgm. zinc daily	On zinc oxide: 24.4 mgm. zinc daily	On zinc oxide: 0.5 to 24.0 mgm. zinc daily	
Adults 325± days		0.038 0.034 0.030	0.042 0.043	0.048 0.040			
			1 to 2 mgm. zinc daily				
450± days	0.038 0.038 0.039 0.039 0.041 0.042		0.043 0.050		0.035 0.047 0.050 0.055 0.061	0.042 0.048 0.052 0.049 0.051 0.061 0.058	Zinc salts rats on doses from age of 6 weeks: zinc oxide rats, from age of 26 weeks
Average.....	0.040	0.034	0.045	0.044	0.050	0.052	

gram) at all ages from approximately twenty-one days of age through an ascending series up to 160 days of age.

Fairhall[1] in this laboratory analyzed six approximately 500-day old rats, which had been for twenty-eight weeks under the strictly controlled régime of a normal zinc balance experiment, and found their total body concentration in zinc to be very uniform—viz., 0.032 to 0.036 mgm. per gram.

The control rat families in our fertility experiments provided us with excellent material for additional study upon the influence of age on zinc concentration in rats. We wished to see, furthermore, what effect, if any, zinc feeding might have on the total zinc concentration at different ages.

Experimental procedures. A few hours after a litter of rats was born, two members of the litter were chloroformed for analysis. A second group of rats was analyzed when sixty days old; and a third group in adult life.

The new-born animals were washed with dilute hydrochloric acid and distilled water, to remove any possible extraneous zinc that they might have picked up from their surroundings. All the other animals had the gastro-intestinal tract removed intact, slit up, and the contents washed out. The hides, and, in the case of the zinced animals, the mouths and throats were also thoroughly washed.

The methods of zinc analysis were those outlined by Fairhall (1926). Zinc, when under 2 mgm. in amount, was estimated by the ferrocyanide turbidimetric method; when over 2 mgm., by the ferrocyanide titration method.

Experimental results. The effect of zinc feeding upon the total zinc concentration of new-born, 60-day old, and fully grown rats is shown in table 2. This table also gives control figures upon the normal zinc concentration of rats of different ages. These latter figures for the total zinc concentration in normal rats are, on the whole, slightly higher than those of Fairhall and of Lutz—an observation which may possibly be accounted for by the fact that at the time of our experiment our stock foods were running higher in zinc than had been the case with theirs.

There is no indication from our figures of any correlation of the age of the animal with the total zinc concentration the average zinc concentration (in milligrams per gram of tissue) of new-born, 60-day old and adult rats being, respectively, 0.038, 0.039 and 0.040 mgm. The individual figures in the three groups range from 0.030 to 0.051 mgm.[2]

[1] Unpublished data. Personal communication.

[2] Our opinion that new-born animals do not show a significantly higher zinc concentration than do adult animals of the same species is further confirmed by determinations of the zinc content of three new-born (placenta still attached) kittens, vigorous but of poor stock, found abandoned in the laboratory. We had no clue as to the mother's history except that she was a stray cat and had not been zinc-fed.

There seems also to be no significant increase in zinc concentration with zinc feeding, except possibly in the case of the adult zinc oxide rats, where both the average and the individual analyses are somewhat higher than those of normal rats of the same age. There are, however, a few cases where new-born and 60-day old rats have reached as high a level, but this was not maintained by the group as a whole. In terms of total zinc in the rat, a control adult with a zinc concentration of 0.040 and weighing 216 grams without intestinal contents had, for example, a total zinc content of 8.56 mgm. A zinc oxide adult of approximately the same weight, 218.6 grams (with a concentration of 0.056), had a total zinc content of 12.14 mgm. The total increase in zinc content during the long period of zinc feeding was, therefore, in this particular case 3.58 mgm., an amount not very significant in terms of storage.

SUMMARY AND CONCLUSIONS

The feeding of organic zinc salt solutions (acetate, citrate and malate) or of zinc oxide suspensions in doses of from 2 to 38 mgm. of zinc daily to albino rats, not only for many weeks previous to mating, but during pregnancy and lactation as well, has no significant effect upon the health of the parents, upon fertility, nor upon the health and early growth of the offspring.

The offspring of zinced parents, weaned at twenty-three days of age and themselves put upon daily doses of zinc, grow at a normal rate and to a normal adult size.

The average normal zinc concentration of albino rats does not vary significantly at different ages, the average total zinc concentrations of new-born, 60-day old, and of adult rats being, respectively, 0.038, 0.039 and 0.040 mgm. of zinc per gram of tissue. This constancy of zinc concentration, regardless of age, is suggestive evidence of a functional importance on the part of this metal.

With the exception of slight increases in total zinc concentration resulting from the feeding of heavy zinc oxide suspensions over long periods of time, the ingestion of zinc in the form of organic salt solutions or of oxide suspensions has no effect upon the total zinc concentration of the albino rat. That is to say, zinc compounds given by mouth to the rat do not usually result in storage of zinc in the animal in amounts above the normal.

Zinc analyses on these kittens gave the following figures for total zinc concentrations: 0.022, 0.023 and 0.030 mgm. per gram. The average zinc concentration of an adult cat according to Lutz (1926) is 0.033; according to our own observations, 0.031. The variation between these sets of figures is well within the normal variations at any one age.

If storage in excess of the normal quota does occur, the amounts stored are very small and have no effect upon the health of the animal.[3]

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[3] A detailed discussion of the absorption, excretion and storage of zinc, with a report of experiments upon the feeding of zinc to cats and dogs, may be found in a recent article by the authors (Drinker, Thompson and Marsh, 1927).

Physiol. Rev. 39(3) (1959)

Biochemistry, Physiology and Pathology of Zinc

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METALLIC ZINC WAS NOT DISCOVERED as early as many of the other metals, but its very important use as an alloy with copper in brass manufacture antedates the earliest records of investigation. Since zinc does not occur in an uncombined state in nature, the discovery of brass presumably was a happy accident of an ancient serendipitous metalsmith who melted copper in the presence of zinc ore and carbon. Hence the name, zinc, from the German "of unknown origin." The desirable physical properties of brass and its physical resemblance to gold made the alloy very desirable.

The Chinese probably were the first to extract zinc metal, though its first description in 1597 by an occidental traveler, Liborius, related that it was observed in India. It was originally sold as 'spialter,' from which the industrial name 'spelter' derives, a term which now applies to less pure zinc.

The element has been estimated to represent 0.004 per cent of the earth's crust and is twenty-fifth in order of abundance, certainly far scarcer than less familiar elements such as zirconium, vanadium, titanium, or strontium. Zinc usually occurs in combination with sulfur or oxygen. Cadmium usually occurs in zinc ores, its name being derived from *cadmia formicum*, furnace zinc, a name applied by early metallurgists to flue dust from working calamine zine ore. The ores of zinc are widely distributed, the principal one being the cubic sulfide, sphalerite.

The element has the atomic number 30 and atomic weight 65.38. There are several isotopes, of which Zn^{65}, with a half-life of 250 days, has been most widely used for biological studies. Zn^{63}, with a much shorter half-life, 38 minutes, has been employed where this property is a desirable factor, as in therapeutic investigations.

The ready reactivity of zinc with negatively charged groups may be pertinent to its significance in biology, where it is aggregated over and above other elements more abundant in the environment.

The presence of zinc in living organisms and its role as an essential nutrient for plants and animals has been recognized ever since it was shown by Raulin (226) to be necessary for the growth of *Aspergillus niger*. Its occurrence in biological matter was first described by Lechartier and Bellamy (144). In the same year, Raoult and Breton (225) described the presence of zinc in human liver. The next 50 years saw only sporadic interest in the biological role of this element (240).

[1] Supported by grants from the National Institutes of Health and the Howard Hughes Medical Institute.

Difficulties in methodology limited most investigations to qualitative efforts. It was realized that the element was a common constituent of plants and animals, yet the evidence for its universal occurrence in all living matter was so fragmentary that it discouraged generalizations as to its biological role.

Critical examination of the existent, sparse literature led Lutz (155) to study the distribution of zinc. He found it present in all organs of the rat, cat and man. The concentrations were constant within the error of the method, and the presence of the element could not, therefore, be attributed to adventitious contamination.

Not until 1934 was conclusive evidence adduced that zinc is essential to normal growth and development of animals. The mouse was studied first (20, 21 85); a year later this conclusion was extended to the rat (268). Almost simultaneously, convincing evidence accrued which implicated the specific deficiency of this element in a variety of pathological states of higher and lower plants, exemplified by chlorosis in maize (12), even though the indispensability of zinc in the growth of corn had been suggested earlier (173).

The isolation and purification of an enzyme, carbonic anhydrase, containing 0.33 per cent zinc as part of its molecule, by Keilin and Mann (129) offered the first concrete explanation of a mode of action of this element. Zinc was demonstrated to be essential to the mechanism of action of this enzyme, which catalyzes the dehydration of carbonic acid and participates in the elimination and incorporation of carbon dioxide.

The recent discovery of zinc in many highly purified enzymes (293) has revealed the diversity of its function in protein and carbohydrate metabolism, owing to its presence in several dehydrogenases of many species, a peptidase, and a transphosphorylase, jointly covering the whole evolutionary spectrum.

The discovery of zinc deficiency as the basis of porcine parakeratosis (288) was the long-expected implication of this nutrient in a natural, pathological process of a higher animal species. The findings of metabolic abnormalities in man, as in postalcoholic cirrhosis of the liver, are the inevitable results of the tedious and difficult fundamental studies (313, 316). The ubiquitous distribution of zinc had been the teleological basis of expectations of such discoveries.

At this juncture, it may be predicted safely that this element, which plays a cardinal role in significant aspects of metabolism, will be shown to be involved crucially in other aspects of human and animal pathology.

Zinc and zinc metabolism have been reviewed repeatedly (1, 101, 294, 295, 298, 343). In the following discussion an effort has been made to appraise the existent literature in the light of our present knowledge of the biochemical function of the metal and to integrate this information wherever possible into the compositional, physiological, pathological, and nutritional data which have accumulated.

Methodology

As in the study of other trace elements, problems of methodology have featured in the investigation of the biological occurrence and function of zinc. The slow progress in this field can surely be attributed to difficulties in the analysis of the small concentrations of zinc present in tissues and fluids. As a consequence

of poor methodology much of the early analytical work is of dubious value (155) and will, therefore, not be recapitulated. This literature and the considerations pertinent to these judgments have recently been reviewed and documented in detail (157, 283, 298).

The physical and chemical methods employed for the determination of zinc and the awareness and avoidance of contamination by the analyst are most important aspects in the evaluation of data and of the conclusions drawn from them. Scrupulous chemical sterility and fastidious laboratory technique, analogous to those considered mandatory in bacteriological work, are essential since zinc is a ubiquitous contaminant.

Convenient biological samples for zinc analysis may be expected to contain microgram amounts of the metal. Thus, almost universally, methods of high sensitivity are called for. While a variety of specialized techniques may be employed, colorimetry, polarography and emission spectrography presently constitute the analytical approaches most likely to be useful to workers interested in analytical methods of tested validity (4, 97, 105, 138, 179, 195, 235, 239, 262, 283, 292, 296, 300).

Fundamental Chemical Considerations

In contrast to the highly colored iron and copper proteins and enzymes, zinc compounds, in general, and zinc proteins, in particular, are colorless and, therefore, do not attract attention to themselves. The red and blue colors of iron and copper proteins seem to have been responsible for their early recognition and systematic study. Possibly due to their lack of visible color, the identification of zinc proteins has been more or less fortuitous.

Biologically, zinc is found to be complexed to organic ligands rather than free in solution as the metallic ion. The physical chemistry of these zinc complexes has received special attention by some investigators to predict biological phenomena or biochemical reactivity on the basis of structural analogies. Most of the work in the field of zinc coordination chemistry, of course, has not been undertaken with such a biological view in mind. Physical chemical information is often quoted out of context, in order to fit a given biological hypothesis, thus doing violence both to physical chemistry and to biology.

The scope of this treatise precludes a detailed discussion of the physical chemistry, biochemistry, and physiological and toxicological implications of the reversible complexation of zinc. The ubiquitous distribution of the metal and the many organic and inorganic radicals with which it can combine, clearly could result in an almost infinite number of interactions.

The association of zinc with the components of proteins has received particular attention both in model systems and in natural products. Much of the effort has been devoted to the localization of the binding sites and to the description of their chemical configuration. It has been the ultimate objective of many of these studies to explore the molecular basis of the action of enzymes and hormones, or to study transport mechanisms. The mechanism of viral invasion has also been studied in this regard (140).

The problems pertinent to the mechanism of chelation and coordination

complexes have been discussed extensively (11, 161), and the significance of such metal interactions to biological activity has been elaborated upon from biochemical (145) and physical chemical points of view (353a). The general interaction of metal ions, among them zinc, with amino acids, peptides and proteins, has received systematic treatment in a recent review (93). The physical chemical studies of model systems have led to the conjecture that imidazole (64, 205) and sulfhydryl groups (5, 215) are possible sites for the binding of zinc ions to the component amino acid of peptides and proteins (93). The existence of zinc purine complexes has been known since 1886 (139). Nucleotides like ADP and ATP have also been shown to form zinc complexes which, however, are relatively much weaker than those of amino acids (127). It has been suggested that such complexation increases the physiological availability of metal ions by solubilizing metal salts (201).

Such model systems have been extrapolated to offer explanations for biochemical and physiological mechanisms; the border line between inductive and deductive reasoning is sometimes discernible with difficulty (343). The considerations leading to the operational distinction between metalloproteins and metalloenzymes on one hand and metal-protein as well as metal-enzyme-complexes on the other have been discussed (293, 297).

Physical Chemistry

Serum Proteins

The hypothesis that zinc binds to the imidazole groups of human serum albumin is consistent with experimental evidence (93). Zinc ions at a concentration of 15 mM will form water-soluble complexes with albumin, α-lipoprotein, α-glycoprotein, alkaline phosphatase, serum esterase and metal-combining globulin. The β-lipoproteins and γ-globulin or fibrinogen are insoluble in water under the same conditions. The proteins can be solubilized with chelating agents by the selective dissociation of the zinc protein complex. In this manner, subfractions of γ-globulins can be prepared (120). The implications of these binding studies to transport of zinc in plasma have not been investigated.

In blood serum, zinc exists in at least two fractions; firmly bound zinc is said to amount to about 34%, and loosely bound zinc, to 66% of the total zinc content. The firmly-bound zinc protein, a globulin, satisfies the criteria of metalloproteins, whereas the loosely-bound zinc protein should be defined as a metal-protein-complex. The firmly-bound zinc protein has not been purified adequately to state with certainty the percentage of the total serum zinc that it contains. The loosely-bound complex appears to be concerned primarily with zinc transport (326). Neither substance has been shown to exhibit enzymatic properties.

Insulin

It has been known for many years that crystallization of insulin, at pH levels near 6, may be accomplished only in the presence of Zn^{++}, Cd^{++}, Co^{++}, or Ni^{++} (95, 243). It was found that the final products alternatively contained .52% zinc, .77% cadmium, and .44% cobalt. These percentages were not changed by re-

crystallization (244). There is sufficient zinc in the pancreas to allow crystallization of insulin without further addition of this metal (79). The dependence of the composition of zinc insulin on pH and ionic strength was detected early (43, 44). At pH 1.7 zinc is removed completely from amorphous insulin which crystallizes at this pH without zinc as acid insulin sulfate and which contains 12 sulfate groups. Starting with material dialyzed at pH 1.7, a large number of crystalline insulin preparations can be obtained with various anions and cations and without zinc. The crystal structure has been determined by x-ray diffraction (71).

Amorphous insulin and zinc form a complex which dissociates below pH 4 but which slowly changes to the crystalline state on standing between pH 5.6 and 6.0, yielding a 1:1 complex. Zinc is bound much more firmly, however, in crystalline insulin than in amorphous insulin (278, 279).

The data suggested that one zinc atom probably chelates two imidazole residues of amorphous insulin, while in crystalline insulin, carboxylate groups were postulated to bind additionally. When zinc is further added to crystalline zinc insulin, a larger amount of metal is bound. This is accompanied by continuous aggregation and the formation of a non-crystalline precipitate from which the metal can be readily removed by dialysis at pH 7.6. These changes coincide with alterations in the ultracentrifuge and electrophoretic patterns. Similar observations were made with cobalt and iron. The interaction of cobalt with insulin is analogous to that noted in the formation of zinc insulin crystals, while iron-insulin differs from both. The hormone preparations employed in these experiments contained 10 and 18 μg of zinc per gram of insulin and were fully active physiologically (50).

Thus insulin can be prepared in both amorphous and crystalline form entirely free of zinc and other metals, but still physiologically active. It cannot be judged from physical chemical or physiological evidence whether an insulin-zinc-complex is necessary for biological activity. The suggestion that such a complex absolutely must form *in vivo* has no valid experimental basis. Moreover, the physical chemical association of zinc with insulin *in vitro*, which has been the experimental basis and intellectual model for the subsequent physiological and histochemical studies, has not been shown to be a necessary compositional or structural feature. If the molecular configuration, as studied *in vitro*, has any bearing on the basis of the hormonal action *in vivo*, a 'no zinc' hypothesis has as much justification as any postulate concerning a 'zinc, cobalt, iron or nickel' insulin. Circular reasoning, as is often used in such speculative syntheses of physical-chemical fact and biological conjecture, is not likely to provide any progress in this regard.

Zinc-Containing Protein of Human Leucocytes

A protein that contains 0.3% zinc per gram of dry weight can be obtained from leucocytes in humans. This zinc protein is responsible for 80% of all the zinc found in human leucocytes and is differentiated clearly from carbonic anhydrase. While the protein is presumably an enzyme, the nature of its enzymatic activity is not known (106, 311).

Biochemistry

Zinc Metalloenzymes

Seven zinc metalloenzymes have been characterized thus far: the carbonic anhydrase of bovine erythrocytes (128); the alcohol dehydrogenase of yeast (302, 303) and of equine liver (282, 304); the glutamic dehydrogenase of bovine liver (312) and the lactic dehydrogenase of rabbit skeletal muscle (308). Recent findings are consistent with the existence of yet additional pyridine nucleotide-dependent metallodehydrogenases (314), although their final inclusion in this group of zinc enzymes awaits their purification from extraneous proteins and metals, and their characterization in this homogeneous state. Carboxypeptidase (306, 307) and alkaline phosphatase (164) have also been found to contain zinc. These problems have been the subject of a review (293) to be brought up to date by the present discussion. Pertinent data concerning these zinc metalloenzymes are summarized in table 1.

Carbonic anhydrase. Carbonic anhydrase was first described as a protein catalyst in 1932 (33) and was purified from erythrocytes shortly thereafter (180–182). It has never been obtained in ultracentrifugally or electrophoretically pure form; the claims of its crystallization (247) have been questioned (129). The molecular weight of carbonic anhydrase from bovine erythrocytes is approximately 30,000 (69, 216, 257). Its ecological distribution, the methods for measurement of its activity, and the procedure for its isolation and purification have been reviewed amply (86, 233, 298, 322, 342).

Zinc was first identified as a constituent of bovine erythrocyte carbonic anhydrase by Keilin and Mann (128), and this was confirmed rapidly (117, 146). Plant carbonic anhydrase also contains zinc (56). The exact molar stoichiometry of zinc in carbonic anhydrase is not known, since adequately purified samples have not been analyzed simultaneously for zinc, extraneous metals, and protein homogeneity. Based on a molecular weight of 30,000, the reported zinc contents of bovine erythrocyte carbonic anhydrase vary from 0.92 and 1.04 (248) to 1.52 (128) and 1.46 (117) gram atoms per mole. The zinc contents of carbonic anhydrase from erythrocytes of the ox, sheep and human may differ (128).

Although the chemical details of the bonding between the metal and the protein are not known, it is reported (131, 321) that the enzyme can be split into thermolabile and thermostable fractions. The latter fraction is described as a zinc-containing tripeptide with 2 glutamic acid and 1 glycine residues, which lacks carbonic anhydrase activity itself, but increases the activity of dilute enzyme solutions (131). In view of the incomplete and preliminary nature of this work, substantiation is necessary before this fragment can be considered a prosthetic group or coenzyme of carbonic anhydrase.

Although the manner of zinc protein binding is obscure, its affinity is great, as shown by electrodialysis (249) and exchange experiments with Zn^{65}, which did not exchange with enzyme zinc over a period of 32 days (289).

It is feasible to estimate the carbonic anhydrase content of human erythrocytes if certain assumptions are made (128); 0.21 gm of carbonic anhydrase is found to be present in 100 ml of packed red cells (298).

TABLE 1. ZINC METALLOENZYMES OF KNOWN METAL CONTENT

ENZYME, ABBREV., REF.	MOL. WT.	% METAL	METAL/PROT.*	COENZYME/PROT.†	METAL/COENZYME*	EMPIRICAL FORMULA
Carbonic anhydrase (bovine erythrocytes) CAD (117, 128, 248)	30,000	0.2–0.3	1 (?2)			$[(CAD)Zn_{1-2}]$
Carboxypeptidase (bovine pancreas) CPD (306, 307)	34,300	0.18	1			$[(CPD)Zn]$
Alcohol dehydrogenase (yeast) YADH (302, 303)	150,000	0.18	4	4(DPN)	1Zn/DPN	$[(YADH)Zn_4](DPN)$
Alcohol dehydrogenase (equine liver) LADH (282, 304, 305)	73,000 (81,000)	0.18	2	2(DPN)	1Zn/DPN	$[(LADH)Zn_2](DPN)_2$
Glutamic dehydrogenase (bovine liver) GDH (2, 312)	1,000,000	0.02–0.03	2–4	2–4(DPN)	1Zn/DPN	$[(GDH)Zn_{2-4}](DPN)_{2-4}$
Lactic dehydrogenase (rabbit skeletal muscle) SLDH (308)	?	0.07				$[(SLDH)Zn_?](DPN)_?$
Alkaline phosphatase (porcine kidney) (164)		0.15				

* Values expressed as gm atoms per mole. † Values expressed as moles per mole.

This enzyme is inhibited by a number of agents known to form complexes with metal ions, such as cyanide, sulfide and azide (182), BAL (341) and thiocyanate (52); these are thought to act by combining with zinc. The inhibition by sulfide is disputed (146, 320). Cyanide is a very potent, reversible inhibitor, 85% inactivation occurring at 4×10^{-6} M, and the inactivations caused by these complexing agents indicate that zinc may be an active enzymatic site (128); no data either on the kinetics of these inhibitions or on the effects of chelating agents have been published. The marked inhibitory action of the sulfonamides (160a) has not been shown to be exerted through the enzyme zinc atoms. Carbonic anhydrase activity in plants has been reported (32, 56, 200, 254, 340). Plant carbonic anhydrase contains zinc (56), but the purest preparations of Sibly and Wood (254), as estimated polarographically, contained only 0.056%, not removable by water. In addition to this low zinc content, the plant enzymes appear to be more resistant to inhibition by KCN, although azide inhibits about 75% at 10^{-3} M.

In studies on oat plants at various stages of their life cycle and on tomato plants, carbonic anhydrase was found only in non-chloroplast fractions of leaves (357). Plants grown in zinc-deficient media showed low carbonic anhydrase activity, zinc and protein nitrogen levels, suggesting this correlation.

Carboxypeptidase of bovine pancreas. Pancreatic carboxypeptidase, first crystallized in 1937 (7), has been purified from pancreatic juice (89) and has a molecular weight of 34,400 (222). Quantitative spectrographic analyses, which have been confirmed by chemical measurements, demonstrate that carboxypeptidase is a zinc metalloenzyme (306, 307). Each enzyme molecule contains 1 atom of zinc, and all other metals are absent or present in stoichiometrically insignificant amounts.

Carboxypeptidase is inhibited by sulfide, cyanide, cysteine (259, 260), α, α'-dipyridyl and others (307). These agents form strong complexes with zinc ions, and, in fact, do not inhibit when first exposed to equimolar amounts of zinc, cupric, or

ferrous ions. The addition of zinc ions to carboxypeptidase, already inhibited by orthophenanthroline, restores enzymatic activity, demonstrating the reversibility of this reaction. When zinc is removed from the enzyme, activity is lost in direct proportion. Readdition of zinc ions fully restores activity (319). The sulfonamides, which strongly inhibit carbonic anhydrase, are without effect on carboxypeptidase.

Alcohol dehydrogenase of yeast. An alcohol dehydrogenase was crystallized from brewer's yeast in 1937 (199) and is usually now prepared from baker's yeast (224). The crystalline yeast alcohol dehydrogenase has a molecular weight of 150,000, binds 3.6 DPN molecules (98) and contains 4 atoms of zinc per molecule (302, 303) as measured by spectrographic and chemical analyses. Recent studies, in which yeast was grown in the presence of Zn^{65}, demonstrate directly the biosynthetic incorporation of zinc to form $(YADH)Zn_4^{65}$; the zinc content of these crystals, as measured both by radioactivity and chemically, is 4.0 gram atoms per mole. These biosynthetically incorporated zinc atoms are not miscible with zinc, present from extraneous sources (318).

Reports that a crystalline alcohol dehydrogenase from brewer's yeast contains 5 atoms of zinc (333), binds 5 DPN molecules and can utilize and incorporate added zinc ions to form up to 31 active enzymatic sites (334), are open to question. The protein purity of these preparations is not stated, nor is the method of protein estimation with biuret corroborated. The accuracy of the zinc method using x-ray fluorescence is not validated in detail (333).

A preliminary report indicates that the enzymes prepared from baker's yeast and that from brewer's yeast are not identical (130). Although they have certain properties in common, their isoelectric points and ultraviolet extinction coefficients differ, suggesting differences in their structure and composition. Immunological studies from the same laboratory further indicate an immunological dissimilarity (8). Both enzymes mutually completely adsorb the homologous as well as the heterologous rabbit antiserum, but the quantitative interactions are not identical. Both enzymes do appear to be zinc metalloenzymes, being inhibited by chelating agents; however, the zinc content of the proteins was not reported (130). This raises questions concerning the identity of the enzymes which different investigators have studied.

Zinc in excess of 4 atoms can be removed, together with other contaminating metals, by dialysis against 0.1 M phosphate buffer at pH 6. The enzymatic activity is increased by removal of these extraneous ions. Exposure of yeast alcohol dehydrogenase to pH levels below 6 results in rapid loss of activity, proportional to the removal of intrinsic zinc from the enzyme. Thus, at pH 5.0, the enzymatic activity and zinc content have been reduced to 50%, and at pH 4.5, all of the zinc is removed from the enzyme and it is totally inactive. The relationship of inhibitors to —SH groups has been studied (108).

Inhibition studies further demonstrate this role; a large number of chelating and complexing agents, capable of combining with zinc, inhibit the enzymatic activity when the enzyme is exposed to them under various conditions (303). The degree of inhibition is dependent on the duration of contact between enzyme and inhibitor prior to measurement of activity, and on the pH and temperature of the preincubated mixture, as well as on the concentration of the inhibitor.

Present knowledge leads to the conclusion that the zinc atom is an active enzymatic site which interacts in some manner, the details of which are as yet undetermined, with diphosphopyridine nucleotide (107). Zinc has also been implicated in the structural integrity of the protein (297) since orthophenanthroline decomposes the enzyme into smaller molecular subunits.

Alcohol dehydrogenase of equine liver. A DPN-dependent alcohol dehydrogenase has been crystallized from horse liver (28, 29) which, though it catalyzes the same reaction, is different in many respects from the yeast enzyme. Liver alcohol dehydrogenase has less substrate specificity than yeast alcohol dehydrogenase, since it also oxidizes vitamin A alcohol (26, 27) and glycerol (112). Equine liver alcohol dehydrogenase has been reported to have molecular weights of 73,000 (281) and 84,000 (68) and binds 2 DPN molecules at neutral pH values. It was predicted, on the basis of studies with yeast alcohol dehydrogenase, that liver alcohol dehydrogenase would be found to contain 2 atoms of zinc per molecule, since its molecular weight is half that of yeast alcohol dehydrogenase and since it binds 2 moles of DPN (302, 303). This prediction was rapidly confirmed (282, 304, 305). The firmness of the zinc binding, the concomitant rise of zinc and activity during purification, accompanied by elimination of extraneous metals (305), and the formation of characteristic absorption spectra (315, 317) all confirm that liver alcohol dehydrogenase is a zinc metalloenzyme. Like yeast alcohol dehydrogenase, liver alcohol dehydrogenase loses zinc and activity pari passu upon dialysis at pH values below 5.5.

Extracts of the livers of cow and pig show a reduction of DPN in the presence of added ethanol, and it appears that there is a bovine liver alcohol dehydrogenase and a porcine liver alcohol dehydrogenase (Hoch and Vallee, unpublished data).

Glutamic dehydrogenase of bovine liver. The DPN-dependent glutamic dehydrogenase crystallized after purification from beef liver (210) differs from the other enzymes of this group in some important respects. The zinc metallodehydrogenase described in this section and other pyridine nucleotide-dependent dehydrogenases all act on primary or secondary alcoholic groups; glutamic dehydrogenase is the only known enzyme of this group which catalyzes the reversible oxidative deamination of an amino acid. Glutamic dehydrogenase, having a molecular weight of 1,000,000, is also a much larger molecule than other pyridine nucleotide-dependent enzymes that have been studied (210).

The glutamic dehydrogenase of beef liver is a zinc metallodehydrogenase; all crystalline preparations contain zinc in stoichiometrically significant amounts and do not consistently contain significant amounts of other metals. The average zinc content of eight different batches of crystalline preparations was 3.4 ± 1.0 atoms per molecule, (2, 3).

The zinc metalloenzyme nature of glutamic dehydrogenase is further shown by the inhibiting effects of metal-binding agents; these have been studied in detail (3, 3a). Orthophenanthroline inhibition of this enzyme, as with yeast and liver alcohol dehydrogenase, is competitive with the oxidized and reduced coenzyme, indicating that the coenzyme is attached to glutamic dehydrogenase at or near its zinc atoms.

The presence of zinc in glutamic dehydrogenase has been confirmed (121),

and the element has also been implicated in the structural integrity of this enzyme (82, 82a, 82b, 262a).

Lactic dehydrogenase of rabbit skeletal muscle. The redox interconversion of lactic and pyruvic acids is catalyzed by a DPN-dependent enzyme isolated and crystallized from rabbit skeletal muscle (13). This enzyme has not yet been completely characterized and its molecular weight is not known. The crystalline enzyme is not homogeneous, showing at least three components of different masses on ultracentrifugation. The heterogeneity of lactic dehydrogenase isolated from the same organ of an animal, from different organs of the same animal, and from different animals, has been demonstrated recently (352).

All preparations of crystalline rabbit muscle lactic dehydrogenase, or perhaps more properly dehydrogenases, which have been analyzed, contain zinc, the average being 637 μg of zinc per gram of protein (308). These preparations have all contained metals other than zinc, but in very variable amounts. With purification, the zinc content alone rises with increasing activity; the concentration of other metals does not follow any consistent course.

Like the zinc metallodehydrogenase discussed above, this enzyme is inhibited either immediately or after preincubation with a number of metal-binding reagents; and some of these inhibitions can be prevented and reversed by Zn^{++} ions. The inhibition of orthophenanthroline is competitive with DPN, indicating that this coenzyme is bound at or near a zinc atom. Apparently, zinc plays a functional role in the activity of rabbit skeletal muscle lactic dehydrogenase.

Rabbit skeletal muscle lactic dehydrogenase thus resembles yeast alcohol dehydrogenase, liver alcohol dehydrogenase, and glutamic dehydrogenase in all major respects. The final establishment of the molar stoichiometry of zinc awaits further purification of this enzyme.

Other dehydrogenases may prove to be zinc or other metalloenzymes, but the data in this regard are only of a preliminary nature (314). Studies of these impure enzymes were performed to outline avenues of approach likely to be profitable in defining further the role of zinc in enzymatic dehydrogenation.

Alkaline phosphatase of porcine kidney. Alkaline phosphatase from swine kidneys has been shown to be a zinc enzyme (164). A linear relationship of specific activity to zinc content was found throughout purification. The best preparations contained 0.15 per cent of firmly bound zinc. It was surmised that one molecule of the enzyme contains several atoms of zinc. The final preparation obtained so far exhibits three components in the ultracentrifuge. The enzyme is inhibited by versene, KCN, α, α'-dipyridyl, and a large number of amino acids. Loosely bound zinc and copper, both of which inhibit, may be removed by dialysis against amino acids acting as complexing agents. This interaction then results in activation due to the removal of the inhibiting metal ions. It was surmised that the magnesium salt of the phosphate ester is the actual substrate for alkaline phosphatase, accounting for the essentiality of magnesium ions in this reaction (164).

Hexokinase of Neurospora crassa. The isolation of a 'zinc-dependent' hexokinase from *Neurospora crassa* led to the suggestion that this is a zinc metalloenzyme (177, 178). The criteria for this conclusion are not identical with those employed to

evaluate the zinc metalloenzymes just described. The hexokinase was not isolated in homogeneous form and, indeed, showed significant phosphoglucose isomerase activity. The evidence for an enzymatic role for zinc is derived from the decrease of hexokinase activity in zinc-deficient mycelia, which is specifically overcome within 12 hours, but not immediately, by the addition of zinc to the culture. The hexokinase extracted and partially purified from zinc-deficient felts is significantly activated by Zn^{++} ions, but that from control felts is not. Zn^{++} ions also protect the enzyme against an SH-reagent, *p*-chloromercuribenzoate. Versene inhibits enzymatic activity, but high concentrations of KCN, NaN_3, α, α'-dipyridyl, and 8-hydroxyquinoline do not. Data on the metal content of the preparations are not given.

These observations, in the absence of further data, may indicate a metal-enzyme-complex rather than a metalloenzyme. The role of zinc in this enzyme requires further study. Apparently, the hexokinase of yeast is not similar to this enzyme. Findings on zinc-deficient tomato plants, where inorganic phosphate accumulated, were interpreted to suggest a possible role of this metal in the hexokinase of those tissues (228).

Zinc-Enzyme Complexes

Table 2 lists a number of enzymes whose activity is increased by the addition of Zn^{++}. The lack of specificity is borne out by the fact that most of them are activated by other ions also. The biological implications of these data cannot, of course, be detailed at this juncture. Though no pretense is made at being complete, the table shows that numerous instances of this type have been observed. Their further study will very likely reveal important and additional evidence concerning the biological function of zinc.

Enzymatic Consequences of Zinc Deficiency in Microorganisms and Plants

The complexity of higher living forms often precludes the identification of the exact biochemical locus of action of a nutrient. Such difficulties may be somewhat simplified when the results of deficiencies are studied in lower organisms. Even further, the findings in such relatively uncomplicated organisms may often then be extrapolated to interpret those in more highly organized species, a principle of transphylar analogy of metabolic patterns. This has been shown to be possible to some extent in the case of *Neurospora crassa*, the common bread mold, which responds to zinc deficiency by displaying typical alterations of its enzyme patterns. Similar alterations are also produced by other cation and micronutrient deficiencies; this may reflect some of the occult interdependences which exist in higher forms.

A wild strain of *Neurospora* 5297.1, has been examined in detail as regards the activity of its tryptophan desmolase system (196). This system is contained in cell-free extracts of mats, and is dependent on the presence of pyridoxal phosphate (290). The growth rate of zinc-deficient mats is 33–50% that of the zinc-supplemented controls; extracts of these mats have only 40% of the enzymatic activity of the controls. The addition of 2×10^{-6} M to 5×10^{-2} M Zn^{++} to the extract fails

TABLE 2. METAL-ENZYME-COMPLEXES ACTIVATED BY ZINC*

ENZYME	ACTIVATING METALS	REF.
Glycylglycine dipeptidase	Zn^{++}	(150)
Arginase	Zn^{++}, Mn^{++}, Fe^{++}, Co^{++}, Ni^{++}, Cd^{++}	(63)
Dehydropeptidase	Zn^{++}	(358)
Alanyl and leucyl glycine dipeptidase	Zn^{++}, Pb^{++}, Cu^{++}, Mn^{++}, Sn^{++}, Cd^{++}, Hg^{++}	(17)
Tripeptidase	Zn^{++}, Co^{++}	(123)
Glycyl-L leucine dipeptidase	Zn^{++}, Mn^{++}	(162) (258)
Carnosinase	Zn^{++}, Mn^{++}	(96)
Amino peptidase	Zn^{++}, Co^{++}, Mn^{++}	(230)
Histidine deaminase	Zn^{++}, Hg^{++}, Cd^{++}	(269)
Alkaline phosphatase	Zn^{++}, Mg^{++}, Co^{++}, Mn^{++}, Ni^{++}, Ca^{++}	(42) (202)
Lecithinase	Zn^{++}, Cu^{++}, Mg^{++}, Co^{++}, Mn^{++}	(359)
Enolase	Zn^{++}, Mg^{++}, Mn^{++}	(335)
Yeast and Clostridium aldolase	Zn^{++}, Fe^{++}, Co^{++}	(336)
Oxaloacetic decarboxylase	Zn^{++}, Mn^{++}, Co^{++}, Cd^{++}, Pb^{++}, Ni^{++}, Fe^{++}, Mg^{++}, Ba^{++}, Cu^{++}	(263) (218)

* Other metals which increase the observed activity are also listed.

to restore activity; indeed, higher concentrations of Zn^{++} cause even further inhibition in a manner similar to the effect of Zn^{++} on known zinc metalloenzymes. Addition of KCN or KCNS to the control extract inhibited tryptophan desmolase activity. Supplementation of the extracts with pyridoxal phosphate increased activity only slightly; the effect of increased pyridoxal in the growth medium was not mentioned. These effects were reported specific for zinc deficiency; iron or manganese deficiency in the growing media, or additions of Cu^{++}, Mg^{++}, or Mn^{++} to the extracts, have no effects. It was concluded that "the enzyme which converts indole and serine to tryptophan might contain zinc or might be produced by a series of steps, one or more of which required zinc." Resolution of these or other possibilities might be accomplished by isolation and analysis of the enzyme or enzymes involved. These studies were consistent with earlier findings in zinc-deficient tomato plants, where the metal was shown to be required for the synthesis of tryptophan, and indirectly, therefore, for auxin production (255, 287).

Further studies on the enzyme patterns of the same strain of *Neurospora* (197, 198) revealed that zinc deficiency leads to alterations in the activity—either increases or decreases—of other enzymes as well, while certain enzymes are not affected at all. The decrease in tryptophan desmolase activity was confirmed, and alcohol dehydrogenase, normally present, was found to be absent in zinc-deficient *Neurospora*. The addition of Zn^{++} to the extracts had no effects on these activities. A 10- to 20-fold increase in activity of DPNase was observed, but fumarase, hexokinase, aldolase, and triosephosphate dehydrogenase activities were normal. These changes were not completely specific for zinc deficiency. Magnesium, manganese, or iron deficiencies did not affect the action of these enzymes. However, deficiencies of other substances did produce results similar to zinc deficiency. Calcium de-

ficiency decreased growth rate and alcohol dehydrogenase activity and increased DPNase and fumarase activity; tryptophan desmolase activity was normal. The results of biotin deficiency were identical with those of zinc deficiency. A combined zinc and biotin deficit potentiated the increase in DPNase activity up to 100-fold. Markedly lowered alcohol dehydrogenase and high DPNase activity were noted in nitrogen or zinc deficiency in a variety of wild *Neurospora* strains.

Recovery from these zinc deficiencies was accompanied by gradual increases of growth rate and the return of normal enzyme patterns when zinc and an appropriate nitrogen source were supplied; a similar response occurred when other curtailed nutrients were added.

The absence of effect when Zn^{++} was added to deficient mat extracts and the cumulative nature of the enzyme activities in zinc-deficient and normal extracts indicate that ionic zinc is not involved here in the process of activation or inhibition of enzymes through formation of metal-enzyme-complexes. The effect of zinc deficiency on alcohol dehydrogenase activity seems directly causal, in view of the findings detailed in the section on YEAST ALCOHOL DEHYDROGENASE.

These findings are consistent with the interpretation that the alcohol dehydrogenase of *Neurospora crassa*, like that of yeast and equine liver, is a zinc metalloenzyme. On the other hand, sufficient evidence is not on hand to interpret either the change in activity of other enzymes or the interdependence between zinc, calcium, biotin and nitrogen in biochemical or structural terms.

The studies on *Neurospora crassa* (197, 198) also showed that protein content is markedly lowered in zinc-deficient mycelia. Protein concentration, or activity of alcohol dehydrogenase or tryptophan desmolase is not increased when the zinc-deficient medium is supplemented with amino acids, vitamins, purines and pyrimidines, although growth returns to normal levels. The failure of *Neurospora* to synthesize proteins was ascribed to the zinc deficiency, and this defect was thought to be the basis of the observed decreases in enzyme activities.

Zinc-deficient tomato plants also show decreased protein content and contain about twice the total amount of amino acids and 10 times the amide content of normal zinc-supplemented controls (219). The increases of extractable glutamine and asparagine were especially marked, and significant amounts of β-alanine appeared which were absent in the controls. Citrulline and ethanolamine, which were present in the controls, were, however, absent in the zinc-deficient plants. Similar but less striking rises in amino acid content were observed in tomato shoots deprived of iron, copper, or manganese. These findings are of interest in connection with those of decreased protein synthesis in *Neurospora*. Similar findings of lowered protein content were reported in zinc-deficient oat plant leaves (357). In these plants and in zinc-deficient tomatoes, carbonic anhydrase activity was also lowered, and the degree of diminution of protein zinc and carbonic anhydrase activity were all highly correlated.

Pyruvic carboxylase activity was absent in extracts of zinc-deficient *Rhizopus nigricans* (81a), and it was concluded that zinc is necessary for synthesis of the enzyme protein itself but that zinc is not a constituent of the enzyme.

The aldolase activity of higher plants is decreased in zinc deficiency, while

copper deficiency does not affect this enzyme (223). This is of interest when compared with the conclusions of Rolinson (231), based on the finding that zinc requirements of *Aspergillus niger* vary for different carbon sources, although zinc is essential for all of them. The metal was thought to be involved in the metabolic events of the Embden-Meyerhof scheme "after the formation of the triosephosphates." While aldolase is responsible for the formation of these esters, a number of metallodehydrogenases would fit the requirements of Rolinson (231).

Zinc Deficiency in Plants

The literature on the effect of zinc on growth of plants has been summarized repeatedly (240, 267, 332). This aspect of the effect of zinc in biological systems has been of particular concern to agronomists because of the immense economic implications of such deficiencies. Thus, considerable financial losses were incurred in California and Texas consequent to zinc deficiencies in citrus groves and pecan trees respectively, and the studies in the Twenty-Mile desert of Australia are well known for the resultant reclamation of land otherwise agriculturally unproductive. The nomenclature of the diseases differs on a geographic basis and is often colloquial. Table 3 summarizes some of the conditions and their signs. Interference with chlorophyll synthesis has been noted in all these conditions but has not been explored extensively (332).

Applications of zinc salts are often effective when the soil is acid; however, neutral or alkaline soils render the zinc salts insoluble, interfere with the absorption and make the therapy ineffective. Sprays of zinc salts applied to leaves or injections into tree trunks overcome the problems of solubility in soil and have generally been found more successful. The original literature should be consulted for the details of these considerations.

Composition of Biological Material

General Comments

The zinc content of various tissues has been studied by many workers during the past 80 years. Lutz in 1926 (155) summarized the data up to this time and concluded that previous workers had employed inadequate methods; he felt that data prior to his writing had to be discounted. He reinvestigated the zinc content of many organs in the rat, cat and man by means of a more sensitive and precise technique, but again this information, carefully gained, must be judged to be of dubious reliability by present standards. The data indicated, however, that zinc occurs in all tissues of these species, varying reproducibly within narrow limits. The methods employed by subsequent workers are also diverse; the precision, accuracy, and the base lines of comparison—dry, ash, or wet weight—vary greatly. Few of the studies employ the zinc/nitrogen ratio; since the metal is usually associated with protein, the zinc/nitrogen would be a more desirable basis of comparison for relating composition to function. The presence of blood in organs also constitutes a potential source of error. In most instances, the organs were not perfused, nor were corrections applied for this source of contamination. These considerations have been detailed elsewhere (298). Zinc data referred to ash weight

TABLE 3. DIAGNOSIS OF ZINC DEFICIENCY DISEASES IN PLANTS

SPECIES	DESCRIPTIVE NAME	SYMPTOMS
Citrus	Mottle leaf	Pigmentation; intervenal chlorosis, necrosis; reduced leaf size; malformation of leaves; sparsity of foliage; 'dieback'; shortened internodes; normal leaves in shaded areas of tree; reduced production of fruit. Symptoms suggest a close relationship of zinc to chlorophyll production.
Apple, pecan	Rosette leaf	Whorls of small, stiff, mottled leaves near tips of current season twigs; terminal rosette, reduced fruit buds, malformed fruit, 'dieback.'
Walnut	Yellows	
Peach, apricot, pear, plum, cherry, grape	Little leaf	Similar to 'rosette' in apple trees. Chlorotic mottling of foliage in late summer; internal chlorosis next spring followed by purple pigmentation and necrosis; reduced and misshapen fruit production.
Tung	Bronzing	Bronzing of terminal leaves, deformation of terminal shoots; necrotic spots, abnormally developed internodes and subsequent 'bunched' foliage. High susceptibility to cold injury. Eventual reduction in size of tree and death.
Maize	White bud White tip	Internal light, yellow streaks followed by necrotic spots. Unfolding leaves are pale yellow or white. Older leaves have slate to dark brown necrotic areas, merging until whole leaf dies. Stunting results from improper growth of internodes.
Cereals		Purpling and death of older leaves. Mature plants have characteristic grayish appearance.
Flax		Rosetting and death of leader shoots. Lateral shoots issue from the bases.
Clover		Dwarfed, rosetted growth.

have generally been omitted in this discussion, since their biological meaning is ascertained with difficulty.

Zinc is present in human and other vertebrate organs, including man, in quantities varying from 10 to 200 μg per gram (137, 147, 298). Most organs, including the pancreas, contain between 20 and 30 μg of zinc per gram wet tissue. Liver, voluntary muscle, and bone contain about double this amount. The zinc content of the liver of newborn rats is 2- to 3-fold higher than that of adult animals. Within 2 weeks post-partum, the concentration decreases to about 100 ppm, which is the content of adult rat livers (16). The dermal appendages of various species have been found to contain substantial amounts of zinc. The differences in organ content of different species is not remarkable, and in view of the analytical uncertainties previously mentioned, the detailed results add little of significant value, though they have been compiled (291).

An extensive study determined the mineral composition of whole human bodies on a fat-free basis (351). From the data obtained on four individuals, the total zinc content of an adult fat-free body weighing 70 kilograms varies from 1.36 to 2.32 grams. The total Fe content is 4.2–6.1 gm, and the Cu content 81–230 milli-

grams. The values for copper and iron are in good agreement with those independently determined by others, and the data for zinc may therefore be considered a good approximation to the true content.

Studies With Zn^{65}

The distribution of the radioisotope, $_{30}Zn^{65}$, in the mouse and dog has been studied as a guide to the distribution of the metal in tissues (193, 251, 252). Animals were serially killed subsequent to the injection of the isotope. The amounts of zinc introduced were negligible as compared with the total amount in the body. Tissue content of Zn^{65} was expressed in terms of percentage of the injected Zn^{65} found in each organ. The organs were not perfused and the data are not corrected for blood content.

In both mice and dogs, early in the experiment, the liver contained the largest fraction of the injected dose. In both species, there was a gradual decrease of radioactivity, more rapid in the mouse than in the dog. Over a period of 160 hours, the most rapid uptake and loss was observed in liver, pancreas, kidney and pituitary. Least activity was found in erythrocytes, brain skeletal muscle and skin. The activities of spleen, gastrointestinal tract, adrenals, lungs, lymph nodes, brain, heart and thymus were intermediate. Red blood cells and bone were the only tissues examined which accumulated zinc. Of the administered dose of Zn^{65}, 6.5% was excreted in pancreatic juice within 5 days (193). These data have been confirmed, and elimination of the metal in bile has also been found (23). Since carboxypeptidase of pancreatic juice is a zinc metalloenzyme (306, 307), at least a fraction of this zinc elimination is accounted for by this enzyme, though other zinc enzymes may also be found to contribute to the total amount of zinc in pancreatic juice.

Unfortunately, in these studies with Zn^{65} the total zinc content of the organs was not measured. Therefore, specific radioactivity cannot be calculated as a guide both to tissue content and to isotope turnover. While the observations only extended over 160 hours, the isotope could be detected for as long as 8 months, and probably a year, in dogs, normal humans, and one patient with myelogenous leukemia (87, 299, 309). The isotope passes across the placenta of the dog. The total stable zinc content of tissues of the offspring is similar to that of adults, though the ratio of radioactive to total zinc in three of the animals killed serially during the first postnatal week shows a redistribution of the isotope, with a decrease in the extremities and a rise in the concentration in the viscera (309). More recently, it has been found that Zn^{65} is absorbed at a very low rate from the gastrointestinal tract of the rat. However, once absorbed, the isotope moves freely from the dam across the placenta to the fetus. More than half of the dose retained is transferred to the young through the milk within 96 hours (75).

The male genital organs and semen, the pancreas, the tissues of the eye and the liver, and body fluids have been investigated intensively; they will be given attention here.

Male Genital Tract

A careful reexamination of the zinc content of the male genital organs of the rat was undertaken, based on early suggestive findings of 'high' zinc contents of

horse testis, seminal vesicle of swine and rat epididymis, as well as human and bull prostate (22). There are 15 to 30 μg of zinc per gram wet weight in the epididymis and seminal vesicles, while 11 samples of the dorsolateral prostate of the rat contain 180.0 ± 45.5 μg per gram compared to 13.7 ± 3.2 μg per gram in 9 samples of ventral prostate (169). On a dry weight basis, there are 874 ± 68 μg of zinc per gram in the dorsolateral rat prostate; the rabbit prostate contains 1296 ± 31 μg per gram, and the normal human prostate, 859 ± 96 μg per gram (170). There are also relatively high concentrations in the epididymis, but the previous report of a large amount of the metal in testicular tissue was not confirmed (67, 147, 169). The zinc content of the dorsolateral prostate of growing rats increases from about 40 μg per gram wet weihtg at 40 days to a maximum of 250 to 300 μg per gram at about 160 days, decreasing thereafter; the content of the ventral prostate remains insignificant and does not appear to undergo similar fluctuations. Experiments with Zn^{65} confirmed the selective accumulation of this isotope in the dorsolateral prostate of the rat (92), but the removal of this organ neither affects fertility nor fecundity (91). Prostatic fistulae in dogs have been employed to detect the appearance of Zn^{65} in the prostatic secretions. The constant and rapid appearance of the isotope in prostatic fluid subsequent to its intravenous injection suggested an integral function of the element in the canine prostate (221).

The activity of carbonic anhydrase increases simultaneously with the zinc content. The amount of enzyme calculated accounts only for 3.5 per cent of the total zinc of the dorsolateral rat prostate; a satisfactory correlation between enzyme activity and metal content could not be made (171). Neither alkaline nor acid phosphatase activities show any apparent relationship to zinc content (77, 104). A significantly decreased zinc content in prostatic carcinomata, 190 ± 61 μg per gram, is suggested, but the number of analyses is as yet insufficient to prove this point (170). Diminished concentrations of zinc are also observed in prostatic hypertrophy and prostatitis (104, 170).

Early observations suggested zinc concentrations of 1–2 mg per gram of dried human semen (22). These reports have been reexamined and confirmed (172). First, middle, and last portions of ejaculate were collected and analyzed separately. Insignificant differences between sperm and plasma were found in all three portions, though a significant but variable percentage of seminal plasma zinc was found dialyzable.

Though present in human semen, carbonic anhydrase activity is insignificant and cannot account for the high zinc content. Surprisingly, the zinc content of aspermic semen does not differ from that of normal human semen. Mann investigated the iron, zinc, and copper content of ram semen (160). The spermatozoa of the starfish, *Asterias amurensis*, contained 210 μg per gram dry weight of zinc (85).

Zinc deficiency in rats, induced by a diet containing less than 0.5 μg zinc per gram, results in degeneration of the testes, hypoplasia of the coagulating glands, seminal vesicles and prostate, and relative or complete decrease in the number of sperm in the epididymes. All changes produced by zinc deficiency, except the testicular atrophy, were reversed when zinc was restored to the diet. When testicular atrophy had occurred, however, neither testis nor epididymis regained normal size, function, or zinc concentration (190). The effects of testosterone and gonado-

tropin administration to these zinc-deficient animals suggested a diminished production of these hormones as a result of zinc deficiency. Gonadotropin specifically increases the size of the prostate, seminal vesicles and coagulating glands, testis and epididymis, and further induces an increase in the zinc content and the rate of uptake of zinc by the dorsolateral prostate. The sole effect of estrogen, on the other hand, is a diminution in size of the prostate (188, 189). These data are in partial disagreement with those of others (90, 126).

Cadmium destroys testicular tissue when administered subcutaneously to rats. When cadmium is injected together with a large excess of zinc acetate, testicular degeneration is prevented. This has been interpreted as 'competition' of these elements for a common molecular site of action, with cadmium forming an inactive complex (212, 213).

There is no functional explanation for the presence of high concentrations of zinc in the male reproductive tract. The female genital tract does not exhibit such unusual zinc concentrations. The observations on zinc content have led to speculations as to the role of zinc in this system, but specific data are not at hand to explicate its role. None of the presently known zinc enzymes are clearly implicated, and neither the biochemical nor the physiological implications of these observations relating zinc to the male reproductive system are discernible presently; rapid progress in this area of investigation may justly be expected.

Pancreas

Zinc and the physiology of insulin. Based on the assumption that zinc is indispensable to the structural and functional integrity of insulin (see above), the occurrence and functional role of this metal in the physiology and pathology of the pancreas have been subjected to particularly intensive investigation. Reports of the crystallization of zinc with insulin and on the zinc and insulin content of normal and diabetic pancreas (78, 79, 245, 246) have evoked much controversy. It was reported that 14 normal pancreatic glands contained 0.14 mg of zinc and an insulin activity of 1.7 IU per gram of tissue respectively. The pancreases of 18 diabetic subjects, by contrast, contained 0.07 mg of zinc and 0.1 to 0.5 IU of insulin per gram of tissue. The diabetic pancreas thus seemed to contain a quarter of the normal amount of insulin and half the normal amount of zinc, and a correlation between the two parameters was implied. The details of the spectrographic technique used are not available, but the data relating to zinc contents of normal pancreas appear three to six times too high by comparison with the results obtained by others.

These investigations prompted others to reexamine the subject (70, 241, 242). Dry and wet weight of the fat-free gland were used as the base lines. The average zinc concentration of normal pancreas was 30.6 μg; that of diabetic pancreas was 25.3 μg per gram of fat-free gland. The apparent differences were caused by sporadically high values in the control group which, unfortunately, was heterogeneous in origin and contained few truly normal cases. The authors concluded that the differences between 'normal' and diabetic pancreas were statistically insignificant and decided that their data did not imply physiological relationships between zinc and insulin. Hypophysectomy did not effect the pancreatic zinc content (114).

The relationship of zinc to the physiology of insulin and of the pancreas has

since received steady attention, based in part on histochemical studies with diphenylthiocarbazone (208) and, later on, with dithizide (209). Variable amounts of zinc were detected in both the α and β cells of the Islands of Langerhans (163, 165). Previous failures to detect alterations by analysis of whole pancreas were attributed to the fact that the Islands of Langerhans constitute only a small percentage of the total mass of the organ. The amount of zinc detected in the β cells appeared to vary as a function of the changes in insulin activity induced by physiological stimuli (167). The injection of dithizone and similar agents produced diabetes in rats (124, 209); and dithizonates could be detected in the pancreas of such diabetic animals (234, 356). A diabetogenic agent, alloxan, reduces the uptake of Zn^{65} by the rat pancreas (151).

All of these histochemical and pathophysiological studies have been interpreted to demonstrate that association of zinc with insulin *in vivo* is an indispensable prerequisite to physiological storage or to the action of this hormone, or both. The chemical and physical chemical relationships of insulin and zinc have been adduced as the model for the formation of intracellular insulin depots (163). Since neither alloxan nor dithizone induces diabetes in the guinea pig, and since the islet tissues of this species does not stain with dithizone (208), these data have been thought to constitute negative confirmation of the zinc-insulin hypothesis in another species (166).

Another approach has taken advantage of the fact that islet tissue of certain teleost fish is anatomically separated from the exocrine pancreas. Quantitative analyses of such islet tissue for both zinc and insulin suggested a parallelism. The zinc content varied from 100 to 1000 μg per gram of fresh islet tissue; the variability was attributed to the physiological states of the animals. The insulin assays were correlated with measurements of zinc (344).

Autoradiography has been employed to distinguish between the uptake of Zn^{65} by acinar and islet cell tissue (175). Zn^{65} concentration of the rat islet cells remained stable throughout the experimental period, whereas the concentration in acinar tissue, initially high, decreased with redistribution and excretion of Zn^{65}. It was estimated that 50 % of the injected Zn^{65} is excreted rapidly and 42 % enters a compartment from which the loss is very slow. The zinc in islet tissue was thought to belong to the latter moiety. No conclusions were drawn concerning the postulated association of zinc with insulin *in vivo*. Though the technique did not permit differentiation of zinc content of α and β cells, it was assumed that the distribution reflected the metabolism of the β cells primarily, since these are preponderant in rat pancreas (175).

The α cells of the pancreas of the duck, as that of other birds (40), are segregated into macroscopically visible, brownish, pinpoint-sized tissue components. Zinc concentrations of such isolated areas were found to be 564, 577, and 810 μg per gram of fresh tissue, about 10-fold the zinc content of the remainder of the pancreatic tissue (349). Since the pancreas of the duck also produces 10 times more glucagon per unit weight than does the vertebrate pancreas (327), this finding was interpreted to imply a relationship between zinc and glucagon akin to that inferred for zinc and insulin (349).

Further studies based on the detection of colored dithizone complexes in the

α cells of the duck subsequent to intravenous injection of this complexing agent, showed localization of color in the α as well as in the β cells of the pancreatic tissue. Staining was diffuse, however, and did not coincide with the distribution of the α or β granules. The distribution of the stain was different when tissue sections were treated with dithizone after sectioning (234). Moreover, it was suspected that several different metal dithizonates had formed.

The recent evidence relating zinc to both the structure and function of the two hormones, insulin and glucagon, is voluminous and impressive. It may be appropriate to assist critical evaluation by commenting on several pertinent features of this long-standing controversy.

This work was initiated on the basis of physical chemical studies whose pertinence to the physiological function of insulin is dubious (see above). Zinc is not an obligatory component of the crystalline or amorphous insulin molecule, and its presence does not seem required for either normal structure or function. Both insulin and glucagon can be obtained entirely free of zinc, yet both are fully active *in vivo*. It has been suggested that zinc-free hormones will associate with zinc *in vivo*. Until a crucial experiment is devised to test this suggestion, it remains hypothetical.

While this hypothesis of synchronous action would appear to have gained considerable support from histochemical studies, it should be emphasized that agents like diphenylthiocarbazone, in addition to complexing zinc, combine with a large number of metal ions to yield variously colored products.

The specificity of the analytical system for a single metal ion is achieved by the critical adjustment of pH and by the elimination, by various means, of interfering elements (239). When evaluated critically, the specificity of some of the methods employed in these studies is found to be low. The conclusions based on such data must, therefore, remain tenuous, and a cautious view would seem justified. A histochemical technique (156), incorporating the features of specificity characteristic of microchemical techniques (300), was compared to earlier nonspecific histochemical methods. While the nonspecific methods provided results similar to the ones already detailed, the specific method completely failed to demonstrate any zinc in the α or β cells of the Islands of Langerhans. By contrast, the specific method identified zinc in the prostate (170, 171), in leucocytes (301, 176) and in the gastric mucosa (158, 159), confirming earlier macrochemical analyses of these tissues. This method was, in fact, capable of detecting zinc in the intestinal mucosa subsequent to intravenous injection of zinc acetate; this tissue does not exhibit zinc staining normally, but had been postulated to excrete the element (174). It was shown further that large concentrations of cobaltous, cupric, and ferrous ions, when introduced into tissue sections, interfere with histochemical zinc determinations, but yield colored products similar to zinc dithizonates (156).

Diphenylthiocarbazone and related compounds are non-polar and virtually insoluble in aqueous solution at physiological pH. By inference, their diabetogenic characteristics are attributed to chelation, but other modes of action cannot be excluded a priori.

Marked changes in serum and urine zinc have been reported as a result of

dithizone diabetes (168). Such variations seem completely out of proportion to the total quantities of zinc which can be mobilized from the pancreas in this manner. Therefore, the suggestion that dithizone exerts its action by combination with zinc lacks experimental verification. It should not be forgotten that pancreatic juice contains zinc in substantial concentration. The exocrine pancreas elaborates at least one enzyme, carboxypeptidase, containing 1 gram atom of zinc per mole. Since 11 per cent of Zn^{65} administered to dogs is eliminated through the pancreatic juice within 2 weeks, one might expect to find reasonable accumulations of the metal in parts of the organ other than the islet cells, an expectation not verified by the type of experiment cited above.

Doubt concerning the validity of the zinc insulin hypothesis has also been raised by another type of experiment. The injection of 2–3 millicuries of Zn^{65} in rats failed to produce diabetes. The dose was thought to be sufficient to irradiate the island cells and inactivate the mechanism responsible for the production of insulin. Administration of Zn^{65} or stable zinc did not result in any changes of the blood sugar content (253).

The most plausible support for the zinc-insulin-glucagon hypothesis is provided by chemical analysis of isolated islet organs of teleost fish (344) and studies of duck cell islands (349). Yet here, as in other instances, it is difficult to reconcile the divergent chemical and functional evidence without inductive reasoning which invokes physical-chemical model systems and equates their implication to *in vivo* phenomena (see above).

There is a marked preponderance of hypotheses and speculations over precise data and crucial experimentation, but the role of zinc in the action of insulin and glucagon has not been settled with finality. The recently renewed activity in the field, however, should ensure a conclusive settlement in the foreseeable future.

Zinc and other hormones. Zinc has been stated to bear a relationship to the action of other hormones. Marked augmentation of the effect of hypophyseal gonadotropic extracts, as judged by production of estrus and changes in the uterus, has been reported (24, 72). Similar effects of copper and zinc on follicle-stimulating hormones and luteinizing hormone in the rat have been noted (76). The addition of zinc to equine gonadotropin, however, decreases its activity (143). The physiological significance of these findings is not clear.

It has been suggested that zinc is an intrinsic and functional constituent of whale corticotropin (111). Subsequent studies demonstrated that peptic hormone digests of swine pituitary bind both zinc and copper, but not a number of other metals. Contrary to insulin, zinc was bound maximally at acid pH; the hormone shared this property with salmine and dihydrostreptomycin (37).

Corticotropin preparations with increased and prolonged physiologic action can be obtained by allowing combination with zinc phosphate or zinc hydroxide in aqueous solutions near neutral pH (113). This procedure has become a practical measure in the pharmacy of ACTH.

As in the case of insulin, the physical-chemical behavior of ACTH with zinc led to physiological studies of the clinically useful hormone-metal complex. The claim that zinc is concentrated by the pituitary followed rapidly on the heels

of the physical-chemical studies (135), although this finding has not been substantiated.

This sequence of events is not unlike that accounting for the present state of confusion regarding the relationship of zinc to endocrine pancreatic function; the evolution of knowledge in this field may be anticipated with analogous curiosity.

Structure of the Eye

The tissues of the eye contain large quantities of zinc (147, 280). The data of Leiner and Leiner (147) showing high zinc contents in perch and trout eyes have been corroborated (30); see table 4. This survey of species has been extended to a large number of vertebrates (345). The precision and accuracy of the methods employed by these different investigators are analogous in all these studies and allow comparison of the data.

The species differences are marked, as is the distribution in different parts of a single eye. The zinc content of lens and aqueous humor is not remarkable in any instance. The choroid and retina of fish and carnivores, however, may contain high concentrations of the metal, as compared with herbivorous animals. The zinc content of the choroid and retina of trout, perch, marten and fox, of the choroid of frog and dog and of the retina of the cat, are particularly outstanding. Divergent data have been obtained by different observers on rabbit eyes (30, 345).

Results are generally expressed as the mean, with or without the range of values; none of the communications, however, contain further statistical computations. Table 4 shows the zinc content of many different species in micrograms per gram dry weight of the lens, aqueous humor, retina, choroid, *tapetum lucidum* and iris.

In cattle, sheep, whale and perch, the zinc in iris and choroid was judged to be associated with a melanin fraction. Acid hydrolysis reduced the zinc content of this chromogen fraction to nearly zero, and trypsin digestion increased that of the otherwise insoluble fraction. The pigment-protein complex constituted 41.6% of the dry weight of the perch choroid, accounting for 74% of the zinc content of this tissue. No functional significance was attributed to these findings (31).

The divergence in the zinc content of the choroid of carnivorous and herbivorous animals has been attributed to the difference in zinc content of the *tapetum lucidum*. There are differences in the anatomy of this anatomical constituent of the eye in herbivores and carnivores. The *tapetum lucidum cellulosum* has been thought to be responsible for the iridescence of the eyes of carnivores (346, 347).

The results shown in table 4 indicate a major aggregation of zinc in the *tapetum lucidum cellulosum*, apparently a function of age, being considerably less in young adults though the exact age distribution is not known. Accurate anatomical dissection of this tissue, followed by analysis, demonstrated a mean zinc content of .7% in the cat, 7.2% in the dog, 7.4% in the badger, 11.6% in the fox and 13.3% in adult seals. Similar studies of the *tapetum lucidum fibrosum* (346, 348) of herbivores, of hoofed animals, and of some fish and of whales, show that this tissue,

TABLE 4. ZINC CONTENT OF COMPONENTS OF EYE IN DIFFERENT SPECIES

SPECIES, REF.	LENS	AQUEOUS HUMOR	RETINA	CHOROID	AREA OF TAPETUM LUCIDUM	BAL. OF CHOROID	ISOLATED TAPETUM LUCIDUM	IRIS
Trout (147)	16.0		540–990	9700–11,350				6900–8700*
Trout (30)	15.7		580	15,100				5990*
Perch (147)	20		1090–1780	24,000–29,600				6200–7710
Perch (30)	15.6		728	23,800				6380*
Frog (31)								
R. temporaria	78.5		500	14,400				
R. esculenta	16.3							
Cattle (31)	37.3	30.0	71.0†	139.0‡				246
Cattle (31, 346)	34.5	38.3	159.0	229.0	97	360		
Calf (345)	31.7	41.8	90.7	91.7				
Sheep (31, 346)	117		80.0†	277‡				436*
Sheep (345)	32.0	97.2	111.0	220.0	269	717		10.5
Goat (345)	29.7	42.6	152.0	275.0				
Horse (345)	28.9	18.6	135.0	270.9	206	664		
Deer (345)	32.1	65.3	86.5	158.5				
Pig (345)	20.8	67.2	132.0	176.0				
Boar (345)	21.2	82.5	254.0	371.0				
Dog (345, 346)	43.6	150.0	474.0	8803.0	46,496	565	61,000–85,000	
Fox (345, 346)	29.8	205.0	775.7	37,811.0	67,233	1308	97,000–138,000	335.5
Cat (345, 346)	32.3	174.0	1368.4	1991.0	4,640	632	4035–9681	389.1
Marten (345)	27.7	388.0	691.7	32,199.8				354.2
Hare (345)	25.7	197.7	495.2	943.0				283.4
Sperm whale (31)	35.2		54.1†	37.2‡				99.5*
Rabbit, colored (31)	15.8			466				127
Rabbit, colored (147)	18.9	9.6	36.3	212				
Rabbit, colored (345)	36.8	252.0	491.0	2000.1				
Rabbit, albino (31)	54.4			86.2‡				54.5*
Rabbit, albino (345)	25.0	142.0	402.2	1512.0				393.1
Seal (350)	23.3	223	201		8,280–40,020		112,900–146,000	
Badger (346)					61,300	8730	24,475	

Values in μg/gm dry wt.
* Plus ciliary body. † Minus pigment epithelium. ‡ Plus pigment epithelium.

equivalent to, but different from the *tapetum lucidum cellulosum*, does not contain outstandingly large amounts of zinc (346).

The intravenous injection of 100 mg/kg body wt. of dithiophenylcarbazone, a reagent which chelates zinc and other metals, causes blindness in dogs within 24 hours. This has been attributed to destruction of the *tapetum lucidum* through formation of a zinc dithizonate, followed by detachment of the retina. Histologically, the chemical events are discernible by irreversible destruction of the morphological detail. Such effects are much less certain with other agents and in other species. The dosage of this reagent required to bring about detachment of the retina in the dog is considerably smaller than that necessary to induce diabetes.

Further investigation of this problem led to the isolation of a zinc-cystein-monohydrate complex. This zinc-cystein-complex was held to account for the high zinc and sulfur content of the *tapetum lucidum cellulosum* of the fox (347). The same

substance was isolated from the *tapetum lucidum cellulosum* of the seal. This species contains exceptionally large amounts of this tissue, increasing with age and eventually accounting for about 10 mg of zinc in the eye of adult animals (348).

The functional significance of the zinc-cystein-complex is unknown. It is presumed to occur as a polymer in nature. The *tapetum lucidum cellulosum* has been postulated to increase the effectiveness of twilight vision, and the participation of the zinc-cystein-complex in luminescent or fluorescent photochemical events has been suggested (347).

Though the zinc content of the retina is not nearly as remarkable as that of the choroid, it is, nevertheless, substantial (see table 4). It has been shown (27) that alcohol dehydrogenase of horse liver can catalyze the redox interconversion of Vitamin A_1 alcohol and aldehyde. Retinene reductase, which catalyzes this reaction in the retina, is apparently identical with alcohol dehydrogenase. Alcohol dehydrogenase of horse liver has been shown to contain 2 atoms of zinc per molecule of apoenzyme (305), and some of the zinc content of the retina may be attributable to its presence. The vitamin A-resistant night-blindness of individuals afflicted with postalcoholic cirrhosis of the liver (214) has been attributed to inactivation of this enzyme in postalcoholic cirrhosis (316).

Blood

Whole blood. The zinc concentration of the blood of normal vertebrates, including the human, has been subjected to increasingly intensive study (15, 35, 39, 51, 58, 134, 136, 155, 256, 300, 313, 325, 326, 354, 355). While difficulties in technique have continued to cause some problems in establishing the norms in man, a series of successive approximations seem to have reached the true values reasonably well.

Table 5 shows the zinc concentration of normal human whole blood, serum or plasma, erythrocytes and leucocytes; serum contains about 12%, leucocytes 3% and erythrocytes 85% of the whole blood zinc. The results shown in table 5 are in fairly good agreement and are those thought by various workers to represent their latest and most reliable data. Earlier results are usually higher (301, 354), presumably due to contamination of needles, syringes, or glassware (326). The normal adult serum zinc concentration is close to 120 ± 20 μg per 100 ml (see table 5); the whole blood content varies from 700 to 800 μg per 100 ml, and the erythrocyte content from 1200 to 1300 μg per 100 ml. The values for erythrocyte content in Vikbladh's data (326) were obtained by difference rather than by direct measurement, probably explaining their marked divergence from those of other observers. Analyses performed on serum and plasma are equivalent, indicating that zinc is not removed by clot formation. There are no seasonal or diurnal variations, nor do there appear to be differences between the sexes. There are no significant variations in pregnancy (325, 326). The plasma zinc content, high at birth, approaches the adult values in the course of the first year. The erythrocyte zinc content in newborn infants is only 25 per cent of the normal adult value, rising progressively over the first 12 years of life (15). This observation correlates

TABLE 5. RECENT DATA OF ZINC (μG/100 ML) OF NORMAL FLUID OR CELLS AND OF LEUCOCYTES AND ERYTHROCYTES

BLOOD	PLASMA OR SERUM	ERYTHROCYTES	ERYTHROCYTES*	LEUCOCYTES*	REF.
811±79	124±15.9	1783±185			(325, 326)
880±200	121±19	1440±270	$1.3 \pm .2 \times 10^{-2}$	$3.7 \pm 1.3 \times 10^{-2}$	(301, 313)
713	120	1180			(134)
704	130±13.9	1300		2.3×10^{-2}	(354, 355)
657	109±20	1244±180			(15)
794±207	239±86		$1.335 \pm .382 \times 10^{-2}$	$3.335 \pm 1.43 \times 10^{-2}$	(51)

Standard deviations are indicated when available.
* Per million cells.

with observations of the low level of erythrocyte carbonic anhydrase previously noted in premature and newborn infants (266).

Thus zinc appears to be a constant constituent of plasma or serum, erythrocytes and leucocytes. The investigations of blood zinc in disease have attempted to correlate clinical entities with either an increased or decreased concentration of the metal, the change being related to the mean of a 'normal' group. The analytical methods, the 'normal' values obtained, and the statistical criteria employed to judge values 'abnormal', vary for different workers. These circumstances complicate interpretation of changes in disease, particularly since some publications contain only the conclusions and do not communicate the detailed data on which they are based (355).

Serum. The exact characteristics, number and function of the serum zinc proteins are not known. Zinc is said to exist in at least two fractions: firmly bound zinc, amounting to about 34%, and loosely bound zinc, amounting to 66% of the total zinc content (see above) (326). When Zn^{65} is administered intravenously, most of it disappears within 24 hours, though a minor fraction can be detected in the blood of patients for many weeks (309). Only 11% of the dose administered to patients with neoplastic disease remains in the plasma after 30 minutes (62). Ten per cent of a dose of Zn^{65} given to steers can still be found in the serum after 4 hours; only 3% can be detected after 10 hours when equilibrium is reached (74). In the dog, 20–30% of an oral dose of the isotope was associated with the serum albumins, 20–30% with α_1 and α_2 globulin, while the balance was distributed equally among the β_1 and γ-globulin fractions. 'Firmly' and 'loosely' bound moieties were present in all serum protein fractions, though zinc seemed most strongly bound to the globulins (355). Other observers noted only two peaks of radioactivity in electrophoretic studies of human and rabbit serum. In man, the larger peak was found in the slow moving part of the albumin fraction. The smaller fraction migrated with the α_2 globulin region, in a position similar to the α_2 peak of lactic dehydrogenase (324).

The discovery of zinc dehydrogenases (324) some of which are present in normal plasma, suggest further studies.

Zinc may combine with the β_1 globulin, transferrin, *in vitro* (272). This reac-

tion does not compete successfully for iron, but has formed the basis of a suggestion that this protein is responsible for the transport of both iron and zinc. The experiments reported in support of this suggestion are unconvincing (326). The physiological significance of the physical-chemical studies is, therefore, uncertain.

The serum zinc concentrations in acute and chronic infections (pneumonia, bronchitis, erysipelis, pyelonephritis) are decreased below the range of normal values and are restored to normal with recovery (326). At maximum temperature elevation, the serum zinc concentrations were thought to reach their lowest values (30–80 μg/100 cc). The inverse relationship between serum zinc concentration and both body temperature and erythrocyte sedimentation rate is thought to be a coincidence by others, since experimentally induced fever is said to increase serum zinc concentrations (355). Statistical considerations do not support either of these interpretations.

Decreased serum zinc concentrations in untreated pernicious anemia have been observed repeatedly (299, 326, 355). Therapy reverses this finding. In all other anemias studied so far, the data either indicate normal values or are so sketchy that no definite conclusions may be drawn (326, 355). A statistically significant decrease in serum zinc has also been observed in myocardial infarction (330). It accompanies changes in a variety of enzyme activities and an increase in copper concentration (329).

Decreased serum zinc values have been observed in various malignancies (326, 355), although a uniform pattern is not discernible.

A large variety of other conditions have been thought to demonstrate 'normal' serum zinc concentrations by one observer (326), while others have found them low. The zinc concentration in lymphomatous disease, e.g., has been reported increased, based on data which are non-Gaussian in distribution (133). These results are not in agreement with those obtained by others (355).

A significant depression of the serum zinc concentration is characteristic of Laennec's cirrhosis (313, 326, 355), although the data are not unequivocal in other diseases of the liver, e.g. infectious hepatitis.

Increases of serum zinc in hyperthyroidism, hypertension, polycythemia vera and eosinophilia, as well as subsequent to administration of adrenaline, thyroxine, thyrotropic hormone, therapeutic x-irradiation, and experimentally induced hyperthermia have been reported (355). Many values called 'abnormal' on the basis of the controls employed would be 'normal' according to the standards of other workers. Some aspects of these considerations have been discussed (206).

Erythrocytes. The zinc content of erythrocytes of several species have been obtained with a polarographic technique of 2–3% precision (256).

Man	13 μg/gm	Rabbit	9 μg/gm
Rat	10 μg/gm	Goose	6.5 μg/gm
Dog	9 μg/gm		

The zinc content of the cytoplasm and nuclei of the goose, turtle, frog, and fish—all animals having nucleated erythrocytes—have also been examined (136,

191). About three times as much zinc was found in the separated nuclei as in the cytoplasm. The apparent increase in the erythrocyte-plasma ratio with phylogenetic evolution was suggested to be a consequence of the accumulation of carbonic anhydrase in erythrocytes (136).

Zn^{65} penetrates the bovine and human erythrocyte readily, though there is no concomitant increase in carbonic anhydrase activity (15, 74). Equilibrium between bovine serum and erythrocytes is achieved in 6 hours (75).

Determinations of zinc content and carbonic anhydrase activity of human erythrocytes have implied that they are mutually dependent variables, though this correlation is not absolute. In a number of pathological conditions, the two parameters vary in a parallel fashion, allowing the interpretation that a large percentage of erythrocyte zinc is an integral part of carbonic anhydrase (310). Since erythrocytes are rich sources of pyridine nucleotide-dependent dehydrogenase, it may be suggested that a fraction of the non-carbonic anhydrase zinc may be associated with some of these enzymes which contain either zinc or other metals (314).

In normal persons as well as those afflicted with anemias, polycythemia vera, secondary polycythemia, leukemia and congestive heart failure, both erythrocyte zinc and carbonic anhydrase activity vary directly with hematocrit level and hemoglobin concentration. In untreated pernicious anemias, however, the erythrocyte zinc concentration and carbonic anhydrase activity are nearly normal, although the hematocrit value, erythrocyte count and hemoglobin levels are markedly decreased. The mean corpuscular zinc concentration and carbonic anhydrase activity are increased several times more than even the high mean corpuscular hemoglobin level, and are out of proportion to the increase in cell size. In remissions from this disease, zinc concentration and carbonic anhydrase activity return to normal. Erythrocyte zinc concentration and carbonic anhydrase activity are also increased in sickle cell anemia (310).

A decrease of 50 per cent in the concentration of zinc in the whole blood of Chinese suffering from protein deficiency, acute and chronic beriberi, and pellagra has been reported. No separate values for erythrocytes and serum were obtained, and no hematological data are given (66).

Leucocytes. Leucocytes contain 25 times as much zinc per cell as do erythrocytes (301). These findings have been confirmed (355). Zinc has been thought to be associated with the granules of polymorphonuclear and eosinophil cells, the eosinophils containing 10 times more zinc than do other leucocytes (156, 355). The identification of large quantities of zinc in Charcot-Leyden crystals, which are derived from human eosinophils, would tend to confirm these findings (34).

Leucocyte zinc has been resolved into at least two moieties, one being soluble, the other insoluble at pH 7.2. The soluble fraction accounts for 81.1 % of the total zinc content of leucocytes (106). Thus far, neither fraction has been shown to have enzymatic activity. Human leucocytes have failed to exhibit a correlation of zinc content and alcohol dehydrogenase, carboxypeptidase, lactic dehydrogenase, malic dehydrogenase, glutamic dehydrogenase and rhodanese activity (Hoch and Vallee, unpublished).

Marked increases of leucocyte zinc have been seen in patients with anemias, refractory to all therapy and accompanied by leucocyte counts below 2,000 per cubic millimeter (301a). The zinc concentration of leucocytes in acute and chronic lymphatic and myelogenous leukemia is decreased to 10% of the normal value. Urethane and x-ray therapy return the zinc concentration to normal. Intravenous administration of zinc gluconate fails to raise the leucocyte zinc concentration or to influence the course of the disease. The phenomenon is apparently independent of the maturity of the cells (87, 355).

Subcellular fractions. Zinc and other metals were measured in the cell fractions obtained by centrifugal fractionation of rat liver (284). The following values were obtained in milligram of zinc per gram nitrogen:

Whole liver	0.78 ± 0.04	Mitochondria	0.35 ± 0.07
Connective tissue	0.80 ± 0.17	Microsomes	1.40 ± 0.28
Nuclei and cell residue	1.1 ± 0.20	Clear supernatant	1.70 ± 0.08

The distribution of the metal in these fractions is consistent with the intracellular localization of the known zinc enzymes (284).

Studies with Zn^{65} have suggested that the element is associated with the desoxyribose nucleoprotein of nuclei from calf thymus and mouse tumor (99).

Marine Organisms

The liver and the respiratory blood pigment, hemosycotypin, of the snail, *Sycotypus*, were reported to contain zinc (183). This finding has not been reexamined. Substantial concentrations of zinc have also been found in oysters, though its exact localization in this mollusc is not known (103, 277).

Polarographic analyses have demonstrated an average of 2.44 μg of zinc per milligram dry weight in the isolated nucleoli of the ova of the starfish, *Asterias amurensis*, compared to an average of .07 μg per gram in the whole oocyte and ovaries. The staining of the chromosomes of this species with dithizone dissolved in 60% ethanol was interpreted to denote the presence of zinc. The participation of the metal in cell division was postulated, although direct evidence has not been given (83, 85).

Snake Venom

Analyses of cobra venom have variously been reported to reveal .56% (58) and .46–.55% of zinc (227). Viper venom has been suggested to contain lower zinc concentrations. These data require substantiation. They have formed the basis of studies on the mode of action of snake and bee venoms (80).

Foodstuffs

The literature on this subject has been summarized successively by Lutz in 1926 (155), Hegsted *et al.* in 1945 (101), and Monier-Williams in 1949 (192). The interested reader is referred to these reviews for details.

Excretion and Absorption

Urine and Stool

The zinc content of normal human urine and stool was first reported in 1927 (60). The data, obtained with a method of questionable validity, indicated that

1 mg is excreted in urine and about 10 mg in the stool in 24 hours. There was a rise in fecal excretion when zinc-rich foods, such as oysters, were consumed. No concomitant changes in urinary zinc excretion occurred. Excess zinc either passed through the gastrointestinal tract without being absorbed or was eliminated by the intestinal mucosa. Most subjects reacted to an intravenous injection of zinc by excreting it through the gastrointestinal canal, though different individuals varied in their response, which was thought to be related to the state of tissue saturation with zinc. This latter suggestion (174) has been enhanced by histochemical studies, showing increased quantities of zinc in the epithelium of the small intestine subsequent to the intravenous injection of zinc acetate (156).

The normal human zinc intake has been stated to be 10–15 mg per day (174). Most of it is excreted in the stool, varying from 5.1 to 10.3 mg per day as a function of intake. Zinc in pancreatic juice and bile contribute to fecal elimination (23, 193). Cattle and rats absorb variable amounts of Zn^{65} from the gastrointestinal tract (74, 75). The urinary excretion is apparently independent of intake. The minimum daily requirement of man is not defined.

The zinc content of almost all foodstuffs parallels that of their iron content: i.e., substances containing large quantities of one element have a similarly high content of the other, and conversely (174).

Two balance studies in pre-school and school children respectively (250, 264) add little to the information on fecal and urinary excretion of zinc reported by McCance and Widdowson (174); the data presented do not permit general conclusions. A balance study in 13 young college women indicated an average intake of 13.2 mg of zinc per day. Of this amount, 6.6 mg was retained, 5.6 mg were found in the stool, and 1.0 mg also formed in the urine, a figure much higher than that of other investigators (174, 313). The data indicate, however, that this amount of dietary zinc is adequate to maintain a positive balance (286).

Normally 440 ± 120 μg of zinc a day are excreted in the urine (174); more recent studies give very similar values, 457 ± 120 μg of zinc per 24 hours. Serial determinations of the daily urine zinc content of one normal person demonstrate that 550 ± 52 μg are excreted per 24 hours. Zinc excretion is independent of urine volume (316).

The first demonstration of zincuria was that which accompanies albuminuria (73). Subsequently, an average of 2.1 mg of zinc per 24-hour collection was reported (174). In both investigations no direct relationship between the amounts of albumin and zinc excreted could be found. While coincidence suggested that zinc was excreted bound to the protein, the data did not bear out the hypothesis. The proposition has not been reexamined critically. A significant zincuria exists in postalcoholic cirrhosis (316). The excretion in 10 patients was 1016 ± 196 μg of zinc per 24 hours. Administration of physiological amounts of oral zinc paradoxically decreases the zincuria to normal.

An increased elimination of zinc also has been postulated in porphyria, though analytical documentation is completely lacking (217). In rats, marked elimination of zinc is induced within 18 hours after administration of ethylenediaminetetraacetic acid (187).

Milk

The concentration of zinc in cow's milk (3–5 mg/l) is about 10-fold greater than that of iron (.3–.6 mg/l), 100-fold greater than that of copper (.05–.15 mg/l), iodine (.03–.07 mg/l) and manganese (.02–.03 mg/l), and 1000-fold greater than that of cobalt (.004–.007 mg/l) (291).

The values for zinc content in milk from different species are comparable (137). For cow's milk a mean of 3.0 mg per liter has been reported (9). A spectrographic study of milk from a pure-bred herd of Guernseys and of commercially available milk failed to reveal significant differences in zinc content (Vallee, unpublished).

It has been noted that colostrum contains a large amount of zinc; the concentration falls significantly as lactation progresses (137). These data have been confirmed by serial analyses of human and cow's milk, starting on the day of delivery and continuing for several months. In both species, the zinc content is as high as 20 mg per liter at the time of delivery; in cow's milk it returns to 3–5 mg per liter within 2 weeks, to remain at this level over a period of 6 months; but in human milk it falls off gradually to reach 650 μg per liter at that time (15). Feeding of zinc to cows increases its concentration in their milk (9). More than half of the dose of Zn^{65} retained by lactating rats is excreted in the milk within 96 hours (75).

The concentrations of zinc in milk are of particular significance in view of the potential and real importance of the element to nutritional disease in the young. The prevalence of such disease recently led Indian workers to investigate the zinc content of the milk of cows in apparent poor health and from animals grazed in areas known to be deficient in this element; this milk is low both in zinc and in other metals (122).

Zinc Deficiency in Animals

Mouse

A series of experiments in 1922 (18) were designed to deprive mice specifically of dietary zinc, although it was realized that the diets lacked other growth factors. The zinc-fed controls lived longer than the zinc-deprived animals, but the members of both groups died during the experiment (19). Similar findings were obtained by others (119).

The experiments were repeated some 10 years later (20, 21), with diets improved by the addition of vitamins. The young mice were separated from their mothers at 21 days. The 'zinc-free' diet presumably contained more than 50 μg of zinc per hundred grams of diet. Animals on this diet failed to grow, lost weight, and succumbed with symptoms of ataxia and posterior column disease within 14 to 23 days. The control animals receiving zinc survived for 57–74 days. It was concluded that zinc is essential for the growth and survival of the mouse. Similar results were obtained almost simultaneously (285) and were contrary to earlier, inconclusive experiments (203). The improvement in purification of the diets probably accounts for the discrepancy.

The studies in mice have been extended (204) to test the hypothesis that

suckling mice depend upon the zinc of colostrum for their normal and adequate supply of the element. The newborn were separated from their mothers within 12 hours after birth and transferred to foster mothers for 13–18 days postpartum. Growth and ossification were retarded, as were the eruption of the incisors and the separation of the eyelids. There were pathological alterations of the dorsal skin (81). Newborn mice were normal when nursed by their own mothers for 2½ to 4 days prior to their transfer to foster mothers. Oral administration of zinc prevented these disorders, leading to the conclusion that zinc in colostrum was, indeed, responsible for the maintenance of physiological homeostasis. The design and execution of these experiments have been questioned (207).

Rat

The rat has also been used as an experimental animal (268). Close attention to preparation of diets results in normal growth of the control animals. The growth of zinc-deficient rats proceeds at one-third of the normal rate. The fur softens and turns from black to gray in 6–7 weeks. The zinc-deficient animals require 52 per cent more ration to gain 1 gram. Two animals fed adequate amounts of zinc after termination of the experiment resumed their previous normal appearance (268). The difference between deficient and adequate diets is surprisingly small. Zinc-deficient rats received 22 μg of zinc per day (115). Feeding of 30 μg of zinc per day caused marked improvement, and 40 μg per day completely prevented the manifestations of zinc deficiency. The controls received 300 μg per day.

The glucose tolerance curves in these zinc-deficient animals are delayed and irregular; there is no glycosuria, however, and the blood sugar and liver glycogen are normal. These findings suggested delayed absorption of glucose from the gastrointestinal tract (115).

Protein metabolism was disturbed also, as evidenced by a distinct delay in the time required for the blood nitrogen to reach a maximum value and by the lower plasma total protein level in the zinc-deficient rats. The efficiency of food conversion to body tissue was much increased by pituitary transplants (115).

Additional studies tried to ascribe the disturbances in carbohydrate and protein metabolism to changes in activites of enzymes thought to involve zinc. Pancreatic amylase and proteolytic activity in zinc-deficient animals is lowered significantly but is not restored by the addition of zinc *in vitro* or *in vivo*. Some of these findings may now be viewed in light of the discovery that carboxypeptidase contains zinc (306, 307). However, similar decreases in enzyme activity are obtained in magnesium deficiency, and here the changes are considered to be the result of general debility (116). Zinc has no effect on crystalline pepsin and trypsin activity. While there are no changes in bone phosphatase, intestinal phosphatase activity in zinc-deficient rats is considerably decreased; this was regarded as a possible explanation for the observed disturbances in amino acid metabolism described above. In all the animals, the zinc content of soft tissues is normal, but that of bone is lowered (118). Though there is a slight anemia, no striking lowering of carbonic anhydrase activity is encountered.

The uric acid content of blood of zinc-deficient rats was found doubled on all

diets tested (328). Carbohydrate, fat, protein, or nucleoprotein content of the diet did not influence the course of zinc deficiency, but the hyperuricemia was not reversed until 5 weeks after the zinc had been added to the deficient diet. The hyperuricemia was thought to be due to increased uric acid synthesis, not lack of destruction, since uricase activity was completely normal; this finding was not surprising, since this enzyme does not contain the element (110, 220).

In another study, the growth of zinc-deficient rats was retarded after 2–3 weeks. In two rats marked eczema, and in a few others alopecia, were noted. The alkaline phosphatase activity of blood was markedly reduced, but no such changes were found in kidney or bone phosphatases.

Carbonic anhydrase activity per unit of erythrocytes is unchanged (54). Later work with mice (53, 55) showed that zinc deficiency causes a decrease of liver catalase activity which cannot be restored by the addition of zinc *in vitro*. Liver esterase activity and riboflavin content of heart, lung, kidney and liver are unchanged. Cadmium does not replace zinc in deficient diets.

Histological studies of zinc-deficient rats (54, 81) reveal hyperkeratinization, thickening of the epidermis and intra- and intercellular edema of the skin and mucous membranes of the esophagus and mouth.

The parakeratosis of the esophagus is particularly striking. The skin shows a thick layer of keratinized cells, hyperkeratinization and thickening of the epidermis with loss of hair follicles but not of sebaceous glands (81). Testicular atrophy and decreased size of the accessory sex organs has been additionally noticed (190). A similar parakeratosis has been observed in mice who showed alopecia, thickening and keratinization of the epidermis, thickening of the corium and hypoplasia of the subcutis. In addition, a rosary-like tail, clubbed digits, and deformed nails were described (204). Most recently, zinc deficiency has been observed in the chick (229) and turkey (141).

Porcine Parakeratosis

The recent discovery that porcine parakeratosis may be a primary or secondary zinc deficiency is a significant step forward in our knowledge of the function of the element in higher vertebrates. Spontaneous parakeratosis is widespread (132). The condition is characterized by dermatitis, diarrhea, vomiting, anorexia, severe weight loss, and eventual death. The increase of its incidence in recent years has been attributed to the practice of adding bone meal to the rations to accelerate growth and ossification. The disease has been observed primarily in hogs receiving rations composed of corn, vegetable protein, vitamins and mineral sources.

In 1955, Tucker and Salmon (288) induced a nutritional disease in hogs which closely resembled the spontaneous and endemic disease of this species described as parakeratosis (132). By a process of systematic elimination, this disease has been defined as a zinc deficiency which is aggravated significantly by a high calcium content of the diet. Symptoms and signs of zinc deficiency have been observed on diets containing as much as 34–44 ppm of zinc. Supplements of zinc carbonate induce prompt recovery.

Experimental parakeratosis has been induced with greatest facility in the presence of large amounts of calcium. At levels of about 30–35 ppm of zinc, a normal dietary calcium intake (0.48–0.82 %) aggravates the symptoms of parakeratosis and inhibits weight gain. These symptoms become progressively more severe when the level of calcium is increased. A total of 80–100 ppm of zinc in the rations markedly reduces the signs of the deficiency and prevents the occurrence of dermatitis; these levels of zinc restore the parakeratotic pig to normal (45, 148, 149, 152, 153, 265).

The addition of 1.33 % of monosodium phosphate has no effect on growth but significantly alleviates the severity of the dermatitis (14, 148), an effect unexplained thus far. Oral administration of vitamin A acetate neither ameliorates the skin condition nor does it increase the serum vitamin A concentration, which has been mentioned as being low (265).

The requirement of zinc by hogs on practical rations appears to be very high compared to the 4–6 ppm found to be essential for the mouse and rat on fortified diets. The zinc content of purified swine rations is usually less than 10 ppm. Yet these animals do not develop parakeratosis, even though the calcium content of their diets is approximately the same as that of practical rations. It would appear that, in addition to calcium, there are yet other unidentified factors in practical hog feeds which are responsible for the apparent high zinc requirement. Studies with Zn^{65} indicate that in experimental parakeratosis the absorption of zinc from the gastrointestinal tracts is normal, and parakeratosis has been induced with diets containing normal levels of calcium (Plumlee, personal communication). Thus, the disease is not explained by postulating an interference with the absorption of zinc.

Chemical analyses of tissues for metals have been performed infrequently, and little is known of biochemical changes in this condition, making any localization of specific metabolic defects difficult.

The addition of calcium to the diet does not affect the zinc content of any of the organs examined; on the other hand, addition of zinc sulfate increases the concentration of zinc in some tissues (109). Both calcium and magnesium are reported present in normal concentrations in the sera of parakeratotic animals. There are small and variable changes in serum phosphorous and in blood glucose and hemoglobin levels, and the serum albumin:globulin ratio is inverted and decreased (265). When the amount of calcium in the rations is decreased from 1.2 % to 0.8–0.5 % (148), the zinc concentrations in liver, kidney, hair and bone rise significantly, but the zinc of plasma, pancreas, skin and intestine does not show a similar change. Increasing the zinc content of the rations to 128 or 1028 ppm produces no increase of zinc in skin, but there are increases of zinc in other organs (109, 148).

Serum alkaline phosphatase activity has been assayed in these animals and is said to be extremely variable in animals receiving rations containing 32–44 ppm of zinc (265). On the other hand, Lucke reported distinctly low serum alkaline phosphatase activities in parakeratotic animals and found increasing activities when such animals receive increased amounts of dietary zinc (157). This may be

viewed in the light of the discovery of zinc in alkaline phosphatase of swine kidney (164). Further investigation of metal levels and of zinc metalloenzyme activities certainly seems to be indicated in parakeratosis.

The failure of pigs with parakeratosis to gain weight and grow, the anorexia, and the skin lesions as well, are analogous to the effects of zinc deficiency in mice and rats. Since the esophagus is markedly keratotic also, it may be conjectured that this alteration in the histological structure of the upper gastrointestinal tract contributes materially to the inanition by interference with deglutition.

Parakeratosis as described so far has all of the characteristics of a conditioned deficiency, though the same disease probably can result from a primary zinc deficiency. The discovery of this spontaneous disease, however, has provided students of the function of zinc with new facilities to explore and delineate its significance in biochemistry, nutrition and pathology. This opportunity should bring about significant gains in the understanding of the contribution of zinc to homeostasis.

The only reports of primary nutritional human zinc deficiency deduced from analyses of blood and tissues are provided by Eggleton (65–67). The zinc contents of toenails, fingernails and skin of Chinese subjects affected with subacute and chronic beriberi are reduced to half the normal value. The blood zinc was subnormal compared to the author's normal controls. Similar changes in the zinc concentration of blood occurred in nutritional edema and semi-starvation. By contrast, zinc and copper contents of the liver and kidney were increased in similar cases. No changes were encountered in diabetes mellitus. This work, however, is as yet inconclusive and fragmentary.

Postalcoholic Human Cirrhosis

Based on the identification of zinc in two mammalian enzymes, liver alcohol and glutamic dehydrogenase, deviations in zinc metabolism were sought in human liver disease. Marked abnormalities of zinc metabolism have recently been described in postalcoholic cirrhosis, a widely prevalent disorder (313, 316). The concentration of zinc in the serum of patients with postalcoholic cirrhosis is markedly decreased; it bears a significant relation to the disease and its fluctuating course. In cases with 'severe' disease, the serum zinc concentration is 66.6 ± 19 as compared to the normal of 121 ± 19 μg per 100 ml. The greatest depressions are noticed in comatose patients; concentrations below 30 μg per 100 ml are a poor prognostic sign. Among the tests of liver function, zinc data correlate best to studies of bromosulphthalein retention (313). The concentration of the element in liver tissue of individuals dying with the disease is also decreased (154, 316). Concentrations of 29.0 ± 7.0 μg zinc per gram wet tissue were found, as opposed to 74.0 ± 23.0 μg per gram in 'normal' individuals. Substitution of dry weight or nitrogen for wet weight did not alter the difference in the two groups. Iron decreased similarly, while the concentrations of calcium, magnesium, aluminum, manganese and copper did not change. These elements, therefore, served as 'internal standards,' suggesting that the abnormal concentrations of zinc and iron are not due to anatomical alterations of the liver, and accounting for such com-

positional changes simply by replacement of liver cells with connective tissue. The data indicate, therefore, that these alterations in zinc and iron content are characteristic of the disease.

Such patients excrete abnormally large quantities of zinc in their urine, 1016 ± 196 μg per 24 hours. Oral zinc sulfate in physiologic quantities tends to restore normal excretory patterns; a tendency toward restoration of normal liver function was also noted.

The data were interpreted in the light of the comparative biochemistry of zinc and ethanol metabolism. It was considered possible that a conditioned zinc deficiency is a feature of this disease, though the conditioning factors can only be speculated upon at present. Such a hypothesis seems consistent with the known historical, pathophysiological and pathobiochemical knowledge of this disease (316).

In summary, zinc is indispensible in mouse, rat and porcine physiology, and several human pathological states are accompanied by, or may be related in some way to, zinc deficiency or imbalance.

Zinc Intoxication

No attempt will be made to cover the pharmacological effects and chemical changes in man or animals given zinc, except to point out that the intravenous administration of 2 mg per kilogram of zinc gluconate is tolerated well by both dog and man (309). Four milligrams per kilogram produce lassitude, decreased tendon reflexes, bloody enteritis, diarrhea and paresis of the hind legs in the dog. The electrocardiographic changes are similar to those observed in potassium intoxication (309).

Daily doses of 300 mg of zinc gluconate were tolerated by a patient with myelogenous leukemia, and without apparent ill effects. Intravenous doses of 15 mg to a dog weighing 15 kilograms temporarily depressed the leucocyte count, and one dose of 100 mg given to a patient with myelogenous leukemia was followed by a fall in white blood cell count from 300,000 to 150,000 over a period of 5 days, after which it rose to the previous level. Further intensive therapy totaling about 1 gram of zinc gluconate failed to affect the disease process permanently (87).

A series of observations on the effect of injected zinc on metabolic processes deserve attention (59).

Three cats of a group fed large doses of zinc developed fibrosis of the pancreas (60). These findings were confirmed (245). Following completely negative results (102) in rats which were fed a diet containing 0.25% of zinc, it was reported that doses as small as 0.1 mg per day of zinc, fed together with iron, assisted hemoglobin regeneration (194). One-half a milligram of zinc, however, retarded regeneration.

These findings were confirmed, and it was noted, moreover, that in rats, 0.5% of zinc per day resulted in impaired production of erythrocytes and retarded growth; termination of the diet restored normalcy. The largest dose tolerated was apparently between .5 and 10% of zinc of the total diet per day. On the latter diet, animals died after 4 weeks. Terminally, the authors detected an excess of

leucocytes and immature erythrocytes in every case. It should be stated clearly, however, that such daily quantities of zinc are far above the likely physiological consumption of the element. Similarly, glycosuria (276) subsequent to high zinc intake by stomach tube is not likely to occur under normal conditions (273–275).

Rats fed a diet containing 1 % of zinc develop severe anemia in 3–5 weeks (261); growth ceases and many die before the sixth week. On a diet containing 0.7 %, severe microcytic, hypochromic zinc anemia is evident in 4 weeks, though the animals continue to live. The additional feeding of copper maintains the hemoglobin at statistically significantly higher levels, and a mixture of iron, copper and cobalt maintains the hemoglobin at close to normal levels. These elements have no effect on the subnormal rate of growth of the zinc-fed animals, but supplements of a liver extract produce a significant growth response. The feeding of zinc supplemented with liver almost doubles the normal leucocyte count. These findings may bear on the presence of zinc in leucocytes and erythrocytes and may possibly be related to maturation of both these cellular elements. Molybdenum and zinc synergistically retard growth (88). An excess of 1–1.5 % zinc in the diet of rats reduces the growth rate and hemoglobin formation as previously observed (61). Heart cytochrome oxidase activity is reduced. The addition of .03 % copper to the diet effects partial restoration of the growth rate, prevents anemia and increases cytochrome oxidase activity beyond that observed normally. Folic acid has no effect (61). Analogous results were noted by others. Dietary levels of 500–700 mg of zinc decrease liver catalase and cytochrome oxidase activities. The addition of 0.2 mg per day of copper to these toxic amounts of zinc restores the activities of both enzymes to normal, but in this study, no amelioration of the growth deficit was noticed. While the data strongly suggest an antagonistic effect between zinc and copper, independent actions were not ruled out (323).

The effect of excess zinc intake on liver and bone metabolism has been studied. An intake of 1 % zinc per day markedly reduces the fat content of the liver of rats fed 10 % casein and 10–30 % fat. The diminished liver fat content is not a consequence of faulty fat absorption since fecal fat content is normal (236–238).

Several other metabolic abnormalities were also noticed. On high zinc diets urinary and fecal nitrogen, fecal phosphorus and sulfur, urinary uric acid, and creatinine contents are all increased, while phosphorous and sulfur content of the urine are concomitantly decreased (237). These results suggest widespread metabolic effects of excess amounts of zinc, but efforts at localization of the enzymatic steps involved have not been revealing.

Toxicology and Industrial Hazards

Zinc poisoning may occur as a result of three distinct processes: *1*) ingestion of toxic amounts of zinc with food or drink; *2*) direct skin contact with zinc or zinc salts; *3*) inhalation of fairly high concentrations of freshly formed zinc oxide fumes (101).

The ingestion of toxic amounts of excess zinc in food produces an acute and transitory illness within a few minutes after ingestion. The symptoms may include

malaise, dizziness, tightness of the throat, emesis, colic and diarrhea (36). Excess zinc intake can usually be attributed to the preparation of acid foods (fruits, juices, stewed fruits) in galvanized containers, resulting in solubilization of zinc. Treatment is symptomatic and supportive; preventive measures will obviate a recurrence.

In general, zinc and zinc salts are well tolerated by the human skin. Several cases of poisoning due to direct contact with zinc or zinc salts have been reported, though it seems to be a relatively rare occurrence. A contact dermatitis, attributable to exposure to zinc chromate, has been observed. It is troublesome, persists long after the exposure, and is quite disfiguring. The chromate is, however, presumed to be the prime offender (94).

The problem of 'metal fume fever' ('galvo,' 'brass-founders' ague,' 'brass chills,' 'zinc shakes,' 'spelter shakes') occupies, by far, the most prominent role among the possible toxic effects of zinc. Increasing attention to the subject has brought about little further insight into the mechanism of action of the metal fumes in the genesis of the syndróme.

Inhalation of zinc oxide fumes results in fever, malaise and depression, and cough which may become violent enough to induce vomiting, excessive salivation and headache. Occurrence of gastrointestinal symptoms is disputed by most authors, though some claim to have observed colic (57). A sweetish taste has usually been noted to accompany the attack. A high white blood cell count is typical. Increases in urinary urobilinogen and zinc excretion have been observed. About 8 hours after exposure, while the temperature and white blood cell count are rising, a chill may occur when the temperature reaches 103–104° F, followed by a drop to normal; the leucocytosis may persist for another 24 hours. The effect of zinc is not cumulative and does not predispose to other disorders of the respiratory tract.

Zinc oxide is not the only metal oxide giving rise to this syndrome. Inhalation of cadmium oxide may also produce a severe reaction which may be fatal, while similar but less severe reactions may be observed due to the inhalation of magnesium oxide fumes. No reaction is observed following the inhalation of ferric or aluminum oxide.

The danger of metal fume fever is encountered wherever zinc oxide is an industrial product or by-product: zinc smelting, the manufacture of zinc oxide and zinc powder, the manufacture of brass, the welding of galvanized iron (41). The disease is self-limited, and its treatment is symptomatic, consisting of general supportive measures.

Obviously, prevention of metal fume fever is more important than its treatment. Preventive measures are easily instituted and aim at concentrations of less than 15 mg of zinc oxide per cubic meter for 8 hours. When exposure for those periods cannot be avoided, respirators should be used.

Zinc and Cancer

Interest in the relation of zinc to cancer developed as a result of reports (58) concerning its presence in many human and vertebrate organs and its particularly

high concentrations in certain tumors (47–49, 142). It has been suggested that malignant tissues contain very high concentrations of zinc, while their content of copper is low. The zinc concentration of all tissues, serum and urine were increased in rabbits inoculated with sarcomata (270, 271). On the basis of present biochemical and chemical knowledge of the techniques used for preparation and analysis of samples, these data must be considered of dubious significance.

In the cock testis, teratomata develop subsequent to the injection of zinc chloride into these organs (185, 186). The tumor rarely occurs spontaneously in these animals. Periods of highly abnormal or artificially induced sexual activity seem to be prerequisite conditions (10). The tumors can be transplanted (6). The physiological significance of these data is questionable, but they may be viewed in the light of the work on the male human genital tract (see above).

The possible carcinogenic role of zinc was investigated further (24, 25). Local zinc injections into the breast of Marsh-Buffalo mice actually inhibit the development of carcinomata when compared with injections of normal saline into controls. The significance of these unconfirmed results is not clear.

Leukemic leucocytes contain only one-tenth of the normal amount of zinc. These low values rise to normal as the leucocyte count falls as a result of therapy (87). Stable zinc compounds fail to influence the disease process or to raise the zinc content of leukemic cells.

The lowered zinc content of leukemic leukocytes may be compared with data on mouse epidermis painted with methylcholanthrene to induce hyperplasia and carcinogenesis (38). The ratio of the metal content to nucleoprotein-bound phosphorus in normal skin and epidermis and following carcinogenesis was studied. The ratio of copper to nucleoprotein-bound phosphorus is lowered to 17% of normal, and the ratio of zinc to nucleoprotein-bound phosphorus is lowered to 32% of normal. Similarly, there is a marked decrease in iron and calcium. It appears that a new chemical equilibrium is established. This work implies that a rearrangement of the cellular metal patterns may occur.

Similar changes of the metal pattern have been reported in experimental rat hepatomata induced by *p*-dimethylaminoazobenzene. Zinc concentrations were said to decrease during the period of liver damage, increasing again just before neoplasia is evident; unfortunately, no data are given (211). In another study of rat hepatomata, minor increases were observed; due to analytical difficulties, these are difficult to interpret. In regenerating liver tissue zinc could be detected spectrographically (16).

Liver tissue, when invaded by metastases, shows an increased zinc content in the uninvolved areas. The invading tumor tissue itself contains very low amounts of zinc (211). Organs invaded by lymphomatoses exhibit a variable zinc content. Apart from an increased content of the metal in bone, no definite conclusions could be drawn (133). An increased zinc content of two carcinomata of the thyroid has also been described, while no significant changes in the zinc content of a kidney tumor and of the uninvolved parts of the organ could be detected when compared with normal controls.

Studies of spontaneous mammary adenocarcinomata and dibenzanthracene-

induced spindle-cell sarcomata of mice led to the conclusion that the tumor tissue of either type took up abnormally large amounts of Zn^{65} (100). A variety of tissues of tumor-bearing mice contained unaltered amounts of Zn^{65}, although the presence of the tumor altered Co^{60} and Cu^{64} metabolism (232). Unfortunately, total zinc was not measured in either study. Polarographic analyses of various tumors and the uninvolved, but invaded tissues yielded data demonstrating differences between them, but an estimate of their analytical significance could not be made (99).

Decreased zinc content of malignant prostate glands has been recorded (170).

Carcinoid tumors have also been analyzed for zinc. Three intestinal tumors contained 127, 312 and 845, and one bronchogenic carcinoid contained 170 μg per gram fresh weight, exceeding the zinc content of comparable normal tissue by several multiples. Though physiological studies with serotonin were undertaken to illuminate a hypothetical relationship, none was found (350).

Neoplastic cells may have a metal distribution quite different from that of normal ones. The available information is often contradictory, the data are sketchy, and multiple factors seem to contribute to the confusion. Standardization of analytical methods, cytological criteria guiding selection of material to be analyzed, and establishment of proper baselines would aid greatly in establishing reliable analytical data. Any conjecture concerning the implications of the reported findings would seem premature.

Zinc and Porphyrins

It seems certain that a zinc uroporphyrin exists. Watson *et al.* (339) have found the zinc complex of the Waldenstrom uroporphyrin (331) to be a constant constituent of the urine and stool in cases of intermittent acute porphyria. In these cases, a zinc uroporphyrin is found in the liver. In fact, the occurrence of the zinc porphyrin complex is characteristic of this type of porphyrinuria, while the congenital type usually excretes the porphyrin in the free state (337–339). A zinc coproporphyrin has also been described (338), and it is found in cases of lead poisoning (184, 337) and in the urine of patients with acute rheumatic fever (125).

Zinc coproporphyrin has been described in bouillon cultures of *C. diphtheriae* (46). The investigators were unable to state whether the zinc porphyrin was present in the bacterium or was formed in the solution after disintegration of the organism by chemical combination of free zinc ions and coproporphyrin. The relationship of zinc to porphyrins is not understood at present, and its physiological significance is unknown.

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Absorption of Cu^{64}, Zn^{65}, Mo^{99}, and Fe^{59} from Ligated Segments of the Rat Gastrointestinal Tract

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ABSTRACT **Portions of rat intestinal tract were ligated to provide the following isolated, in vivo, segments: stomach: duodenum, mid-section, or ileum. In successive studies, Cu^{64}, Zn^{65}, Fe^{59}, or Mo^{99} was injected into one of the ligated segments. After a predetermined period of time, the rats were killed and tissue samples were taken for isotope analysis. On the basis of these tissue counts, the following relative rates of absorption were indicated. For Cu^{64}, absorption was greatest from the stomach and declined as the isotope was placed further away from the pylorus. Zn^{65} and Fe^{59} were taken up most rapidly from the duodenum, somewhat more slowly from the ileum and the mid-section, and with the least absorption occurring from the stomach. Mo^{99} was absorbed readily from the stomach and all 3 intestinal segments. Absorption from the 3 intestinal segments was at similar rates and all three yielded significantly higher rates than the stomach.**

Little is known of either the site or the mechanism for the absorption of copper from the intestine. However, a number of other ions, both chemically similar and chemically dissimilar, have been shown to affect the metabolism of copper. Of these, zinc has been shown to be antagonistic in the rat (1–3) and in the chick (4). Molybdenum has been shown to exert an effect on copper metabolism of sheep (5) and of rats (2, 6–8) and iron has also been shown to be interrelated with copper metabolism (10–12).

The considerations which preceded the work reported herein were as follows. If the interrelationships are mediated at the gut level, it could be postulated that they are the result of interactions in or on the intestinal mucosa. Either alternative could indicate a common absorptive site for the Cu and the interrelated ion(s). The studies reported here were therefore undertaken in an attempt to determine the site or sites in the intestinal tract where Cu, Zn, Mo, and Fe, respectively, are most rapidly absorbed. Zinc and molybdate represent a cation and an anion, respectively, which interfere with copper metabolism (1–8), whereas, iron is an example of a cation that enhances the utilization of copper (10, 11).

EXPERIMENTAL

Rats of the Sprague-Dawley strain were used in these experiments. They were housed in stainless steel batteries with raised wire floors and were fed a stock chow diet.[2] This diet was found, by chemical analysis, to contain 7 to 10 ppm of copper, 45 ppm of zinc, 0.4 ppm of molybdenum, and 200 ppm of iron.

For individual experiments, the procedure was as follows. After an overnight fast, the rats were anesthetized with ether and the peritoneal cavity was opened by a 2.5- to 3.5-cm midline incision. The segments, with the exception of the stomach, were removed from the body cavity and ligated in 2 places. The second ligature was left loose, the needle was inserted through the ligature into the lumen of the gut, the ligature was tightened and the isotope used[3] was injected into the following segments: 1) stomach; 2) duodenum (about 0.5 to 7.5 cm distal to the pyloris); 3) mid-section (about 33 to 40 cm proximal to the ileocecal juncture); and 4) ileum (about 2 to 9 cm proximal to the ileocecal junction). The stomach was ligated at the pylorus but the esophagus was left open. After the injection had been made, the segment was returned to the

Received for publication February 27, 1965.

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[2] Big Red Dog Chow, Agway, Ithaca, New York. Trade names and company names are included for the benefit of the reader and do not infer any endorsement or preferential treatment of the product listed by the U. S. Department of Agriculture.

[3] All isotopes were obtained from Union Carbide Nuclear Company, Oak Ridge, Tennessee.

body cavity and the incision was closed with sutures and stainless steel wound clips. After 2 hours for those animals that had received Mo^{99} and 3 hours for those animals that had received Cu^{64}, Zn^{65}, or Fe^{59}, the rats were anesthetized with ether, decapitated, and tissues were taken for direct gamma counting. Results are expressed as a percentage of the dose per tissue. Values for blood were arrived at by multiplying the counts per minute per gram of blood by 0.07 times the body weight of the animal, and values for kidneys were obtained by doubling the count obtained from one kidney. The data were subjected to an analysis of variance and means were compared using a multiple range test (13). Statements of significance are based on odds of at least 19 to 1 ($P \leqq 0.05$).

Details of dosage and other essential information will be given in conjunction with the results of the individual experiments to which they pertain.

RESULTS

Cu^{64} experiments. In these experiments, male rats weighing 250 to 400 g were used. The doses of copper ranged from 0.01 to 0.06 μmoles per rat because of differences in the specific activity of different batches of Cu^{64}. The isotope was received as $Cu(NO_3)_2$ in 1 N HNO_3. Upon receipt, it was diluted with physiological saline and the pH was adjusted to 5.0 to 6.0. Final dilution was to 10 ml, and each rat received 0.3 ml of the resulting solution. The results of these experiments are shown in table 1. The trend for the uptake of Cu^{64} from the various segments was as follows: stomach > duodenum > mid-section > ileum. This trend held regardless of which tissue was sampled and also held for the sums of the means of the sampled tissues. Statistical analyses indicated that all of the tissue uptakes were significantly higher ($P \leqq 0.05$) when the isotope was placed in the stomach than in the ileum. If the sums of the sampled tissues are used as an index of uptake, then absorption from the stomach is significantly higher than from the ileum at the 1% level of significance.

Zn^{65} experiments. In these experiments, male rats weighing from 200 to 350 g were used and these rats were allotted at random into replicates on the basis of body weight. The Zn^{65} was obtained as $ZnCl_2$ in 1 N HCl and had an initial specific activity of 7936 mc/g. Upon receipt, it was diluted with physiological saline and adjusted to pH 6.0. The final solution contained 0.3 μmoles of Zn/ml, and each rat received a dose of 0.15 ml of this solution. The results are presented in table 2. Analysis of the sums of the means for all the tissues reveals that Zn^{65} was taken up more quickly ($P \leqq 0.01$) from the duodenum than from any of the other segments. The tissue uptake from the ileum was higher ($P \leqq 0.05$) than from the stomach in all tissues except blood. Then, a rank of the means for total Zn^{65} uptake by the sampled tissues has the following order: duodenum > ileum > mid-section > stomach. Some difficulty was encountered in determining the exact dosages of Zn^{65}; hence the absolute values for tissue uptake are subject to this measurement error and are probably high. However, each rat received an identical dose; hence the relative rates of uptake from the different segments would be unaffected by errors in calculating the absolute dosage.

Mo^{99} experiments. Female rats with a weight range of 175 to 200 g were used in

TABLE 1

Uptake of Cu^{64} from various segments of the intestinal tract

Segment	% of dose/tissue [1]				
	Blood	Heart	Kidneys	Liver	Sum [2]
Stomach [3]	2.26[a]	0.058[a]	2.34[a]	6.78[a]	11.43[a]
Duodenum	1.74[ab]	0.042[ab]	1.57[ab]	4.52[ab]	7.87[ab]
Mid-section	1.37[ab]	0.032[ab]	1.18[ab]	4.21[ab]	6.80[ab]
Ileum	0.53[b]	0.014[b]	0.31[b]	1.45[b]	2.33[b]

[1] Each tissue value is the mean of 7 observations.
[2] Sum of the means for blood, heart, kidneys and liver.
[3] Values in any column not followed by the same letter are significantly different ($P \leqq 0.05$).

these studies. The Mo^{99} was obtained as NH_4MoO_4 and had an initial specific activity of 99 mc/g. Prior to use, it was diluted with physiological saline and the pH was adjusted to 7.0 with HCl. Each rat received 0.2 ml of the resulting solution which corresponds to 2.7 μmoles of Mo/rat. The results of these experiments are shown in table 3. The sum of the means shows that total uptake by these tissues from the stomach was lower ($P \leqq 0.05$) than from any of the other segments. Uptake by the liver was greater ($P \leqq 0.01$) from the duodenum than from the other segments, and uptake by the kidneys and the blood was higher ($P \leqq 0.5$) from either the duodenum or the ileum than from the stomach. The means for uptake by the various tissues fall in the following order: duodenum > ileum > mid-section > stomach. An exception to this is blood where the mean for the ileum was slightly higher than that for the duodenum.

Fe^{59} experiments. Male rats weighing 250 to 400 g, and female rats weighing 175 to 250 g were used in these experiments. Rats were randomized into replications on the basis of sex and body weight. The Fe^{59} was received as $FeCl_3$ in 1 N HCl and had an initial specific activity of 25,454 mc/g. This original solution was diluted with physiological saline, and ascorbic acid was added to reduce the iron to the more soluble ferrous state. The final solution was adjusted to pH 6.0 with NaOH and contained 0.06 mmoles of ascorbic acid and 0.07 μmoles of iron/ml. Each rat received a dose of 0.15 ml. The results are presented in table 4. If the sums of the means of all the tissues are ranked,

TABLE 2

Uptake of Zn^{65} from various segments of the intestinal tract

	% of dose/tissue [1]				
Segment	Blood	Heart	Kidneys	Liver	Sum [2]
Stomach [3]	0.05[b]	0.005[c]	0.06[c]	0.34[c]	0.46[c]
Duodenum [4]	5.41[a]	0.525[a]	7.78[a]	38.09[a]	51.80[a]
Mid-section	0.66[b]	0.063[bc]	0.93[bc]	5.61[bc]	7.26[bc]
Ileum	2.04[b]	0.152[b]	2.38[b]	12.90[b]	17.48[b]

[1] Each value is the mean of 4 observations.
[2] Sum of the means for blood, heart, kidneys and liver.
[3] Values in any column not followed by the same letter are significantly different ($P \leqq 0.05$).
[4] Only 3 observations in blood mean.

TABLE 3

Uptake of Mo^{99} from various segments of the intestinal tract

	% of dose/tissue [1]				
Segment	Blood	Heart	Kidneys	Liver	Sum [2]
Stomach [3]	1.99[b]	0.046[a]	0.44[c]	1.12[c]	3.60[b]
Duodenum	4.91[a]	0.397[a]	1.34[c]	2.82[a]	9.46[a]
Mid-section	3.99[a]	0.098[a]	0.81[bc]	1.85[b]	6.75[a]
Ileum	5.17[a]	0.115[a]	0.98[ab]	2.02[b]	8.28[a]

[1] Each value is the mean of 5 observations.
[2] Sum of the means for blood, heart, kidneys and liver.
[3] Values in any column not followed by the same letter are significantly different ($P \leqq 0.05$).

TABLE 4

Uptake of Fe^{59} from various segments of the intestinal tract

	% of dose/tissue [1]					
Segment	Blood	Heart	Kidneys	Liver	Spleen	Sum [2]
Stomach [3]	0.52[c]	0.007[c]	0.34[b]	0.17[b]	0.02[b]	1.06[c]
Duodenum	6.34[a]	0.064[a]	1.15[a]	1.35[a]	0.16[a]	9.06[a]
Mid-section	2.28[bc]	0.024[bc]	1.00[a]	0.58[b]	0.13[a]	4.01[bc]
Ileum	3.11[b]	0.044[ab]	1.30[a]	0.67[b]	0.14[a]	5.26[b]

[1] Each value is the mean of 9 observations.
[2] Sum of the means for blood, heart, kidneys, liver and spleen.
[3] Values in any column not followed by the same letter are significantly different ($P \leqq 0.05$).

they fall in the order: duodenum > ileum > mid-section > stomach. The total uptake by the sampled tissues was higher ($P \leq 0.05$) from the duodenum than from any of the other segments and uptake from the ileum was higher than that from the stomach. The retention by individual tissues generally followed the same trend as the total uptake by all of the sampled tissues.

DISCUSSION

Copper was absorbed relatively quickly from the stomach with the absorption rate from the small intestine decreasing as the isotope was placed further away from the pylorus. The quantitative importance of the absorption of Cu^{64} from the stomach is difficult to evaluate, since it is not known how long a given portion of food remains in contact with the stomach under normal conditions. However, Cu^{++} was the only one of the cations tested which was absorbed from the stomach faster than from the intestinal segments. Sacks et al. (10) observed that when Cu was placed in an upper jejunal loop, serum copper values increased slightly but little or no difference was observed with distal or middle jejunal segments. Tompsett (14) postulated that Cu is absorbed from the upper part of the intestine where the contents are acid. However, to our knowledge the results reported herein are the first direct evidence for the absorption of Cu from the stomach.

For Zn, the duodenum had, by far, the highest rate of absorption, the ileum and the jejunem followed, in that order, and almost no Zn was absorbed from the stomach. Iron absorption followed a pattern qualitatively similar to that for Zn, namely, the duodenum and ileum were most active and little absorption occurred from the stomach. In an earlier study on the absorption of iron from various parts of the intestinal tract, Hahn et al. (17) noted that when radioiron was introduced into the terminal segment of a jejunal fistula, the amount absorbed was less than 50% of that retained when the dose was given in the stomach. This suggests that the greatest uptake occurs in the stomach and duodenum, but does not differentiate between them. Our results indicate that, of the two, the duodenum is the more important. With respect to Zn, there is little information on the major absorptive sites, but such information as is available indicates some similarity between the absorption of Zn and that of Fe. This agrees well with the results reported here.

Molybdenum was absorbed readily from both the stomach and the intestinal segments. The intestinal segments were more active in this respect than the stomach and little difference was observed between the various segments. Little information is available in the literature on the sites(s) of Mo absorption. However, the observation that all segments of the small intestine and, to a lesser extent, the stomach were functional with respect to absorption of the hexavalent Mo ion is consistent with reports of rapid absorption of the molybdate ion from the gut (15, 16).

Direct comparisons between the various isotopes are difficult to make because of widely different rates of absorption. This difficulty can be alleviated by comparing ratios rather than absolute values. Ratios were therefore calculated from the sums in tables 1 through 4. For each isotope, the ratio of uptake from a given segment of small intestine to uptake from the stomach was calculated and these are presented in table 5. This table serves to point up further the wide difference between the elements. The ratios range from less than one, in the case of Cu, to over one hundred for Zn. Zn^{65}, Mo^{99}, and Fe^{59} yielded qualitatively similar absorption patterns, namely, duodenum > ileum > mid-section > stomach; however, comparison of the ratios for these elements indicates that they are quantitatively different.

To recall briefly, the primary purpose of this work was to determine the part of the intestinal tract from which the selected isotopes were absorbed most rapidly. The

TABLE 5

Ratios of isotope uptake from the duodenum, the mid-section and the ileum to uptake from the stomach

Isotope	Ratio of isotope uptake		
	Duodenum-to-stomach	Mid-section-to-stomach	Ileum-to-stomach
Cu^{64}	0.7	0.6	0.2
Zn^{65}	112.6	15.8	38.0
Mo^{99}	2.6	1.9	2.3
Fe^{59}	8.5	3.8	5.0

results are relatively conclusive in this respect. It was also thought that these experiments might show whether the Zn-Cu, the Mo-Cu, or the Fe-Cu interrelations were mediated at the gut level. The results reported here do nothing to vindicate this idea, but a conclusive answer will require more direct experiments.

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Arch. Biochem. Biophys. 46: 337-44 (1953)

Effects of Excessive Dietary Zinc in the Rat and the Interrelationship with Copper[1]

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Received April 6, 1953

Introduction

An appreciation of the metabolic interrelationships among the food elements is becoming increasingly important to the understanding of the functioning of any one nutrient. In mineral nutrition, the interaction of calcium, phosphate, and vitamin D is well known. Also, the *peat scours* disease of cattle has been shown to occur not only with a deficiency of soil copper, but appears to be associated with excessive molybdenum. The interrelationship between copper and molybdenum has been studied by Marston (1) and Cunningham (2) who concluded that these elements are reciprocally antagonistic in animal metabolism. A similar interrelationship may be true for copper and zinc. Following the observation of Sutton and Nelson (3) that excessive dietary zinc caused an anemia in rats, Smith and Larson (4) found that this effect could be overcome by supplementing the diet with added quantities of copper sulfate. The zinc–copper interrelationship suggested an investigation of the effects of excessive dietary zinc on various enzymes in the liver of rats. The results reported here indicate an impairment of the enzymes, catalase and cytochrome oxidase, with little influence at similar dietary zinc levels on diphosphopyridine nucleotidase, isocitric dehydrogenase, and alkaline phosphatase. Supplementation of the zinc toxic diet with small quantities of copper sulfate resulted in liver catalase and cytochrome oxidase activities of a magnitude equal to that of the controls.

[1] Contribution No. 49 of the McCollum-Pratt Institute, The Johns Hopkins University. This work was supported in part by a contract, AT(30-1)-933, between the U. S. Atomic Energy Commission and The Johns Hopkins University.

Experimental Procedure and Materials

The male albino rats of the Wistar strain used in these studies were obtained from a commercial source at 4–5 weeks of age. The basal diet had the following percentage composition: casein, 20.0; glucose, 69.0; corn oil, 6.6; salt mixture U.S.P. XIV, 4.0; choline chloride, 0.1; 2500 international units (I.U.) vitamin A, 250 I.U. vitamin D, and 15 mg. α-tocopherol in corn oil, 0.3. Other vitamins were provided in the following quantities, mg./100 g. diet: thiamine· HCl, 0.5; riboflavin, 0.8; niacin, 4.0; pyridoxine· HCl, 0.5; Ca pantothenate, 4.0; biotin, 0.04; folic acid, 0.2; menadione, 0.5; vitamin B_{12}, 0.003, inositol, 10.0; and *p*-aminobenzoic acid, 10.0. Zinc carbonate was added to the basal diet to provide the desired level of dietary zinc. In the first series of experiments the level of zinc was varied from 300 to 1000 mg./100 g. diet. Four rats were in each group, and the experiment was repeated once. When it was found that 500 mg. per cent zinc produced toxic effects without causing rapid death of the animal, this level was used in most cases.

After 4–5 weeks on the experimental regimen, determinations were made of hemoglobin, liver catalase, cytochrome oxidase, diphosphopyridine nucleotidase, isocitric dehydrogenase, and alkaline phosphatase.

The livers were prepared for enzyme assay in the following manner: The animals were lightly anesthetized with ether, decapitated, and exsanguinated. The livers were removed rapidly and cooled. The tissues were immediately homogenized in a Ten Brock glass homogenizer with about five times their weight of cold 0.1 *M* phosphate buffer at pH 7.0; when alkaline phosphatase activity was to be measured, cold water was used for homogenization. Just prior to assay, the homogenates were diluted to give the enzyme concentration necessary for the procedures.

Catalase activity was estimated at 37°C. by the perborate method of Feinstein (5). The usual system was as follows: 8.0 ml. of 1.5% $NaBO_3 \cdot 4H_2O$ at pH 6.8, 1.5 ml. of 0.1 *M* phosphate buffer at pH 6.8, and 0.5 ml. of enzyme preparation. Catalase activities are reported as millimoles perborate degraded in 20 min./mg. protein.

Cytochrome oxidase was estimated by the spectrophotometric method of Smith and Stotz (6) in which the rate of oxidation of reduced 2,6-dichlorobenzenoneindo-3′-chlorophenol is followed. The system had the following components: 1.0 ml. of 0.2 *M* phosphate buffer at pH 6.8, 1.0 ml. of approximately 0.001 *M* reduced dye, 0.5 ml. of 2×10^{-4} *M* cytochrome c, and 0.5 ml. of the homogenate. Reaction rates are expressed as increase in log (I_o/I)/min./mg. protein.

Diphosphopyridine nucleotidase (DPNase) was estimated using the cyanide addition reaction of Colowick, Kaplan, and Ciotti (7). Activities are expressed as micromoles of diphosphopyridine nucleotide (DPN) split in 7.5 min./mg. protein at 37°C.

Alkaline phosphatase was estimated using the method of Bessey, Lowry, and Brock (8), in which the breakdown of *p*-nitrophenylphosphate to *p*-nitrophenol is measured. Activities are expressed as micromoles phosphate released in 30 min./mg. protein.

Isocitric dehydrogenase activity was estimated by the spectrophotometric procedure of Grafflin and Ochoa (9), in which the increase in optical density at

340 mμ, due to the reduction of triphosphopyridine nucleotide, is observed. Activities are expressed as the increase in log (I_o/I)/min./mg. protein.

Protein content of the homogenates was determined using the biuret procedure of Robinson and Hogden (10).

Cytochrome c (Sigma) was assayed spectrophotometrically and diluted to 2×10^{-4} *M*. 2,6-Dichlorobenzenoneindo-3′-chlorophenol (Eastman) was titrated against ascorbic acid and made to approximately 0.001 *M* and reduced with hydrogen and palladium asbestos. DPN (Sigma "65") was used without purification. *p*-Nitrophenylphosphate, disodium (Sigma), was extracted twice with *n*-butyl alcohol and twice with ethyl ether before use. *p*-Nitrophenol (Sigma) was used without purification for preparation of a standard curve. DL-Isocitrate was prepared from DL-isocitric acid lactone (California Foundation for Biochemical Research) with sodium hydroxide. Triphosphopyridine nucleotide (TPN) (Sigma "10") was purified to 75% purity by a procedure involving ion-exchange chromatography.[3]

Results and Discussion

Effect of Dietary Zinc on Liver Enzymes

The results of enzyme assays on liver homogenates from control and zinc-toxic animals are presented in Table I. Of the enzymes observed, the iron-containing catalysts, cytochrome oxidase and catalase, were most markedly affected. At a dietary level of zinc of 500–700 mg./100 g. diet, the catalase activities of the homogenates were reduced to 50% of the controls. Similarly, the activities of cytochrome oxidase were reduced to 33% of the controls. While it has already been demonstrated that the hemoglobin content of the blood is reduced in zinc toxicity (3, 4, 11), these determinations were also made as a point of reference. Our findings showed a 59% reduction in hemoglobin at 700 mg. per cent zinc and a 36% reduction at 500 mg. per cent.

The specific activities of DPNase and isocitric dehydrogenase of the liver homogenates were not detectably different in the zinc-toxic and control animals. Alkaline phosphatase activity, likewise, did not appear to be affected when the dietary level of zinc was 500 mg. per cent; however, at a level of 700 mg. per cent the alkaline phosphatase activity of the liver homogenates was 2–3 times the normal value. While these experiments were in progress, Sadasivan (12) reported increased alkaline phosphatase when zinc-toxic diets were fed. The level of zinc oxide fed in his experiments was 1%. The physiological significance of the alteration in alkaline phosphatase at the higher levels of zinc is not clear.

[3] Kornberg, A., and Horecker, B. L., unpublished.

The Nature of the Zinc Toxicity Effect

The mechanism by which excessive dietary zinc causes decreased liver catalase and cytochrome oxidase activities was considered. Several possibilities are apparent: (*a*) formation of an inhibitor of catalase and/or cytochrome oxidase activity; (*b*) a direct inhibition of enzyme activity by the zinc ions; or (*c*) a breakdown in the mechanism by which the enzymes are formed.

TABLE I

Enzymatic Activities of Liver Homogenates

Enzyme	Control	Zn, 700 mg. %	Control	Zn, 500 mg. %[a]
Catalase, mmoles perborate degraded/mg. protein	5.7 ± .1	2.9 ± .4	5.6 ± .3	3.8 ± .3
Cytochrome oxidase, ΔO.D./mg. protein			1.89 ± .22	0.62 ± .10
DPNase μmoles DPN split/mg. protein	0.13 ± .01	0.12 ± .03	0.12 ± .01	0.12 ± 0.02
Isocitric dehydrogenase ΔO.D./mg. protein	0.15 ± .01	0.12 ± .01	0.13 ± .02	0.12 ± .01
Alkaline phosphatase μmoles phosphate liberated/mg. protein	0.069 ± .002	0.179 ± .040	0.054 ± .006	0.057 ± .002

[a] Values represent means of four or five assays.

In order to determine whether an inhibitor of either catalase or cytochrome oxidase was produced in the zinc-toxic state, equal quantities of liver homogenates from the control and zinc-toxic animal were mixed and the enzyme activity was compared with that of the individual homogenates. As indicated by the data in Table II, no inhibitor of either catalase or cytochrome oxidase could be detected by these means.

The direct effect of zinc salts on the activity of the enzymes was tested by making the zinc concentration in the reaction mixture as high as 10^{-3} *M* with zinc chloride. The effect of the added zinc salt was slight, and only resulted in a 6% reduction in the catalase activity (Table II).

These observations, while not conclusive, suggest that the observed effect of dietary zinc is mediated neither through the formation of enzyme inhibitors nor by direct inhibition by zinc ions, but probably through some obscure means by which the active enzymes are prevented from forming.

The Influence of Added Dietary Copper on Zinc Toxicity

Schultze (13) has reported that in a copper deficiency there is a gradual decrease in rat liver cytochrome oxidase activity which can be restored by the feeding of small quantities of copper. In an iron deficiency, however, the activity was not decreased providing adequate copper was fed. In the case of catalase, either an iron or copper deficiency has been reported to cause diminished enzymatic activity of the liver preparations (14). Since Smith and Larson (4) have reported that the anemia produced by feeding zinc salts can be overcome by supplementing the diet with 0.2 mg. copper/day, but not with other metal ions, a similar study seemed advisable in respect to the enzymatic anomalies observed.

TABLE II

Catalase and Cytochrome Oxidase Activities in Tests for Inhibitors and Presence of Zinc Ions

Preparation	Relative activities: Cytochrome oxidase	Relative activities: Catalase
	ΔO.D./min.	*ml. 0.05 N $KMnO_4$*
Control	0.321	10.00
Zn toxic	0.143	3.80
Mixture: ½ control + ½ toxic	0.243	7.50
Control	0.280	11.70
Control plus 10^{-4} *M* Zn	0.286	10.95

Four groups of four male albino rats were used in these studies. The first group was fed the basal diet, the second group was given the basal diet plus 500 mg. per cent zinc in the form of the carbonate, the third and fourth groups were fed the zinc-toxic diet, but were also provided 0.2 and 0.4 mg. copper/day, respectively. The copper supplementation was given in 3 ml. of milk and, for control, groups 1 and 2 were given the milk only. One repetition of the experiment was performed. After 4–5 weeks, the animals were sacrificed as described previously, and assays for catalase and cytochrome oxidase were made. The administra-

tion of copper to the animals fed the zinc-toxic diet resulted in liver catalase and cytochrome oxidase activities that were considerably higher than in the unsupplemented zinc-toxic animals (Table III). As has been reported previously by others, the anemia produced by the excessive zinc was improved also under the influence of copper supplementation.

While the observations on hemoglobin concentration, catalase activity, and cytochrome oxidase activity strongly suggest an antagonistic relationship between copper and zinc, there remains the possibility that these are independent effects. Thus, if the dietary copper were not at a high enough level to support maximal catalase and cytochrome oxidase formation, the stimulation of enzyme activity during zinc feeding might be a reflection of inadequate copper nutrition in the basal diet, rather than an antagonistic effect upon the zinc toxicity. The adequacy of the basal diet was tested, therefore, by estimating the catalase and cytochrome oxidase activities of liver homogenates when the diet was supplemented with various levels of copper. On the basis of the copper in the salt mixture used, the basal diet contained a minimum of 80 μg. copper/100 g.

TABLE III

The Influence of Added Dietary Copper on Zinc Toxicity

Dietary conditions	Mean cytochrome oxidase[a]	Mean catalase[a]
	ΔO.D./mg. protein	*mmoles perborate/mg. protein*
Basal diet	1.65 ± 0.14	5.9 ± 0.2
500 mg. per cent zinc	0.66 ± 0.09	3.3 ± 0.7
500 mg. per cent zinc plus 0.2 mg. copper daily	1.58 ± 0.21	6.3 ± 1.0
500 mg. per cent zinc plus 0.4 mg. copper daily	1.70 ± 0.10	6.4 ± 1.0

[a] Each value represents mean of four assays.

A control group of four rats was fed the basal diet, while four other groups of four rats per group were given supplemental copper sulfate at levels of 0.2, 0.4, 0.8, and 1.6 mg. copper/day. The copper sulfate was provided in small quantities of milk as previously described. At the two higher levels all of the copper supplement was not consumed each day, but no correction for this has been taken into account. The livers were assayed for catalase and cytochrome oxidase activity after 5–6 weeks on the diets; the results are presented in Table IV. The data indicate that as far as catalase and cytochrome oxidase are concerned, the basal diet

used in these experiments contained adequate copper to support maximal formation of the enzymes.

Growth Studies

A marked depression of growth below control values was observed when 300 mg. per cent or more of zinc was incorporated into the diet.

TABLE IV

Cytochrome Oxidase and Catalase Activities of Liver Homogenates Following Various Levels of Copper Supplementation

Diet	Cytochrome oxidase[a]	Catalase[a]
	ΔO.D./mg. protein	*μmoles perborate/ mg. protein*
Basal	2.04 ± .27	5.9 ± .2
Basal plus 0.2 mg. copper/day	1.93 ± .08	6.3 ± .5
Basal plus 0.4 mg. copper/day	2.21 ± .06	6.6 ± .9
Basal plus 0.8 mg. copper/day	2.28 ± .12	6.2 ± .2
Basal plus 1.6 mg. copper/day	2.27 ± .28	5.9 ± .4

[a] Each value represents mean of four assays.

TABLE V

Food Consumption on Various Diets

Diet	Mean daily food intake	Diet	Mean daily food intake
	g.		*g.*
Basal	11.2 ± 0.4	Basal	10.6 ± 0.8
Basal + 0.3% Zn	9.5 ± 0.7	Basal + 0.5% Zn	7.6 ± 0.5
Basal + 0.5% Zn	8.6 ± 0.3	Basal + 0.5% Zn + 0.2 mg. Cu daily	9.6 ± 0.8
Basal + 0.7% Zn	9.0 ± 0.7	Basal + 0.5% Zn + 0.4 mg. Cu daily	9.2 ± 0.1
Basal + 1.0% Zn	8.9 ± 1.2		

In terms of percentage of the weight response of the control group, the rats fed 300, 500, 700, and 1000 mg. zinc/100 g. diet weighed 70, 65, 55, 53 %, respectively. The maximum growth-depressing effect was thus observed at 700 mg. per cent zinc with no added effect at higher levels. While the supplementation of the zinc-toxic diet (500 mg. per cent) with copper sulfate resulted in improved liver catalase and cytochrome oxidase activities, the copper had little influence on the growth-depressing action of zinc. At least two distinct effects of feeding zinc are thus apparent: (*a*) an effect on iron metabolism mediated through copper utilization, and (*b*) some obscure influence on growth which as yet has not been elucidated. Smith and Larson (4) have reported that the growth-

depressing action of excessive dietary zinc can be overcome by supplementing with a crude liver extract. The mechanism by which this occurs is still not clear.

The food intakes for the various experiments are presented in Table V. No record was kept of food intake when copper supplementation of the basal diet was studied. The addition of 0.5 % zinc to the basal diet resulted in about a 25 % reduction in food intake during a 4-week period. On the basis of 80 μg. copper/100 g. of basal diet, the control rats consumed an average of 9.0 μg. copper/day, and the rats fed 0.5 % zinc, 6.9 μg. copper/day. On the basis of the decreased copper intake alone, it does not appear likely that a severe copper deficiency would be produced in 4 weeks. The marked reduction of hemoglobin, catalase, and cytochrome oxidase activity and the effect of added copper suggests an antagonistic action between the two metal ions.

Summary

1. The effects of feeding excessive zinc on liver enzymes of the rat were studied.

2. A dietary level of 500–700 mg. per cent zinc resulted in a marked reduction in liver catalase and cytochrome oxidase activities.

3. Copper feeding, along with toxic zinc levels, resulted in an increase of liver catalase and cytochrome oxidase activities to normal values.

4. While copper supplementation influenced the liver enzymes, it had no effect in correcting the growth inhibition produced by zinc.

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PART IV. METHODOLOGY AND PROBLEMS IN ANALYSIS OF TRACE ELEMENTS

ACTIVATION ANALYSIS

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INTRODUCTION

Since its beginning in 1936,[1] activation analysis has become a widely used analytical technique. Although it is best known for the high sensitivity that is possible in the determination of many trace elements, it has other positive characteristics that are often of equal or greater importance. It also has a number of limitations which are usually less publicized than its advantages. Activation analysis should be considered another tool for the analysis of chemical elements, not a panacea for all analytical problems.

The modern analytical laboratory of today must balance analytical needs with cost effectiveness. To do this, the chemist must determine which analytical technique is most cost-effective for the solution of an analytical problem involving a particular element in a particular matrix. Because of its inherently high cost, activation analysis should be limited to those problems in which it offers distinct advantages. For example, one can determine lead by neutron activation analysis using a pulsed nuclear reactor. In general, however, this method will cost over $300 per sample. Also, because of limitations on the number of times per day a particular reactor can be pulsed, only a very limited number of samples can be analyzed per day. In the practical sense this means that although it is technically possible to determine lead by neutron activation, it is far more efficient to use some other method such as atomic absorption. The analysis of mercury is a similar but less extreme example. Activation analysis and atomic absorption are probably the two best techniques for the determination of mercury. Although activation analysis has a greater sensitivity, in most environmental monitoring applications the extra sensitivity is not required. In surveillance programs, the investigator is generally interested in whether or not the element of interest is above or below a certain level. If the level of concern is 0.5 ppm, the investigator does need a sensitivity of 0.0001 ppm. For this application, one would generally choose atomic absorption over activation analysis because of lower cost and the capability to process more samples per day. On the other hand, activation analysis would serve as an excellent method with which to cross-check results.

Although there are many specific applications in which activation analysis will be the method of choice, two broad areas of application are probably of general significance: those analytical problems requiring high accuracy at very low levels, and those problems in which a number of elements are to be determined in a single sample. These two areas of application will be discussed after consideration of the general procedure for activation analysis and its advantages and limitations.

SAMPLE COLLECTION AND PRESERVATION

As in all analytical methods, the first step in activation analysis is to obtain a representative sample. The sample should accurately reflect the characteristics of the larger unit on which information is desired. The sample should be collected, preserved and analyzed in a manner that will provide data that are significant with respect to the reason the sample was collected in the first place. In other words, the results for a particular sample that is analyzed in the laboratory may be correct for that sample but, because of incorrect sampling or handling of the sample prior to the actual analysis, may be greatly in error with respect to the larger unit from which the sample was collected. Since water samples present a particularly difficult problem, they will be used to illustrate some of the pitfalls that may be encountered.

The first step in the collection of water samples is to consider how the data are to be used. For certain applications it may be necessary to analyze a number of samples from the body of water, whereas for other applications significant results at a lower cost may be obtained by making a single composite sample from a number of samples. One must also decide whether sediments in the water are of significance or whether the primary interest is in those elements in solution. If the sediments are of significance, care must be taken to insure that the sample which is analyzed in the laboratory contains a representative distribution of such sediments. If only soluble elements are of concern, one must remove the sediments without losing significant amounts of soluble elements. For many elements at trace levels filtration can result in significant loses. For many applications, centrifugation will remove the sediments without significant losses of elements in solution.

The preservation of the sample between collection and analysis is also of considerable importance. In the case of water particularly, there is the possibility of loss of trace elements by adsorption on the walls of the container or of contamination of the sample by leaching of elements from the container walls. One solution is to add transistor grade nitric acid to the sample. This will inhibit the adsorption of certain elements but increase the possibility of leaching elements from the container walls. A sample of the acid added to the sample should also be analyzed in order to make appropriate corrections. Our group prefers to freeze the sample shortly after collection and to maintain the sample in a frozen state until it is ready to be analyzed. This technique greatly decreases the possibility of loss or contamination. However, even with freezing, it is important to clean the container carefully before use. TABLE 1 shows the sample collection, preservation and preparation technique used in our laboratory.

MATERIALS AND METHODS

Neutron activation analysis is based on the transformation of a stable isotope of a chemical element to an isotope that is radioactive. This is most often done by bombarding the sample with neutrons produced in a nuclear reactor, although other neutron sources may be used. The transformation in a neutron flux of a stable isotope, A, to the radioactive isotope, B, which subsequently decays to stable nuclide, C, may be represented as

$$A\,(n, x)\,B \xrightarrow{\lambda} C$$

where λ is the disintegration constant of the radioactive isotope B and x and B are the reaction products. Qualitative identification of A can be made from the disintegration constant and from the types and energies of the radiations emitted by B as it decays to C. In most cases the gamma-ray emission of B would be measured by the analyst. For quantitative measurements, a standard containing the element of interest is irradiated along with the sample. Although quantitative measurements can be made from calculations using basic nuclear data, the use of a standard cancels out inaccuracies in the nuclear and in some experimental parameters and is generally much more accurate.

The samples and standards are generally packaged in cleaned quartz or polyethylene vials for irradiation in the reactor. The time of irradiation, the neutron flux and the decay period between the end of irradiation and the beginning of radiation measurement can be optimized for the particular matrix and element of interest. Following irradiation, the sample and standard are transferred to nonradioactive containers. In some cases it may be necessary to separate the element of interest chemically. This is often the case for the analysis of trace elements or when certain constituents emit radiations which

TABLE 1

SAMPLE COLLECTION FOR WATER ANALYSIS

1. Wash 250 ml P. E. bottle:
   a. With acetone to remove mold release agent
   b. With 4 *N* Transistor grade HNO_3
   c. Three times with deionized water
   d. Fill with deionized water and let stand for 24 hr.
2. Collect sample and freeze within two hr after collection.
3. Centrifuge water sample for 15 min at 32,000 × g and discard sediment.
4. Package sample in quartz or polyethylene.

obscure the radiations from the element of interest. For example, sodium, bromine, chlorine and potassium are activated to such an extent in blood serum as to make chemical separation necessary for the determination of all elements except selenium, rubidium, zinc, cobalt and iron.

The gamma-ray radiations emitted by the sample and standard are measured using either a NaI(Tl) detector or a solid state Ge(Li) detector. The Ge(Li) detector provides much better energy resolution and can permit instrumental analysis of samples which would have to be subjected to radiochemical separations before they could be analyzed using NaI(Tl). NaI(Tl) detectors are, however, quite useful for the instrumental analysis of a few elements in a relatively simple sample. NaI(Tl) is usually the detector of choice for measuring radioactivity following the radiochemical separation of an element because of its higher efficiency relative to Ge(Li). Both detectors are typically used with multichannel pulse height analyzers, although in general a much more sophisticated system is required for optimum utilization of a Ge(Li) detector. Also, the electronic modules (preamplifier, amplifier and analog-to-digital converter) for the Ge(Li) detector must meet much higher standards with respect to electronic noise, linearity and stability than is necessary for NaI(Tl) detec-

tors. For both detectors, the pulse height analyzer provides the data in a gamma-ray energy vs. gamma-ray intensity configuration. The gamma-ray energy provides the qualitative determination of the radionuclides in the sample, while the intensity or total counts collected for a given energy provides the quantitative determination.

In the usual method of analysis, the radioactivity of the sample and a standard of known weight are measured under identical experimental conditions. The weight of the element of interest in the sample can then be calculated using the equation

$$W_x = \frac{A_x W_s}{A_s}$$

where W_x = weight of the element in the sample, W_s = weight of the element in the standard, A_x = net counts under the gamma-ray peak of the sample, and A_s = net counts under the gamma-ray peak of the standard. Depending on the design of the experiment, it may be necessary to normalize the number of counts obtained from both the sample and standard for one or more of the following parameters: neutron flux, irradiation time, decay time, counting time and dead time. When only a few elements or a few samples are to be analyzed, such calculations are usually done using a calculator. However, for large numbers of elements or samples, computer data reduction is usually used. This is particularly true when Ge(Li) detectors are used because of the large number of gamma-ray peaks in the spectrum (often over one hundred).

ADVANTAGES OF ACTIVATION ANALYSIS

Undoubtedly, the most widely known advantage of neutron activation analysis is the very high sensitivity that is possible using a high flux nuclear reactor. At a thermal neutron flux of 5×10^{13} n $cm^{-2}sec^{-1}$ over two dozen elements can be determined at a concentration of less than 0.1 parts per billion [2] with an irradiation of one hour or less. At the present time, there are several newer analytical techniques which also offer high sensitivity for trace element analysis. The most broadly applicable of these is spark source mass spectroscopy. Atomic absorption and flame emission spectroscopy and anodic stripping voltametry can also provide excellent sensitivities, although on a more limited range of elements. Of these techniques, however, activation analysis provides by far the greatest freedom from reagent and laboratory contamination. For analyses near or below the parts per billion level, this is probably the most significant characteristic of activation analysis. Since only the material that is made radioactive is measured ultimately, any trace amounts of the element to be determined that are present in the reagents or which may contaminate the sample during any chemical processing following irradiation will not affect the accuracy of the analysis.

Another advantage of the activation method is the fact that nondestructive analysis of the sample is often possible. It should perhaps be emphasized that when a nuclear chemist speaks of a technique as being nondestructive, he means that the elemental composition of the sample is unchanged and that additional analyses for other elements in the same sample are possible. Certain physical and chemical changes may take place during irradiation; but unless the flux

cross-section and irradiation time are high, no significant change in elemental composition will occur.

Other advantages of activation analysis include: high accuracy and precision even at parts per billion concentrations; high specificity for individual elements due to the production of radionuclides having characteristic decay constants and radiations; the elimination of the need for quantitative separation by use of carriers; and the ability, in many cases, to provide quantitative data on many elements in a single sample.

Limitations of Activation Analysis

The primary limitation of neutron activation analysis is the need for a high intensity source of neutrons for maximum sensitivity. Because of the cost of building and operating a nuclear reactor, high-sensitivity neutron activation analysis is generally available in only a few highly specialized facilities. Although neutron generators and isotopic neutron sources permit the establishment of an investigator's own activation analysis capability, the relatively low neutron flux available severely limits the analytical sensitivity that can be obtained. In addition, the sophisticated nuclear instrumentation needed to take advantage of the full capabilities of the method is not only expensive but requires highly specialized personnel. For the investigator who needs an in-house capability for the analysis of a wide range of elements at low cost, atomic absorption and flame emission are preferred. Another limitation of neutron activation analysis is that its practical application is limited to approximately 65 elements. Of these elements, however, all but four can be determined with a sensitivity of greater than one part per million.

Although there are a number of other limitations to the activation method, most of these can be minimized by taking suitable precautions. These limitations include: possible damage or volatilization of the sample due to heat; possible structural damage or decomposition due to the high gamma-ray and neutron flux; inhomogeneity of the neutron flux on the sample and standard; primary, secondary or second-order interfering nuclear reactions and self-shielding of samples containing elements having high neutron cross-sections.

Applications of Activation Analysis

Although activation analysis can be applied effectively to many analytical problems, there are two primary areas in which it offers distinct advantages. For a large number of elements, activation analysis can provide high accuracy at very low levels. Our group at the University of Missouri routinely determines chromium in blood sera at levels ranging from 0.5–6.0 ppb. Samples are packaged in high purity quartz vials and irradiated for 100 hr at a flux of 2×10^{14} n $cm^{-2}sec^{-1}$. Following irradiation, chromium is radiochemically separated and the 0.32 MeV gamma-ray measured using a NaI(Tl) detector. The second primary area in which activation analysis can offer significant advantages is in the determination of many elements in a single sample. It is in this area of application that activation analysis is most cost-effective. Based on the cost per element per sample the method can often compete effectively with atomic absorption. This area of application is illustrated below

with examples of multielement analyses currently being done at the University of Missouri.

Acid Extracts of Soils

Neutron activation analysis has been used to determine 20 elements in acid extracts of soils. Concentrations of these elements ranged from <0.001 ppm to 3.9%. A 5 g sample of soil is shaken in a 25 ml 0.1 *N* transistor grade HNO_3 solution. The mixture is then centrifuged and the supernatant decanted. A ½ ml sample is irradiated for one min and counted after decay times of 3 min, 10 min and 30 min. A 0.1 ml sample is then irradiated for one hr and counted after a decay of 24 hr. Mixed standards containing similar quantities of those elements detected qualitatively in the sample are also irradiated and counted. From these two irradiations, concentrations of Na, K, Mn, V, Al, Mg and Ca are calculated. A ½ ml sample is then irradiated for 50 hr and counted after a seven-day decay. Standards are irradiated and counted in the same manner. From this irradiation, concentrations of Sm, Ce, Ba, Eu, Yb, Br, Sb, Tb, Zn, Ca, La, Sc and Fe are calculated. The results for extracts of 12 Yugoslavian soils are shown in Table 2. All measurements were made using a 45 cm^3 Ge(Li) detector and 8192 channel pulse height analyzer.

Table 2

Elemental Abundances of Acid Extracts from 12 Yugoslavian Soils

Element	Average (ppm)	Range (ppm)
Na	56.7	21–132
V	0.650	0.1–2.3
Ca	6,044	2,430–31,000
Ba	32.5	21–44
Br	0.25	0.16–0.37
Zn	6.84	1–14
Sc	0.012	<0.001–0.031
K	222.6	73–500
Mn	64.8	44–110
Sm	0.144	<0.001–0.25
Eu	0.015	<0.001–0.028
Sb	0.014	0.002–0.05
Co	0.841	0.06–1.82
Fe	67.0	<10–260
Mg	570.3	335–850
Al	345.0	6–550
Ce	2.01	0.7–5.9
Yb	0.083	<0.005–0.15
Tb	0.043	<0.01–0.09
La	1.60	0.07–4.2

TABLE 3

ELEMENTAL CONCENTRATIONS IN WALNUT WOOD BASED ON NEUTRON ACTIVATION ANALYSIS OF 63 INDIVIDUAL GROWTH RINGS

Element	Average (ppm)	Range (ppm)
Na	104	53–332
Mg	543	273–1,770
Al	2.8	0.4–16.0
Cl	68	20–330
K	1,100	471–3,470
Ca	2,510	1,420–14,000
Mn	0.32	not detected–1.4
I	0.19	not detected–0.72
Ba	30	not detected–160
Br	0.41	not detected–1.4
Sr	19	16–25
La	0.025	not detected–0.28
Au	0.012	not detected–0.053

Soil Samples

Twenty-three elements have been determined in composite soil samples from southern Illinois. One gram of soil is homogenized in a vibrating agate ball mill for two 30-sec periods. The sample is dried to constant weight at 110°C. Approximately 50–70 mg of soil are irradiated for one min and counted with a Ge(Li) detector after decay times of 20 min and two hr. Standard solutions are irradiated and counted in the same manner and concentrations of Al, Mn, Mg, Ba, Ca, Na and K are calculated. Next, 400–600 mg of soil are irradiated for 10 hr and counted after decay times of 6 days and 30 days. Concentrations are calculated for La, Br, Eu, Co, Fe, Ta, Sc, Rb, Cs, Zr, Sb, Hf, Cr, Th, Yb and Ce.

Walnut Wood Growth Rings

A series of 63 individual growth rings from a central Missouri walnut tree were analyzed for 13 elements using instrumental neutron activation analysis. Samples weighing 50–150 mg were dried to constant weight at 110°C and packaged in polyethylene vials for irradiation. The sample was first irradiated for 1 min and counted after decay times of 3 min, 10 min and 30 min. A mixed standard was irradiated and counted in the same manner, and concentrations were calculated for Na, Mg, Al, Cl, K, Ca, Mn, I and Ba. The sample was then irradiated for one hour and counted after decay times of 20 min and 24 hr. Concentrations for Br, Sr, La and Au were calculated. The results of this study are shown in TABLE 3.

CONCLUSION

Neutron activation analysis is a very sensitve and versatile method for trace element analysis. It is particularly effective for applications requiring high accuracy at very low concentrations and where a number of elements are to be determined in a single sample. In choosing an analytical technique one should consider the sensitivity and accuracy needed to solve the problem at hand, and determine which technique will most efficiently provide an optimum balance of these two parameters.

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BULLETIN OF THE GEOLOGICAL SOCIETY OF AMERICA
VOL. 63, PP. 435-484 MAY 1952

BIOGEOCHEMICAL INVESTIGATIONS IN THE PACIFIC NORTHWEST

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ABSTRACT

Copper and zinc are so widespread in soils and vegetation that any attempt to apply biogeochemistry in search for buried ore bodies of these metals necessitates an accurate knowledge of the distribution of these elements in the absence of mineralization.

In this paper normal and abnormal, or anomalous, contents of copper and zinc in different organs of various ages have been determined and tabulated for the more common trees and lesser plants of the Pacific Northwest.

Some ways by which biogeochemical data may be used are described, including a brief discussion of statistical diagrams and profiles. Some common errors are discussed. Two sets of diagrams illustrating successful applications of biogeochemical data are also given.

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INTRODUCTION

Leyton (1948) has pointed out that when a tree retains more than one year's foliage there may be differences in composition not only between the various organs of that tree but also between the same organs of varying age.

As we have stated (Warren, Delavault, and Irish, 1949) it is most desirable to follow one technique throughout any one investigation. Unfortunately this is a counsel of perfection which it is not always practical to adopt. Most biogeochemists have a field season extending into months, and a technique applicable in May will usually be inappropriate in July. If biogeochemistry is to progress as a tool in the

search for hidden ore bodies it must be developed to the point where an investigator can move into a new area and, after sizing up the general geography and geology of that area, decide on the type of field work most likely to yield profitable results. The problem of choosing an appropriate field method is, in our opinion, most important; on it may depend the success or failure of the whole investigation.

To those who have not carried out biogeochemical investigations, some of the details discussed may sound involved. We think they are neither more nor less involved than any controversy concerning the selection of an appropriate method for a geophysical examination. Most geophysicists agree that the selection of an inappropriate geophysical method greatly lessens the chances of success in any investigation. To enable a biogeochemist to choose intelligently the methods which will best serve his needs, it would seem wise to explore as many biogeochemical techniques as possible, to compare their strong and weak points. We are satisfied that some of the indifferent results obtained by early workers were due to the use of inappropriate techniques rather than to any weakness in biogeochemistry as a tool. Any geophysicist knows that a gravimetric survey might be doomed to failure if used where a self-potential survey might produce useful results. Similarly the use of spruce buds where Douglas Fir twigs were available could, under some circumstances, vitiate a biogeochemical survey. The collecting of willow or alder leaves close to a lake shore when Devil's Club stems are available a short distance away might ruin an investigation.

By making available some of our results from the past five field seasons, and pointing out some of the pitfalls into which we have fallen, we hope we may hasten the development of biogeochemical techniques. We have obtained some results we cannot satisfactorily explain. We are presenting a few of these contradictory results in the hope that others may be able to provide the explanation.

Lastly we present a few by-products of our investigations and suggest a few new lines of promising research.

ACKNOWLEDGMENTS

The authors must emphasize above all their indebtedness to The Geological Society of America for various grants, commencing in 1946, from the Penrose Bequest. These grants have made this research possible. Less substantial but extremely welcome support has come from the University of British Columbia President's Committee on Research.

Many of our colleagues have assisted in various ways. Professors J. Davidson, T. M. C. Taylor, and D. J. Wort of the Department of Biology and Botany; Professor A. M. Crooker of the Department of Physics; Professors D. G. Laird, V. C. Brink, and C. A. Rowles of the Department of Agronomy; and Professor B. G. Griffith of the Faculty of Forestry, all of the University of British Columbia, have been particularly helpful.

During the past few years the following mining companies have co-operated either by permitting us to publish the results of research on their claims or by financing our researches and permitting us to publish many of the results: Consolidated Mining and Smelting Co. Ltd., Britannia Mining and Smelting Co. Ltd., Highland-Bell Ltd., Pioneer Gold Mines of B. C. Ltd., The Granby Consolidated Mining, Smelting and Power Co. Ltd., Kelowna Exploration Co. Ltd., New Jersey Zinc Explorations Ltd., and the British Columbia International Engineering Co. Ltd.

Several individuals were particularly gracious and helpful, and particular mention must be made of Professor M. Y. Williams, the Late Dean Daniel Buchanan, Dr. Alex. Smith, and Dr. J. Rousseau. Many of our friends acted as collectors, and to them we gladly acknowledge our indebtedness.

The botanical terms used are largely taken from *Native trees of Canada*, Bulletin 61, Fourth Edition, Ottawa, published by the Dominion Forest Service of Canada. A few of the lesser plants are taken from *A pocket guide to the trees and shrubs of British Columbia* by B. G. Griffith for the British Columbia Department of Lands, Victoria, B. C. These sources were considered eminently reliable, and they are usually more likely to be available to biogeochemists and geologists in the field than the *Index Kewensis*.

Collecting Problems

Leyton (1948) has pointed out that the time of day when the sample is taken may affect the mineral composition of a sample. In practice we have not found any evidence to suggest that such variations are great enough to interfere with biogeochemistry where applied to prospecting for zinc or copper. Detailed investigations may necessitate reconsideration of this problem.

Another aspect which probably merits more attention is the direction and grade of the slope on which a survey is being made. In many areas the vegetation on north and south slopes varies markedly. Limited resources have prevented our tackling this problem, other than empirically. However, we have discovered anomalies on both north and south slopes. In reconnaissance work our chief interest in this problem has been to try to find a widespread species which could be found growing in critical areas.

The age of a tree scarcely affects the mineral content of young organs. Furthermore this problem usually solves itself: one must take the available trees, and our experience, well supported by results, suggests that it is better to use one species of tree of varying age than to mix species in an effort to get trees of comparable age. We have not found substantial differences between high and low branches.

If we are permitted to bypass for the present the above problems, our survey is reduced to manageable proportions. Our present survey therefore consists of a report on the merits, as tools in biogeochemistry, of different species of trees, and their variously aged organs.

Notes on Analytical Problems

The analytical methods employed were essentially those described in a previous paper (Warren and Delavault, 1949, p. 539–540). Experience has given us a greater measure of confidence in the efficacy of this method and indicates that a precision of 2 to 5 per cent may be expected if enough copper is present in the sample. This precision is better than that likely to be obtained from any normal sampling technique. We no longer find it necessary to run controls using classical methods on a micro scale. The following points may be helpful to any who may wish to adopt the techniques we have used.

(a) Size of sample. In most cases the minimum amount of metal to be expected in a sample is known. The weight of sample to be used in an analysis should be such that it should contain a minimum of 5 γ of copper, and the aliquot carrying the zinc, or the whole sample if zinc only is needed, must contain at least 2 γ of zinc. This usually necessitates a 2-gram sample for copper and zinc and a decigram sample if zinc only is to be determined.

(b) Ashing. Most of our ashing was done in an automatically controlled electric oven at 620°C. We agree with those who state that losses may occur during ashing, but these losses seem to depend upon factors other than the temperatures finally reached. Possibly the main point is to prevent the sample from catching fire; the initial temperature of the oven should be low enough and the rise in temperature slow enough to prevent this. In the field a small gasoline stove, such as the Coleman stove used by the U. S. Geological Survey, gives good results. However, if such a stove is used care should be taken to distill off all volatile material, using a lid on the crucible if necessary, before raising the temperature to a red heat.

(c) Treatment of ash. The ash is transferred to a small beaker, preferably 20 ml., where it is moistened with a few drops of water and then attacked with about 1 ml. of 3 Normal hydrochloric acid for each gram of sample. By evaporating slowly to dryness in 10 to 20 minutes the metals are dissolved and oxidizing compounds destroyed. The beaker is then removed from heat, about 0.2 ml. of 3N hydrochloric acid added, next a few ml. of water, and, after a few minutes, the solution is passed into a graduated 50-ml. cylinder having a ground glass stopper, and there made up to a known volume, usually 10 ml., and titrated as indicated in the previous paper. The zinc may be titrated on an aliquot. However, we now prefer as a buffer a solution containing 250 grams of sodium acetate and 10 ml. of acetic acid per liter in place of the 10 per cent acetate solution previously recommended. If no cop-

per determination is needed, the copper in the solution can be complexed by adding a 50% sodium thiosulphate solution—about 10% of the total volume—after which only zinc can react on the dithizone solution.

(d) Unburnt material. Unburnt material can lead to low results because of a loss of the metal occluded in it. Furthermore, charcoal can be a powerful oxidizing catalyzer and can change dithizone into a yellow compound which cannot compensate the color of the red metal complex. More dithizone than is needed is used, and the final mixed color is not neutral gray but yellowish gray or even chocolate brown. Consequently it is wise to filter off any appreciable amount of charred material. The best ashless filter papers contain copper in appreciable amounts, and just before use they should be treated with Normal hydrochloric acid which is washed off with pure water. This treatment makes the filter brittle and consequently it cannot be given in advance.

(e) Ferric iron. Very large amounts of ferric iron, 1 milligram per ml. or more, may, even in acid solutions, cause oxidization of dithizone. Should vanadium be present even less ferric iron can prove troublesome. This trouble can be mitigated by a judicious use of hydroxylamine salts, or by removing the ferric iron with ether.

(f) Pure water. In the field clear natural water is usually purer, as far as heavy metals are concerned, than water distilled in the laboratory.

(g) An alternative method for copper determination. Frère Sylvestre C.S.C. (1942) has described for copper analyses a method which is more involved but which is better adapted to untrained workers.

General Remarks Concerning Tables

In the tables in this paper the term "negative" refers to an area where bedrock is well exposed and mineralization of the element being investigated is not present. "Positive" indicates that mineralization of the element occurs near by, although this mineralization may not be commercially significant.

Copper and zinc are expressed both as p.p.m. (parts per million) of dry-plant material, and in some tables as p.p.m. of ash; the latter figure is bracketed. The p.p.m. in ash figure is in many instances determined directly from the absolute amounts of copper and zinc extracted from the sample during the making of the analyses. This method of calculation and the fact that the number of significant figures is everywhere reduced to a minimum may make some slight discrepancies apparent to those who examine the tables closely. However, it is believed that the results as presented are in no case distorted with respect to the general principles being evaluated.

In a previous paper (Warren and Delavault, 1948) we discussed our reason for not studying wood, bark, or cones.

A. The Gymnospermae

Family Pinaceae

The Gymnospermae as a group have many features to commend them for biogeochemical work in the Pacific Northwest. There are few areas where one or more genera are not present, and their identification, except possibly for some species of spruce, is comparatively simple. Our data suggest that, even if a species is wrongly identified, no harm will be done, provided the genus is determined correctly.

The Gymnospermae may be determined in winter as well as in summer. This may make it possible in some areas to carry on biogeochemical work even where there is deep snow on the ground.

Our work has dealt only with Pinaceae, but we have found that the genera of this family we have investigated differ significantly in the trace-element contents of their variously aged organs.

A consideration of the copper and zinc content of the tips, stems, and needles of the species which we have investigated follows:

Genus *Pseudotsuga*

Pseudotsuga taxifolia (Poiret) Britton (Douglas Fir)

We have not attempted to distinguish this species from the subspecies or variety *glauca* (Mayr) Sudworth.

The Douglas Fir is also known locally as British Columbia Fir, red fir, yellow fir, Doug-

as spruce, Douglas pine, Oregon pine, and Oregon spruce. The variety *glauca* is sometimes called Blue Douglas Fir and is more rarely referred to as Mountain Douglas Fir and Rocky Mountain Douglas Fir.

Douglas Fir is one of the most useful species of trees for biogeochemical investigations in the Pacific Northwest. It is widespread in southern Alberta and British Columbia, in Montana, Idaho, Washington, and Oregon. It grows close to Juniper and Sagebrush on the one hand and in proximity to Cedar and Hemlock on the other. We have found it at elevations of 6000 feet in southern British Columbia, and even here its copper and zinc content appeared comparable with that of specimens growing close to tide water.

Douglas Fir is readily identifiable wherever cones are available because of their conspicuous three-forked bracts.

For these reasons we have paid special attention to this species. In many instances it is convenient to use it as a yard stick in discussing other species.

Fortunately needles and stems are readily separated in Douglas Fir. If the specimen be allowed to dry the needles fall off naturally. However, if field analyses are being made and "green" or undried samples are being run, needles can easily be stripped from a stem.

In general stems of one full year's growth are preferred where Douglas Fir is being used for biogeochemical investigations.

However, the ease of collecting, as opposed to the ease of analyzing, may tip the scales in favor of buds when the collecting is being done by several people of varying degrees of competence. A high-school child can be taught to collect buds effectively. A lazy or indifferent adult can be expected to mix second- and third-year stems. By merely glancing at a bag of buds it is possible to size up whether or not the collection is satisfactory. It is relatively easy to overlook an appreciable amount of older stems in a collection of second-year material.

One key point arises from the evidence presented in Tables 1, 2, and 3. Heavy mineralization—Sample No. 48.47—could be detected biogeochemically whatever organ or whatever aged material was collected. Weak mineralization—Sample No. 48.37—produces anomalies comparable with the variations found when various organs are compared. In other words it is possible to detect major anomalies using almost any method of collecting and analyzing, but minor anomalies, such as those which are liable to be of importance in tracing down most buried ore bodies, can be found only by careful collecting and attention to details.

In Table 1 the copper, zinc, and ash content of needles and stems are compared.

These samples were collected from trees growing in widely differing climatic zones and over widely differing country rock. Although the ash content of Douglas Fir stems is normally lower than that of the needles growing on them, the copper and zinc content of the stems is almost twice that of the needles. The lower ash content of the stems relative to the needles causes these differences to be even more marked in the ash than it is in the dry plant material.

Because the needles contain much less copper and zinc than the stems it is good practice to select stems for analytical work: this we prefer to do with this species.

In the early part of the growing season a collector is tempted to take young Douglas Fir tips or buds. They readily break off from the growth of the previous season and can be plucked easily by thumb and forefinger. However, these tips contain a large percentage of needles and, because *needles contain less copper and zinc* than the twigs bearing them, young buds should contain less copper and zinc than the stems which bear them.

Table 2 illustrates the differences which may be found if the copper and zinc content of buds and stems bearing them are investigated.

In Table 2 although all the samples are zinc negative a few are copper positive. The copper positive samples show the same general relationship between bud and stem as do the copper-negative specimens. Furthermore, the copper content of the positive samples does not appear to be related to their zinc content in any way.

In Table 3 several useful points emerge:

(1) The copper and the zinc content of dry needles falls off markedly from year to year. A tendency for the ash content of the needles to decrease with age is not sufficiently great to obscure a falling off in the copper and zinc content of the ash of needles.

TABLE 1.—*PSEUDOTSUGA TAXIFOLIA* (DOUGLAS FIR)
Comparison between Needles and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Needles	Stem	Needles	Stem	Needles	Stem
48.C	3 (100)	7 (250)	18 (1000)	36 (1300)	1.8	2.7
48.H	3 (50)	5 (230)	28 (470)	40 (1700)	6.0	2.3
48. 2	2 (70)	4 (140)	13 (430)	30 (1100)	3.0	2.5
48. 4	2 (60)	3 (120)	15 (460)	37 (1370)	3.2	2.7
48. 5	3 (82)	9 (330)	31 (850)	32 (1300)	3.6	2.8
48. 6	2 (74)	5 (200)	21 (620)	37 (1500)	3.4	2.5
48. 7	3 (110)	3 (150)	16 (490)	50 (2100)	3.2	2.4
48. 8	1 (40)	4 (140)	15 (600)	49 (1700)	2.9	2.5
48. 9	4 (130)	4 (170)	28 (890)	50 (2100)	3.1	2.3
48.10	4 (130)	3 (150)	33 (970)	66 (3000)	3.3	2.2
48.11	4 (120)	5 (160)	37 (970)	68 (2000)	3.8	3.4
48.12	5 (130)	6 (170)	34 (880)	50 (1400)	3.8	3.5
Average...	3 (90)	5 (180)	24 (720)	45 (1700)	3.4	2.7

TABLE 2.—*PSEUDOTSUGA TAXIFOLIA* (DOUGLAS FIR)
Comparison between Tips (Buds) and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Tip	Stem	Tip	Stem	Tip	Stem
48.21	4 (160)	8 (290)	22 (880)	44 (1300)	2.5	3.0
48.22	4 (140)	8 (200)	16 (550)	30 (750)	2.9	4.0
48.23	7 (190)	9 (290)	28 (770)	53 (1600)	3.6	3.2
48.24	3 (70)	8 (250)	20 (440)	39 (1200)	4.5	3.2
48.28	4 (110)	7 (200)	24 (700)	50 (1400)	3.6	3.5
48.29	7 (200)	14 (400)	28 (750)	44 (1300)	3.7	3.5
48.37*	6 (270)	13 (380)	24 (1020)	50 (1400)	2.3	3.5
48.40	7 (250)	7 (280)	23 (750)	42 (1600)	3.0	2.6
48.41	5 (210)	10 (430)	17 (720)	40 (1700)	2.3	2.4
48.42*	12 (460)	14 (420)	27 (1000)	51 (1500)	2.6	3.3
48.45*	5 (180)	10 (380)	24 (890)	49 (1900)	2.7	2.6
48.46*	6 (230)	13 (450)	29 (810)	45 (1600)	2.6	2.9
48.47*	14 (480)	20 (680)	20 (680)	42 (1400)	3.0	2.9
48.48	5 (150)	9 (280)	28 (850)	39 (1200)	3.3	3.3
48.49	8 (250)	9 (260)	30 (950)	44 (1300)	3.1	3.4
48.50	9 (360)	7 (290)	25 (940)	50 (1900)	2.6	2.6
Average....	7 (230)	10 (340)	24 (790)	44 (1400)	3.0	3.1

* Sample now known to be copper positive.

Samples 48.28, 48.29, and 48.40, 48.41 were also originally taken as copper negative but as a result of biogeochemical data they are now believed to be copper positive. Drilling will be necessary if this belief is to be substantiated.

(2) The ash content of stems falls off rapidly as the stems age, and this is sufficient to cause, for at least 1 or 2 years, an appreciable increase in the copper and zinc content of the ashed twigs. The copper in the dry twigs tends to decrease between the first and second year, while the zinc remains more nearly constant.

Although there is probably no such thing as

TABLE 3.—*PSEUDOTSUGA TAXIFOLIA* (DOUGLAS FIR)
Comparison between Tips (Buds) and Older Stems and Needles

(a) TIPS AND STEMS

Sample No.	Copper			Zinc			Ash Per cent		
	Tips	2nd Yr. Stems	3rd Yr. Stems	Tips	2nd Yr. Stems	3rd Yr. Stems	Tips	2nd Yr.	3rd Yr.
48.15	7 (140)	5 (150)		32 (640)	25 (760)		5.0	3.3	
48.17	4 (120)	4 (130)		32 (1000)	41 (1300)		3.2	3.1	
48.19	9 (240)	7 (320)		25 (670)	38 (1700)		3.7	2.2	
49. 1	10 (230)	8 (310)	4 (170)	42 (1000)	41 (1600)	38 (1600)	4.3	2.6	2.4
49. 2	6 (160)	4 (180)		36 (940)	45 (1800)		3.8	2.5	
49. 3	12 (300)	8 (400)	8 (410)	45 (1100)	45 (2200)	42 (2200)	4.1	2.1	1.9
49. 5		7 (230)	5 (280)		23 (750)	20 (1100)		3.0	1.7
Average.....	8 (200)	6 (250)	6 (290)	35 (890)	37 (1440)	33 (1630)	4.0	2.7	2.0

(b) TIPS AND NEEDLES

Sample No.	Copper			Zinc			Ash Per cent		
	Tips	2nd Yr. Needles	3rd Yr Needles	Tips	2nd Yr Needles	3rd Yr. Needles	Tips	2nd Yr. Needles	3rd Yr. Needles
48.15	7 (140)	1 (29)		32 (640)	18 (510)		5.0	3.5	
48.17	4 (120)	2 (60)		32 (1000)	29 (900)		3.2	3.2	
48.19	9 (240)	2 (60)		25 (670)	18 (600)		3.7	3.1	
49. 1	10 (230)	3 (100)	3 (86)	42 (1000)	17 (580)	17 (480)	4.3	3.1	3.5
49. 2	6 (160)	2 (67)		36 (940)	22 (730)		3.8	3.0	
49. 3	12 (300)	5 (220)	2 (79)	45 (1100)	14 (570)	14 (440)	4.1	2.4	3.1
49. 5		6 (230)	5 (160)		27 (970)	12 (390)		2.8	3.1
Average.....	8 (200)	3 (110)	3 (110)	35 (890)	21 (690)	14 (440)	4.0	3.0	3.2

a normal Douglas Fir with a normal ash and mineral content, it is expedient for illustrative purposes to indicate what may be taken as normal. The following summarizes Tables 1, 2, and 3 and indicates what may be considered normal variations.

	Copper	Zinc	Ash per cent	Cu:Zn
Needles.....	3(90)	24(720)	3.4	.13
	± 50%	± 30%	± 75%	± 50%
Tips or Buds.....	8(250)	25(800)	3.1	.30
	± 50%	± 30%	± 75%	±50%
Stems.....	6(240)	40(1600)	2.7	.15
	± 50%	± 30%	± 75%	± 50%
(1 year's complete growth)				

Specimens from 34 suites which were both copper and zinc negative and which came from widely differing areas, half from near the West Coast and half from the interior of British Columbia (Warren, Delavault, and Irish, 1949), give the following average results:

	Copper	Zinc	Ash per cent	Cu: Zn
Coast (Stems 1 year's growth).....	4.4(195)	38(1620)	2.3	.12
Interior (Stems 1 year's growth)....	5.7(200)	45(1520)	2.8	.13

Variations outside the limits indicated previously we tabulate as anomalous. Using stems as an example we consider as possibly anomalous anything over 9(360) for copper, 60(2400) for zinc, and anything outside the limits .07 to .23 for the Cu:Zn ratio.

Genus *Pinus*

Several species of Pine occur in the Pacific Northwest, but we have found only three species widespread enough to be of value in our preliminary biogeochemical investigations. All the information collected suggests that there are no significant variations in the copper and zinc content of the various species of pine. White (1950) carried out one investigation in which *Pinus ponderosa* and *Pinus albicaulis* were mixed indiscriminately apparently without impairing the results.

In any one area it is usually relatively easy to determine the species of Pine present, and the recognition of the genus presents no difficulty. All pines appear to have two great advantages over other genera: they are exceptionally easy to collect, and variations between needles and stems bearing them is much less than in Douglas Fir and the Spruces.

The Pines as a genus have some drawbacks which occasionally are serious. In many areas they have no low branches. They take longer to dry than do many other genera. At some seasons they are sticky, and this makes salting much more likely. Lastly Pines frequently grow on eskers. These eskers are in some areas more than 100 feet thick; where this is the case the chances of obtaining any indications of economic mineralization are slim.

(a) *Pinus contorta* Douglas var. *latifolia* Engelmann

This species has several local names including lodgepole pine, black pine, jack pine, western jack pine, white pine, and cypress. It is found in a wide range from the southern Yukon to Northern Colorado, Northern Utah, and Northern California. It is locally abundant from the Pacific Coast eastward to Saskatchewan and Eastern Colorado. Botanists disagree as to whether this species and "Shore Pine" represent varieties of the same species or two separate species. We suggest that biogeochemists treat both as one species. The species is most abundant at altitudes from 2000 to 5000 feet. It is adapted to a wide range of sites but makes its best growth on deep, moist, well-drained loam.

Table 4 shows a comparison between needles and stems. As with Douglas Fir, stems tend to carry more copper, both in dry material and in ash, than do the needles they bear. The zinc content of stems and needles is similar and in this respect differs markedly from the distributions noted in Douglas Fir.

The copper content of this species tends, organ for organ, to be a little greater than that in Douglas Fir; however, the zinc content is a little less.

There is little difference in the ash content of needles and stems, and both are lower than their Douglas Fir counterparts.

In Table 5 (a) and (b) the older stems show a lower ash content and a relatively unimpor-

tant decrease of copper and zinc in dry plant material and an increase of these metals in ash.

If only the negative copper and zinc samples be considered we can set up the following normal compositions.

TABLE 4.—*PINUS CONTORTA* (LODGEPOLE PINE)
Comparison between Needles and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Needles	Stems	Needles	Stems	Needles	Stems
48.14	2 (100)	8 (460)	32 (1700)	27 (1500)	1.9	1.7
48.15	12 (250)	9 (230)	36 (750)	47 (1300)	5.1	3.6
48.16*	13 (450)	12 (400)	39 (1400)	40 (1200)	2.8	3.6
48.17	3 (120)	5 (210)	44 (1800)	40 (1700)	2.4	2.4
48.20*	12 (600)	11 (500)	35 (1700)	29 (1300)	2.1	2.2
49.8	4 (260)	6 (350)	41 (2400)	27 (1600)	1.7	1.7
49.9	5 (290)	7 (410)	28 (1600)	23 (1400)	1.7	1.7
49.10	5 (310)	9 (430)	32 (2100)	32 (1500)	1.5	2.1
49.11	3 (210)	9 (510)	47 (2800)	26 (1500)	1.7	1.7
Average....	6.5 (290)	8.5 (390)	37 (1800)	32 (1400)	2.3	2.3

* Sample now known to be copper positive.

	Copper	Zinc	Ash per cent	Cu: Zn
Needles..	5(120) ± 50%	38(1600) ± 30%	2.3	.13
Tips.....	6(240) ± 50%	42(1700) ± 30%	2.5	.14
Stems....	6(300) ± 50%	36(1800) ± 30%	2.0	.17
(1 year's complete growth)				

Tables 5 (c), (d), and (e) indicate clearly the type of results to be expected in actual field work. Table 5 (d) represents a reconnaissance suite of samples; mineralization, some of commercial interest, is known in the vicinity of virtually all these positive samples.

Table 5 (e) consists of three groups of samples from the Sullivan Mine area, representative of the groups which we have studied from that vicinity.

(b) *Pinus ponderosa* Lawson (Yellow Pine, *Ponderosa* Pine)

This species has many local names including Western yellow pine, bull pine, yellow pine, British Columbia pine, jack pine, and *Ponderosa* pine. In Canada the tree is confined to the drier portions of the southern interior of British Columbia. It extends south to portions of Montana, Idaho, Oregon, and Washington. It grows on dry well-drained slopes or plateaus, usually between elevations of 1000 and 2500 feet, and appears to demand better-drained soils than the Lodgepole pine.

A comparison between needles and stems is shown in Table 6.

The copper and zinc relationships between needles and stems approximate much more closely the relationships noted in Lodgepole pine than those observed in Douglas Fir. The absolute amount of copper in both needles and stems of yellow pine is only about half that in the Lodgepole pine, and the zinc also is somewhat less. We consider this apparent relative deficiency of no particular significance. The trees which are taken as representative were sampled over a wide area under varying conditions, both of climate and of underlying geology. However, agriculturists recognize the area as one in which there are some definitely recognizable mineral deficiencies of which zinc is one. Copper may also be in short supply.

The only justifiable conclusion is that in yellow pine as in Lodgepole pine the needles and stems tend to be much more similar in their copper and zinc content than do the needles and stems of Douglas Fir.

In Table 7 tips and stems are compared, and the results are comparable with those obtained from negative Lodgepole pines.

TABLE 5.—*PINUS CONTORTA* (LODGEPOLE PINE)

(a) COMPARISON BETWEEN TIPS (BUDS) AND STEMS BEARING THEM

Sample No.	Copper		Zinc		Ash	
	Tips	Stems	Tips	Stems	Tips	Stems
48.21	4 (210)	7 (380)	33 (1700)	34 (1800)	1.9	1.8
48.23	9 (300)	7 (280)	97 (3000)	57 (2300)	3.2	2.5
48.28*	7 (290)	15 (650)	46 (1900)	51 (2100)	2.4	2.4
48.29*	7 (300)	12 (400)	41 (1800)	56 (1900)	2.3	3.0
48.30*	8 (270)	12 (470)	27 (900)	23 (900)	3.0	2.6
48.31*	2 (89)	6 (290)	30 (1300)	35 (1700)	2.3	2.1
48.32*	7 (300)	11 (480)	20 (890)	26 (1100)	2.3	2.3
48.33*	7 (340)	15 (840)	43 (2000)	45 (2400)	2.2	1.8
48.34	6 (290)	9 (430)	36 (1700)	33 (1500)	2.1	2.2
48.35	6 (320)	9 (530)	21 (1100)	25 (1400)	1.8	1.8
48.36	3 (140)	7 (330)	34 (1400)	35 (1700)	2.4	2.1
48.37*	7 (310)	11 (560)	39 (1600)	21 (930)	2.4	2.0
48.38*	7 (350)	9 (510)	50 (2300)	38 (2000)	2.1	1.8
48.39*	4 (210)	8 (460)	36 (1700)	23 (1200)	2.1	1.8
48.40*	8 (380)	17 (810)	38 (1800)	31 (1500)	2.1	2.1
48.41	4 (270)	10 (510)	43 (2900)	43 (2200)	1.5	1.9
48.42*	7 (340)	13 (530)	38 (1700)	36 (1500)	2.2	2.4
48.45*	8 (420)	7 (310)	37 (2000)	48 (2000)	1.8	2.4
48.46*	7 (370)	5 (260)	41 (2200)	33 (1700)	1.9	1.9
48.47*	8 (470)	10 (360)	30 (1800)	29 (1100)	1.7	2.5
48.48	7 (390)	7 (250)	55 (3100)	60 (2100)	1.8	2.8
Average.....	6 (300)	10 (460)	40 (1850)	37 (1670)	2.2	2.2

(b) COMPARISON BETWEEN TIPS (BUDS) AND STEMS OF PREVIOUS YEAR'S GROWTH

Sample No.	Copper		Zinc		Ash	
	Tips	Stems	Tips	Stems	Tips	Stems
49.6	9 (320)	10 (540)	35 (1200)	52 (2800)	2.8	1.8
49.7	13 (490)	13 (600)	53 (2000)	42 (1900)	2.6	2.2
49.8	8 (270)	6 (350)	48 (1600)	27 (1600)	3.0	1.7
49.9	7 (200)	7 (410)	43 (1200)	23 (1400)	3.5	1.7
49.10	11 (360)	9 (430)	37 (1200)	32 (1500)	2.6	1.7
49.11	9 (370)	9 (510)	43 (1700)	26 (1500)	2.8	1.8
Average.....	9 (330)	9 (470)	43 (1480)	34 (1780)	2.9	1.8

(c) COPPER AND ZINC CONTENTS OF A NUMBER OF REPRESENTATIVE NEGATIVE SAMPLES OF WELL-DEVELOPED TIPS

Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
20.49	7 (300)	44 (1900)	2.3	0.16
24.49	4 (250)	30 (1700)	1.8	0.15
26.49	4 (200)	30 (1400)	2.2	0.15
27.49	5 (260)	37 (1900)	1.9	0.14

* Sample now known to be copper positive

TABLE 5—*Continued*

(c) COPPER AND ZINC CONTENTS OF A NUMBER OF REPRESENTATIVE NEGATIVE SAMPLES OF WELL-DEVELOPED TIPS—*Continued*

Sample No.	Copper	Zinc	Per cent Ash	Cu:Zn
28.49	4 (240)	29 (1500)	1.8	0.16
31.49	5 (240)	38 (1700)	2.3	0.14
32.49	6 (290)	34 (1600)	2.1	0.18
33.49	5 (240)	40 (1900)	2.1	0.12
34.49	4 (270)	33 (2000)	1.6	0.14
35.49	7 (200)	49 (1400)	3.4	0.14
38.49	5 (300)	32 (1700)	1.8	0.17
39.49	4 (200)	32 (1600)	2.0	0.12
41.49	4 (220)	35 (1900)	1.8	0.11
50.49	3 (170)	26 (1500)	1.7	0.12
52.49	6 (330)	35 (1900)	1.8	0.17
60.49	6 (260)	28 (1200)	2.3	0.21
96.49	10 (350)	50 (1800)	2.8	0.20
97.49	9 (370)	52 (2000)	2.5	0.18
114.49	3 (220)	33 (2100)	1.6	0.11
115.49	8 (400)	32 (1500)	2.1	0.27
117.49	5 (250)	37 (1800)	2.0	0.13
Average.....	5.4 (260)	36 (1700)	2.1	0.16

(d) COPPER AND ZINC CONTENTS OF A NUMBER OF REPRESENTATIVE COPPER-POSITIVE AND ZINC-NEGATIVE SAMPLES OF WELL-DEVELOPED TIPS

Sample No.	Copper	Zinc	Ash	Cu:Zn
22.49	9 (490)	31 (1700)	2.2	0.29
23.49	7 (390)	36 (2000)	1.8	0.19
36.49	5 (200)	38 (1600)	2.4	0.13
37.49	10 (380)	37 (1000)	2.6	0.37
40.49	7 (250)	41 (1300)	3.0	0.18
61.49	12 (930)	20 (1500)	1.3	0.61
98.49	8 (460)	33 (1900)	1.7	0.24
Average.....	8 (440)	34 (1600)	2.1	0.29

(e) COPPER AND ZINC CONTENTS OF A NUMBER OF REPRESENTATIVE COPPER-NEGATIVE AND ZINC-POSITIVE SAMPLES OF BOUGHS (TIPS AND SOME SECOND-YEAR GROWTH)

Sample No.	Copper	Zinc	Ash	Cu:Zn
54.46	4 (230)	57 (3000)	1.9	0.077
56.46	3 (170)	60 (3600)	1.7	0.048
57.46	4 (210)	48 (2500)	1.9	0.083
58.46	4 (240)	46 (2500)	1.8	0.096
60.46	5 (260)	86 (4200)	1.1	0.063
Average.....	4 (220)	60 (3200)	1.7	0.07
1.46	5 (250)	360 (16000)	2.2	0.015
6.46	6 (250)	100 (4500)	2.2	0.056

TABLE 5—*Concluded*

(e) COPPER AND ZINC CONTENTS OF A NUMBER OF REPRESENTATIVE COPPER-NEGATIVE AND ZINC-POSITIVE SAMPLES OF BOUGHS (TIPS AND SOME SECOND-YEAR GROWTH)

Sample No.	Copper	Zinc	Ash	Cu:Zn
15.46	4 (200)	90 (4000)	2.2	0.050
18.46	3 (140)	200 (9400)	2.4	0.026
20.46	5 (190)	140 (2700)	2.5	0.032
50.46	4 (170)	220 (8300)	2.6	0.020
52.46	5 (180)	170 (6500)	2.5	0.028
53.46	4 (210)	280 (14000)	2.1	0.016
Average.....	4 (190)	190 (8200)	2.3	0.03
7.46	5 (110)	160 (4000)	4.0	0.029
8.46	4 (190)	200 (9600)	2.1	0.020
10.46	13 (220)	200 (3500)	5.8	0.060
12.46	6 (340)	86 (5400)	1.6	0.063
16.46	3 (270)	40 (3700)	1.0	0.074
22.46	4 (180)	90 (3700)	2.4	0.049
25.46	5 (220)	160 (7600)	2.1	0.029
26.46	4 (220)	110 (6200)	1.8	0.035
28.46	5 (280)	160 (9200)	1.7	0.030
43.46	4 (250)	54 (3200)	1.7	0.078
Average.....	5 (230)	130 (5600)	2.4	0.05

TABLE 6.—*PINUS PONDEROSA* (YELLOW PINE PONDEROSA PINE)

Comparison between Needles and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Needles	Stems	Needles	Stems	Needles	Stems
48. 2	4 (140)	3 (150)	28 (1000)	24 (1200)	2.8	2.0
48. 3	4 (120)	3 (190)	25 (780)	18 (940)	3.2	1.6
48. 4	6 (200)	4 (120)	26 (850)	23 (690)	3.0	3.3
48. 5	2 (96)	3 (70)	33 (1300)	35 (1100)	2.6	3.2
48. 7	3 (170)	2 (90)	32 (1500)	26 (1100)	2.0	2.3
48. 8	4 (150)	2 (200)	40 (1500)	32 (1300)	2.6	2.4
48. 9	4 (200)	6 (270)	37 (1800)	30 (1400)	2.0	2.2
48.10	3 (170)	4 (190)	34 (1700)	30 (1300)	2.0	2.3
48.11	5 (230)	4 (150)	60 (2800)	40 (1500)	2.1	2.6
48.12	2 (90)	2 (90)	28 (1000)	33 (1500)	2.7	2.1
Average...	4 (160)	3 (150)	34 (1420)	29 (1200)	2.5	2.4

Table 8 lists the copper, zinc, and ash content of a few stems of different ages. A familiar pattern again may be noted. The ash and the copper and zinc content of dry plant material tends to decrease with age. However, the amount of copper and zinc in the ash of second-year stems is usually higher than it is for first-year material. Usually there is a dropping off of the zinc and copper content, even of ash, after the second year, but in some instances this dropping off is not apparent until after the third year.

The reasons for these variations are not of primary interest to the biogeochemist. The important point is that in any investigation a worker must remember the importance of using stems of only one age.

We have no positive samples of yellow pine and consequently we consider it unwise to attempt to suggest normal and anomalous contents for this species.

(c) *Pinus albicaulis* Engelmann (White Bark Pine)

This species is also known as white-stemmed pine and scrub pine. It grows sparingly in British Columbia, usually at altitudes from 3000 to 7000 feet. Its range extends into Washington, Oregon, Idaho, and Montana.

TABLE 7.—*PINUS PONDEROSA* (YELLOW PINE PONDEROSA PINE)
Comparison between Tips (Buds) and Stems Bearing Them

Sample No.	Copper		Zinc		Ash	
	Tips	Stems	Tips	Stems	Tips	Stems
48.27	6 (280)	8 (380)	25 (1100)	33 (1500)	2.1	2.2
48.48	9 (410)	4 (180)	31 (1300)	25 (1100)	2.3	2.2
48.49	9 (330)	8 (350)	42 (1500)	66 (2700)	2.7	2.4
48.50	10 (330)	12 (430)	28 (910)	30 (1000)	3.0	2.9
Average....	8 (340)	8 (330)	31 (1200)	38 (1600)	2.5	2.4

TABLE 8.—*PINUS PONDEROSA* (YELLOW PINE, PONDEROSA PINE)
Comparison between Stems of Different Ages

Sample No.	Copper			Zinc			Ash		
	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.
49.1	7 (230)	5 (230)		53 (1600)	28 (1300)		3.2	2.2	
49.2	5 (170)	4 (220)		37 (1100)	29 (1400)		3.2	2.0	
49.4	5 (120)	5 (170)	3 (160)	19 (460)	29 (910)	30 (1300)	4.1	3.2	2.2
Average...	6 (170)	5 (210)	3 (160)	36 (1100)	29 (1200)	30 (1300)	3.5	2.5	2.2

TABLE 9.—*PINUS ALBICAULIS* (WHITE BARK PINE)
Comparison between Needles and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Needle	Stem	Needle	Stem	Needle	Stem
48.15	6 (220)	7 (180)	32 (1200)	33 (880)	2.7	3.7
48.17	5 (160)	8 (260)	41 (1300)	58 (1900)	3.1	3.0
48.19	6 (190)	5 (170)	26 (800)	26 (900)	3.2	2.9
Average.....	6 (190)	7 (200)	33 (1100)	39 (1230)	3.0	3.2

In Table 9 needles and stems are compared. A similarity between the copper, zinc, and ash contents of needles and stems of *Pinus albicaulis* and *Pinus ponderosa* is quite apparent.

In Table 10 tips and stems of the previous year's growth are compared. The ash content of *Pinus albicaulis* appears consistently higher than that of *Pinus ponderosa*. However, considering only the zinc content of the dry plant material, it is reasonable to assume that the zinc content of stems tends to be higher than that of the tips.

TABLE 10.—*PINUS ALBICAULIS* (WHITE BARK PINE)
Comparison between Tips (Buds) and Stems of Previous Year

Sample No.	Copper		Zinc		Ash Per cent	
	Tip	Stem	Tip	Stem	Tip	Stem
49.Y. 10	5 (150)		26 (700)	27 (1600)	3.7	1.8
49.Y. 113	5 (140)		29 (800)	39 (2200)	3.6	1.8
49.Y. 259	9 (280)		47 (1500)	64 (1800)	3.1	3.5
49.Y. 274	4 (150)		26 (830)	44 (1500)	3.0	2.8
49.Y. 279	6 (170)		33 (960)	32 (1300)	3.5	2.4
49.Y. 281	7 (110)		26 (770)	31 (1300)	3.3	2.4
49.Y. 288	5 (130)		30 (810)	37 (1200)	3.7	3.0
49.Y. 290	20 (590)		29 (850)	52 (1900)	1.7	2.8
49.Y. 293	13 (400)		31 (1000)	48 (1500)	2.3	3.1
49.Y. 347	9 (230)		33 (870)	40 (2000)	3.8	2.0
Average.....	8 (230)		31 (910)	41 (1630)	3.2	2.6

TABLE 11.—*PINUS ALBICAULIS* (WHITE BARK PINE)
Comparison between Needles and Stems of Different Ages

Sample No.	Copper		Zinc		Ash Per cent	
	1st Yr.	2nd Yr.	1st Yr.	2nd Yr.	1st Yr.	2nd Yr.
(a) STEMS						
48.15	7 (180)	6 (170)	33 (880)	26 (700)	3.7	3.8
48.17	8 (260)	6 (250)	58 (1900)	54 (2200)	3.0	2.4
48.19	5 (170)	4 (150)	26 (900)	39 (1500)	2.9	2.6
Average...	7 (200)	5 (190)	39 (1230)	40 (1470)	3.2	2.9
(b) NEEDLES						
48.15	6 (220)	3 (180)	32 (1200)	15 (880)	2.7	1.7
48.17	5 (160)	2 (100)	41 (1300)	25 (1700)	3.1	2.0
48.19	6 (190)	2 (150)	26 (800)	22 (1600)	3.2	1.4
Average...	6 (190)	2 (140)	33 (1100)	21 (1390)	3.0	1.7

Table 11, in which needles and stems of different ages are compared, tends to confirm the above observation. The ash content of both stems and needles falls off in the second year, but the falling off is much more conspicuous in needles than in stems.

Genus *Picea*

The spruces are widely distributed throughout the cooler parts of the northern hemisphere. Four species are important in the Pacific Northwest, but we have collected data on only three—*Picea glauca*, *Picea Engelmanni*, and *Picea*

The mineral-bearing habits of all the spruces seem to parallel one another closely.

Even a newcomer can learn to recognize a spruce without difficulty. However the determination of the species may be difficult unless cones can be found. Sometimes it is possible to use geography to determine species, as is the case with *Picea sitchensis* which usually grows at elevations below 1000 feet and in consequence is rarely found far inland.

Usually some low branches are available, but in places their lack may present a problem to the biogeochemist.

The collecting of spruce would be particularly

easy if young tips could be used with confidence.

Unfortunately the work we have done suggests that such is not the case, and indeed our limited data hint that, especially for copper, spruce tips, particularly very young ones, are quite unreliable.

than are the needles. The ash content of picea *Engelmanni* consistently runs a little higher than that of Douglas fir or the pines.

Table 13 (a) compares tips and stems bearing them, and Table 13 (b) contrasts tips and stems of previous year's growth. The tips of Table 13 (b) are very young tips, barely having enough growth to enable effective collecting, whereas the tips of Table 13 (a) are 2 to 3 months old. Eliminating the samples known to be positive we may tentatively conclude that:

(1) Very young tips have the greatest ash content.

(2) Developed and older needles have a lower ash content than the stems bearing them.

(3) Older stems are considerably lower in ash than are stems 1 year old.

(4) Stems which contain 1 year's growth appear to be the most reliable for biogeochemistry. We have no explanations for some of the obvious exceptions to the above generalization.

If we eliminate all the known positives it is possible tentatively to propose some normal contents:

	Copper	Zinc	Ash per cent	Cu: Zn
Needles..	4(120) ± 50%	40(1200) ± 30%	3.4	.10
Tips.....	10(260) ± 50%	45(1300) ± 30%	3.6	.22
Stems....	9(320) ± 50%	60(1800) ± 30%	3.2	.15
(1 year's complete growth)				

On the basis of Tables 12 and 13 (a) and (b) tips are most unreliable, especially with respect to copper. Indeed in Table 12 samples Nos. 16 and 19 appear perfectly normal and one or

TABLE 12.—*PICEA ENGELMANNI* (ENGELMANN SPRUCE)
Comparison between Needles and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Needle	Stem	Needle	Stem	Needle	Stem
48.C.	2 (50)	10 (320)	34 (870)	57 (1800)	3.9	3.1
48.14	5 (130)	6 (150)	69 (1800)	77 (1900)	3.8	4.1
48.16	6 (150)	13 (260)	40 (1000)	79 (1500)	4.0	5.1
48.17	6 (160)	9 (170)	40 (1100)	70 (1200)	3.5	5.7
48.19	5 (150)	12 (300)	36 (1300)	65 (1600)	3.3	4.0
49. 3	4 (120)	4 (110)	43 (1300)	60 (1700)	3.2	3.6
49. 5	6 (160)	17 (370)	57 (1500)	62 (1400)	3.7	4.7
49.10	4 (200)	11 (480)	23 (1000)	64 (2700)	2.2	2.4
49.11	2 (86)	10 (430)	28 (1000)	38 (1600)	2.9	2.3
Average...	4 (130)	10 (290)	41 (1200)	64 (1700)	3.4	3.9

Most of our work has been with *Picea Engelmanni*, and all our results must be built on and around this species.

(a) *Picea Engelmanni* Parry

Although generally called Engelmann spruce it is also known as Mountain spruce, Rocky Mountain spruce, and western white spruce.

Insofar as biogeochemistry in the Pacific Northwest is concerned, *Picea Engelmanni* is by far the most important of the spruces. It does not occur west of the coast mountains nor north of the Nechako River and the Big Bend of the Fraser River. However, it is found from the Nechako to Colorado and Utah in the mountains. It favors elevations from 3000 to 5000 feet in the central part of its range but descends to 1000 in the north and ascends to 8000 in Colorado. Its best growth is reported from deep rich loamy soils with a high moisture content. Occasionally its shares new burns with lodgepole pine.

In Table 12 the ash and mineral content of needles and stems may be compared. The relationship between needles and stems is similar to that in Douglas fir, not to that in the pines; the stems are richer in both copper and zinc

TABLE 13.—*PICEA*

Spec. No.	Copper Tips	Copper Stems	Zinc Tips	Zinc Stems	Ash Per cent Tips	Ash Per cent Stems
(a). PICEA ENGELMANNI (Engelmann Spruce) Comparison between Tips (Buds) and Stems Bearing Them						
48.21	2 (100)	6 (230)	27 (1400)	47 (1800)	1.9	2.6
48.22	5 (170)	7 (270)	42 (1400)	33 (1300)	2.9	2.5
48.23	11 (410)	7 (240)	53 (2000)	62 (2000)	3.7	3.1
48.24	7 (190)	11 (250)	51 (1400)	52 (1200)	3.6	4.7
48.25	5 (180)	8 (250)	29 (1000)	42 (1200)	2.8	3.4
48.28	9 (300)	21 (570)	26 (800)	32 (870)	3.2	3.7
48.29	7 (220)	16 (460)	36 (1100)	42 (1200)	3.1	3.4
48.31*	7 (220)	16 (520)	31 (970)	35 (1100)	3.2	3.1
48.33*	6 (190)	13 (380)	38 (1200)	68 (2000)	3.1	3.4
48.35	4 (120)	6 (200)	38 (1050)	39 (1200)	3.6	3.2
48.36	6 (190)	7 (160)	39 (1200)	50 (1200)	3.1	4.2
48.39	6 (170)	10 (260)	49 (1400)	77 (1900)	3.7	4.0
48.40	4 (150)	9 (260)	28 (1000)	50 (1400)	3.7	3.6
48.41	6 (210)	16 (640)	40 (1400)	48 (1600)	2.8	2.9
48.47*	11 (340)	18 (590)	34 (1000)	36 (1200)	3.4	3.0
Average.....	6 (210)	11 (350)	37 (1220)	48 (1410)	3.2	3.4
(b). PICEA ENGELMANNI (Engelmann Spruce) Comparison between Tips (Buds) and Stems of Previous Year's Growth						
49. 3	7 (160)	4 (110)	49 (1100)	60 (1700)	4.4	3.6
49. 7	25 (570)	12 (560)	72 (1700)	100 (4400)	4.3	2.2
49. 8	8 (170)	9 (330)	65 (1400)	72 (2700)	4.6	2.7
49.10	29 (670)	11 (480)	62 (1400)	64 (2700)	4.3	2.4
49.11	10 (300)	10 (430)	50 (1500)	38 (1600)	3.3	2.3
49.Y.33†	16 (230)	5 (210)	95 (1300)	115 (4800)	7.0	2.4
49.Y.43†	25 (540)	5 (190)	90 (2000)	102 (3900)	4.6	2.6
49.Y.101	3 (70)	5 (250)	49 (1200)	52 (2600)	4.2	2.0
49.Y.147	13 (270)	13 (520)	94 (2000)	112 (4500)	4.8	2.5
49.Y.210	12 (240)	8 (250)	76 (1500)	86 (2700)	5.0	3.2
49.Y.424†	6 (200)	6 (200)	56 (1900)	64 (2200)	3.0	2.9
Average....	14 (310)	8 (320)	69 (1550)	79 (3070)	4.5	2.6

(c). PICEA SITCHENSIS (Sitka Spruce)
Suite of Samples from Vicinity of Buried Copper Mineralization, Fairview Mine (Britannia), B.C. (Boughs)

Suite 46B Sample No.	Copper	Zinc	Ash	Cu:Zn
1	15 (580)	28 (1100)	2.6	0.5
2	14 (500)	31 (1100)	2.8	0.5
3	6 (190)	32 (1000)	3.1	0.2
7	16 (640)	32 (1300)	2.5	0.5
8	10 (450)	24 (1100)	2.2	0.4
9	4 (150)	10 (380)	2.6	0.4

* These samples are now known to come from copper-positive areas. Samples 48.28, 48.29, 48.40, and 48.41 were all taken as copper negative but may now be considered as probably copper positive.

† These samples are now definitely known to come from zinc positive areas. It is possible that some of the other high zincs should be considered zinc positive.

(c). PICEA SITCHENSIS (Sitka Spruce)—*Continued*

Suite 46B Sample No.	Copper	Zinc	Ash	Cu:Zn
10	21 (650)	49 (1500)	3.2	0.4
12	7 (330)	26 (1200)	2.1	0.3
14	9 (390)	31 (1300)	2.3	0.3
16	7 (370)	35 (1800)	1.9	0.2
17	20 (740)	26 (960)	2.7	0.8
18	20 (770)	23 (880)	2.6	0.9
19	10 (370)	28 (1040)	2.7	0.4
21	5 (210)	30 (1200)	2.4	0.16
22	25 (800)	27 (870)	3.1	0.9
24	5 (160)	35 (1100)	3.2	0.14
25	5 (170)	34 (1100)	3.0	0.15
26	3 (100)	28 (930)	3.0	0.11
Average.....	11 (420)	29 (1100)	2.7	0.40

(d). PICEA GLAUCA (White Spruce)

Copper, Zinc, and Ash Content of Normal Tips

Sample No.	Copper	Zinc	Ash	Cu:Zn
49. 20	6 (230)	48 (1800)	2.6	0.12
49. 24	5 (190)	38 (1400)	2.6	0.19
49. 25	4 (120)	43 (1300)	3.3	0.09
49. 26	5 (170)	48 (1500)	3.1	0.12
49. 27	4 (160)	32 (1100)	2.8	0.14
49. 28	5 (180)	33 (1200)	2.7	0.15
49. 31	6 (190)	49 (1600)	3.1	0.12
49. 32	8 (210)	51 (1300)	3.8	0.16
49. 34	3 (160)	35 (1600)	2.2	0.10
49. 35	4 (130)	50 (1500)	3.3	0.09
49. 38	4 (170)	38 (1600)	2.4	0.11
49. 39	4 (190)	34 (1400)	2.4	0.13
49. 40	7 (230)	45 (1400)	3.2	0.17
49. 41	5 (200)	29 (1000)	2.8	0.19
49. 60	5 (200)	30 (1100)	2.7	0.18
49.114	3 (160)	28 (1300)	2.2	0.12
Average.....	5 (180)	39 (1400)	2.8	0.14

(e). PICEA GLAUCA (White Spruce)

Copper, Zinc, and Ash Content of Some Copper-Positive Tips

Sample No.	Copper	Zinc	Ash	Cu:Zn
49.21	8 (270)	43 (1500)	2.9	0.19
49.22	10 (430)	40 (1700)	2.3	0.25
49.23	8 (330)	31 (1300)	2.4	0.26
49.29	6 (210)	48 (1600)	3.0	0.14
49.30	13 (430)	48 (1600)	3.0	0.27
49.36	12 (370)	38 (1200)	3.2	0.32
49.37	10 (380)	44 (1700)	2.6	0.23
49.46	4 (130)	40 (1100)	3.5	0.11
49.98	11 (410)	43 (1600)	2.7	0.26
Average.....	9 (330)	42 (1500)	2.8	0.23

two samples, which to the best of our knowledge are both copper and zinc negative, give positive results.

In Tables 13 (a) and (b) the results are more obvious and, on the whole, are satisfactory. Recent development work in the vicinity of some of the "negative" samples suggests they may better be considered as positive, a conclusion obviously indicated when the collection was analyzed.

(b) *Picea sitchensis* (Bong.) Carrière
Sitka spruce

This species is also known as Menzies spruce, tideland spruce, and coast spruce. It extends along the coast from Alaska to Oregon and is the largest and most imposing of the spruces and is particularly important in the Queen Charlotte Islands.

Unfortunately we have as yet been unable to make any detailed investigations of normal members of this species.

However, we have one excellent suite from a copper-positive area; this is given in Table 13 (c). Although many individual samples are low the suite as a whole is relatively high. Furthermore this is a suite of "boughs" which includes many older needles and therefore may safely be assumed to be relatively low in copper. These samples all came from an area of heavy rainfall, possibly as much as 80 inches annually, and thus the area is apt to be relatively highly leached.

(c) *Picea glauca* (Moench) Voss
White spruce

This spruce is also known as single spruce, skunk spruce, northern spruce, pine, cat spruce, yellow spruce, and others.

The white spruce is the most important of the Canadian spruces. In the Pacific Northwest it is abundant in Alaska, the Yukon, and British Columbia but does not extend into Washington and Oregon. With black spruce and tamarack it forms the northern limit of tree growth. It grows best on well-drained, moist, gravelly soil along streams and around the borders of swamps and lakes.

We have had no opportunity to collect systematically from known areas. Fortunately a large collection of buds taken during exploration work is available, and from our present knowledge the samples in Table 13 (d) may, with reasonable assurance, be assumed to be normal.

The specimens whose analyses are given in Table 13 (e) are all from suites now considered copper positive. The differences in the copper content of the two sets of specimens are marked although the copper content of some of the specimens in Table 13 (e) is normal.

None of the samples taken for Table 13 (e) was considered zinc positive, but some of the suites of which they were a part were determined to be zinc positive. The failure of these spruce trees to respond to zinc in the same manner as their associates is not explained. We think the explanation will have something to do with the fact that many of these spruces were growing near the main water table of the lakes near where the samples were taken. Zinc is both more abundant and more soluble than copper, and smaller percentage variations are significant for zinc than for copper. By inference, copper anomalies might in some conditions be easier to spot than zinc abnormalities.

A discerning reader may now be asking why Table 13 (b) which contains tips of normal *Picea Engelmanni* specimens should run so high in copper in comparison with the normal samples in Tables 13 (a) and (d).

Further work must be done before this problem can be answered. Three possibilities have been considered: (1) Marine sediments are reported to contain abnormally large amounts of copper, and marine sediments occur near where some of the high-copper-bearing tips were collected. (2) Many samples dealt with in Table 13 (b) come from well-mineralized areas. Commercially the mineralization is zinc, and the copper content is insignificant. However, the sphalerite in the area contains copper, and this copper content may be sufficient to cause some of the anomalies. (3) Many of the samples reported in Table 13 (b) were very young, with extremely small tips. Fortunately the size of many of the tips was noted during collection. Thus we know that many of the high-copper tips did correspond with the earliest growth which could be collected. This possible relationship between high copper and early growth might be investigated further.

Once again all these unanswered problems serve to show how careful a biogeochemist must be when he starts work in a new area. Constants and normals must be established, if possible on a statistical basis, before any results can be interpreted intelligently.

Genus *Thuja*

(a) *Thuja plicata* (Donn)

Western Red Cedar

The arbor-vitae are usually referred to as cedars in North America. Actually the true cedars belong to the genus *Cedrus*, and none are native to North America.

The western red cedar is the member of the genus native to the Pacific Northwest. The species is found on the Alaska panhandle, along the coast of British Columbia, Washington, and Oregon, and in the valleys of the interior wet belts of British Columbia, Washington, Idaho, and western Montana. It prefers a deep moist porous soil on cool slopes and in gulches.

Thuja plicata is known locally by many other names including giant arbor-vitae, red cedar, British Columbia cedar, British Columbia red cedar, giant cedar, and western cedar.

Unfortunately the results given in Tables 14, 15, and 16 represent our only data on this species, and as may be noted we have no copper-positive suites at all.

Cedar does not tolerate too dry conditions, and it therefore is frequently missing from many areas where mineralization is being investigated.

About the only justifiable conclusions at this time are:

(1) The zinc content of the stems even with some scales still adhering is normally much lower than that of any of the other evergreens yet reported.

(2) The tips seem useless as a guide to zinc concentrations, but the stems appear to be satisfactory.

As a foundation for further studies we suggest that the following may be considered normal:

	Copper	Zinc	Ash per cent	Cu: Zn
Needles..	4(110) ± 50%	12(340) ± 30%	3.5	.33
Tips.....	4(110) ± 50%	14(390) ± 30%	3.6	.29
Stems....	4(100) ± 50%	26(700) ± 30%	3.7	.15
(1 year's complete growth)				

TABLE 14.—*THUJA PLICATA* (WESTERN RED CEDAR)
Comparison between Needles and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Needles	Stems	Needles	Stems	Needles	Stems
48 H.	3 (100)	2 (80)	12 (400)	15 (600)	3.0	2.5
48 I.	3 (90)	2 (90)	8 (200)	14 (550)	4.0	2.6
Average.....	3 (95)	2 (85)	10 (300)	14 (575)	3.5	2.5

Genus *Juniperus*

(a) *Juniperus scopulorum* Sargent

Rocky Mountain Juniper

This variety is also known as the Rocky Mountain red cedar, red cedar, and western juniper.

It is found in the foothills of Montana and western Alberta and westward in Idaho, Washington, Oregon, and southern British Columbia. It is usually found at higher altitudes on dry rocky or sandy soils but grows best in moist canyon bottoms.

(b) *Juniperus communis* L. var. *depressa* Pursh

Dwarf Juniper

The authors have found it impractical to separate this species from a closely related variety *Juniperus horizontalis* Moench. Both are widely distributed in western Canada in relatively dry areas, and in Washington and Idaho. Table 18 may contain both varieties.

Tables 17 and 18 present the limited data we have on these species. No zinc-positive samples are available.

TABLE 15.—*THUJA PLICATA* (WESTERN RED CEDAR)
Comparison between Tips and Stems of Previous Year

Sample No.	Copper		Zinc		Ash Per cent	
	Tips	Stems†	Tips	Stems†	Tips	Stems†
49.Y. 13	5 (120)	4 (100)	17 (380)	38 (950)	4.4	4.0
49.Y. 42*	6 (180)	4 (100)	20 (590)	79 (2200)	3.4	3.6
49.Y. 46*	2 (70)	4 (100)	15 (500)	37 (1000)	3.4	3.7
49.Y. 49	7 (200)	4 (100)	15 (400)	27 (800)	3.7	3.5
49.Y. 52	6 (220)	3 (90)	14 (480)	13 (400)	2.4	3.3
49.Y.179	6 (160)	3 (80)	18 (440)	32 (840)	4.0	3.7
49.Y.182	4 (110)	4 (100)	13 (350)	27 (650)	3.7	4.0
49.Y.200*	3 (70)	3 (70)	14 (340)	77 (1700)	4.1	4.4
49.Y.237*	3 (90)	3 (90)	12 (350)	43 (1300)	3.4	3.4
49.Y.406*	3 (110)	4 (100)	13 (410)	72 (1800)	3.3	3.9
Average.....	4 (130)	4 (90)	15 (420)	44 (1160)	3.6	3.7

* Samples known to be zinc positive.
† With base of green scales adhering.

TABLE 16.—*THUJA PLICATA* (WESTERN RED CEDAR)
Comparison between Stems of Different Ages

Sample No.	Copper			Zinc			Ash, Per cent		
	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.
49.2	1 (50)	2 (85)	3 (100)	9 (310)	11 (400)	12 (330)	2.9	2.9	3.6

TABLE 17.—*JUNIPERUS SCOPULORUM* (ROCKY MOUNTAIN JUNIPER)
Comparison between Tips and Stems Bearing Them
(Some 2nd year material included)

Sample No.	Copper		Zinc		Ash Per cent	
	Tips	Stems	Tips	Stems	Tips	Stems
48. 9	4 (70)	2 (40)	13 (230)	12 (250)	5.7	4.8
48.12	5 (110)	4 (90)	17 (360)	14 (310)	4.6	4.6
48.27	9 (220)	6 (170)	15 (370)	13 (360)	4.0	3.6
48.40*	5 (150)	5 (130)	18 (490)	8 (190)	3.7	4.2
48.41*	21 (520)	5 (110)	17 (420)	9 (200)	4.0	4.6
48.47*	8 (130)	13 (180)	9 (130)	7 (95)	6.7	7.2
48.50†	18 (380)	8 (170)	16 (360)	10 (220)	4.7	4.6
49.50"A".	7 (160)	6 (200)	18 (420)	9 (300)	4.3	3.0
Average.....	10 (220)	6 (140)	15 (350)	10 (240)	4.7	4.6

* These samples are now thought to be copper positive.
† The unusually high copper content of this tip caused a check sample to be taken. This check sample gave the results reported for sample 49.50"A". Salting may be responsible for the high copper in 48.50.

In spite of this limited data the following conclusions seem valid:

(1) Both junipers have a relatively high ash content.

(2) Both junipers in terms of ash have a markedly higher copper and zinc content in their tips than in the stems bearing them.

(3) Both junipers contain copper in amounts

TABLE 18.—*JUNIPERUS COMMUNIS* (DWARF OR COMMON JUNIPER)
Comparison between Tips and Stems Bearing Them
(Some 2nd year material included)

Sample No.	Copper		Zinc		Ash Per cent	
	Tips	Stems	Tips	Stems	Tips	Stems
48. 8	6 (140)	3 (60)	15 (310)	23 (450)	4.8	5.1
48.10	5 (140)	2 (60)	14 (390)	27 (860)	3.5	3.1
48.16	8 (120)	6 (120)	15 (330)	14 (270)	4.2	5.2
48.17	3 (60)	5 (85)	18 (380)	16 (280)	4.7	5.8
48.19	5 (140)	5 (95)	21 (600)	12 (230)	3.5	5.2
48.21	4 (90)	3 (55)	19 (460)	12 (220)	4.1	5.3
48.22	6 (150)	13 (230)	11 (290)	13 (230)	3.8	5.6
48.23	7 (200)	10 (110)	24 (690)	18 (210)	2.9	9.0
48.31	13 (390)	8 (200)	19 (570)	23 (590)	3.3	3.9
48.34	3 (80)	5 (120)	18 (470)	20 (410)	3.8	4.7
48.35	4 (120)	5 (130)	18 (550)	17 (380)	3.3	4.3
48.38	3 (100)	4 (75)	20 (670)	19 (350)	3.0	5.4
48.40	7 (220)	5 (130)	19 (600)	17 (440)	3.2	3.9
48.42	16 (430)	6 (120)	27 (710)	28 (580)	3.8	4.8
Average.....	6 (170)	6 (110)	18 (500)	18 (390)	3.7	5.1

TABLE 19.—*TSUGA HETEROPHYLLA* (HEMLOCK, "ALASKA PINE")
Comparison between 1st year Stems and 1st and 2nd year Needles

Sample No.	Copper		Zinc		Ash Per cent	
	Needles	Stems	Needles	Stems	Needles	Stems
49.Y. 17	2 (70)	7 (300)	19 (520)	38 (1700)	3.6	2.2
49.Y. 37*	2 (100)	3 (120)	10 (400)	61 (2400)	2.6	2.5
49.Y. 47*	3 (80)	5 (170)	20 (510)	83 (2800)	3.5	2.9
49.Y. 48*	3 (110)	6 (250)	12 (440)	53 (2300)	2.7	2.3
49.Y. 50*	3 (140)	8 (330)	11 (450)	63 (2600)	2.5	2.4
49.Y. 146	3 (120)	7 (330)	9 (390)	42 (1900)	2.4	2.2
49.Y. 149	3 (110)	7 (250)	12 (450)	46 (1600)	2.7	2.8
49.Y. 299	2 (90)	6 (210)	9 (420)	61 (2300)	2.2	2.7
49.Y. 323	2 (65)	9 (350)	9 (310)	53 (2200)	3.0	2.4
49.Y. 402*	2 (90)	5 (280)	15 (670)	48 (2700)	2.2	1.8
Average.....	2.5 (100)	6 (260)	13 (460)	55 (2250)	2.7	2.4

* Samples from areas now considered to be possibly zinc positive.

comparable with other genera of the Gymnospermae.

(4) The zinc content of both tips and stems is the lowest of any of the Gymnospermae we investigated.

(5) The response in both species of juniper to known copper mineralization is erratic; in a few samples it is marked but in other cases is not noticeable.

The junipers are not a genus particularly well suited for biogeochemical prospecting. It is difficult to select growth of a particular organ of a precise age from a juniper, and because of the very nature of its occurrence one would expect to have to be close to mineralization before excess metal would become available to a tree.

Bearing in mind the above we suggest that for tips and stems the following be considered normal:

	Copper	Zinc	Ash per cent	Cu: Zn
Tips.....	5(120) ± 50%	17(400) ± 30%	4.2	.30
Stems.... (Young)	5(100) ± 50%	15(300) ± 30%	4.8	.33

TABLE 20.—*TSUGA*

TSUGA HETEROPHYLLA (Hemlock, "Alaska Pine")

Comparison between Stems of Different Ages

Sample No.	Copper		Zinc		Ash Per cent	
	1st Year	2nd Year	1st Year	2nd Year	1st Yr.	2nd Yr.
49.Y. 17	7 (300)	6 (300)	38 (1700)	32 (1500)	2.2	2.0
49.Y. 37*	3 (120)	3 (130)	61 (2400)	51 (2100)	2.5	2.5
49.Y. 47*	5 (170)	6 (210)	83 (2800)	74 (2800)	2.9	2.7
49.Y. 48*	6 (250)	6 (190)	53 (2300)	68 (2300)	2.3	3.0
49.Y. 50*	8 (330)	9 (430)	63 (2600)	62 (3000)	2.4	2.1
49.Y. 146	7 (330)	7 (300)	42 (1900)	67 (2700)	2.2	2.5
49.Y. 149	7 (250)	4 (180)	46 (1600)	54 (2200)	2.8	2.4
49.Y. 299	6 (210)	4 (170)	61 (2300)	72 (2700)	2.7	2.4
49.Y. 323	9 (350)	7 (330)	53 (2200)	44 (2100)	2.4	2.1
49.Y. 402*	5 (280)	6 (330)	48 (2700)	56 (3200)	1.8	1.7
Average.....	6 (260)	6 (260)	55 (2250)	48 (2460)	2.4	2.3

(a). TSUGA HETEROPHYLLA (Hemlock)

Copper, Zinc, and Ash Content of Boughs, Britannia

(Less than 150 feet below copper plant)

Suite 46B Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
27	90 (2400)	200 (5300)	3.8	0.4
28	96 (3100)	198 (6400)	3.1	0.4
29	54 (1700)	37 (1200)	3.2	1.5
30	23 (880)	23 (880)	2.6	1.0
38	690 (19700)	1300 (37000)	3.5	0.5
39	150 (5600)	93 (3400)	2.7	1.5
40	190 (6100)	120 (3900)	3.1	1.6
41	295 (8400)	180 (5100)	3.5	1.6
44	190 (4600)	110 (2700)	4.1	1.8
45	80 (2600)	74 (2400)	3.1	1.1
Average.....	190 (5500)	230 (6800)	3.3	1.1†

(b). TSUGA HETEROPHYLLA (Hemlock)

Copper, Zinc, and Ash Content of Boughs, Britannia

(More than 150 feet below copper plant)

Suite 46B Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
31	31 (800)	32 (800)	3.9	1.0
32	54 (1500)	33 (890)	3.7	1.6
33	42 (970)	51 (1200)	4.3	0.7
34	36 (820)	49 (1100)	4.4	0.7
35	60 (1700)	58 (1700)	3.5	1.0
36	22 (840)	23 (880)	2.6	1.0
37	22 (650)	52 (1500)	3.4	0.4
46	110 (3200)	110 (3200)	3.4	1.0
47	78 (2700)	45 (1600)	2.9	1.7
48	23 (680)	53 (1600)	3.4	0.4
49	41 (1200)	32 (970)	3.3	1.3
50	9 (300)	32 (1100)	3.0	0.3
51	9 (400)	20 (900)	2.2	0.4
Average.....	41 (1200)	45 (1300)	3.4	0.9

* Samples from areas now considered to be possibly zinc positive.

† Cu:Zn ratio from averaged totals = 190/230 = 0.8

(c). TSUGA HETEROPHYLLA (Hemlock)

Copper, Zinc, and Ash Content of Boughs, Britannia
(Different drainage from that below copper plant)

Suite 46B Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
54	4 (170)	59 (2500)	2.4	0.07
55	5 (180)	64 (2400)	2.7	0.08
56	11 (340)	35 (1100)	3.2	0.30
57	13 (430)	34 (1100)	3.0	0.40
Average.....	8 (280)	48 (1800)	2.8	0.2

(d). TSUGA HETEROPHYLLA (Hemlock)

(Tips)

Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
49. 35	5 (190)	12 (430)	2.9	0.44
49. 48	8 (250)	20 (620)	3.1	0.41
49. 49	4 (180)	13 (620)	2.2	0.30
49. 52	6 (260)	21 (840)	2.5	0.31
49. 76	4 (160)	13 (530)	2.4	0.31
49. 78	5 (200)	19 (760)	2.4	0.27
49.108	5 (180)	17 (560)	3.0	0.32
49.112	6 (230)	14 (540)	2.6	0.43
49.113	5 (190)	12 (460)	2.6	0.42
Average.....	5 (200)	16 (600)	2.6	0.36

(e). TSUGA MERTENSIANA (Mountain Hemlock)

(Tips)

Sampl No.	Copper	Zinc	Ash Per cent	Cu:Zn
49.63	3 (120)	12 (450)	2.8	0.28
49.66	4 (190)	19 (880)	2.1	0.22
49.67	4 (170)	14 (560)	2.4	0.30
49.76	4 (160)	15 (520)	2.9	0.30
49.79	4 (190)	11 (550)	2.1	0.35
Average.....	4 (170)	14 (590)	2.5	0.29

Genus *Tsuga*

Two species of hemlock occur in the Pacific Northwest. They may be classed as tolerant trees because they can grow in the shade of, and in competition with, other trees. Both trees demand much moisture.

(a) *Tsuga heterophylla* (Rafinesque-Schmaltz) Sargent Hemlock

This species also is known as British Columbia hemlock and as Alaska pine. It is a large and relatively slow-growing tree and hence has never been a popular tree for biogeochemical investigations; the time consumed in collecting a satisfactory sample usually is longer than for other species or genera growing in the same area.

Hemlock occurs throughout the coastal and wet belts of southern Alaska, British Columbia, Washington, Idaho, and Oregon. All the samples in Tables 19 and 20 come from a wet belt in the southern interior of British Columbia, and possibly the results are not typical of this species elsewhere.

Tables 19 and 20 show that in many respects

it resembles Douglas fir. Hemlock needles are much lower in both copper and zinc than the stems which bear them, regardless of whether they are compared on the basis of ashed or dry-plant material. Similarly both hemlock and Douglas fir appear to have a higher ash content in their needles than in the stems bearing them.

Fortunately there seems to be no marked difference in the copper and zinc content of the first- and second-year stems. This may be important because it may simplify the problem of getting satisfactory samples where the growth is small.

Tentatively we suggest as normal for hemlock

	Copper	Zinc	Ash per cent	Cu: Zn
Needles..	3(100) ± 50%	13 (460) ± 30%	2.7	.23
Stems....	6(260) ± 50%	50(2100) ± 30%	2.4	.12
(One full year's growth)				

A slight change between the above figures and those in Tables 19 and 20 has been made because after the preliminary results were prepared drilling and development work showed, as one might have hoped, that some of the supposedly negative samples were positive.

We have already described three suites of specimens taken under abnormal conditions but under conditions which enable us to show the amounts of copper and zinc this species of tree can absorb without exhibiting any ill, or indeed any visible, effects (Warren and Delavault, 1949). Table 20 (a) shows samples taken from within 150 feet of the copper recovery plant (by precipitation on scrap iron). Table 20 (b) shows samples taken beyond 150 feet of the plant. Table 20 (c) shows samples taken near by but belonging to a different drainage area. The zinc anomalies decreased within short distances; the copper anomalies extend well beyond the 150-foot suite.

Again we must point out that all the specimens in Tables 20 (a), (b), and (c) are of boughs and that these boughs are normally much lower in metal content than are the twigs.

In the light of our present knowledge, the samples in Table 20 (c) suggest that there might be zinc and/or copper in the vicinity, but not in the quantities found near the copper plant.

During 1949 a number of samples of *Tsuga heterophylla* were collected in Tweedsmuir Park. Table 20 (d) represents a typical collection of normal tips.

(b) *Tsuga Mertensiana* (Bong) Carrière Mountain Hemlock

This species is also called black hemlock. It is found throughout much the same range as *Tsuga heterophylla* but at higher altitudes, usually near timber line at altitudes of 2500 to 6000 feet, but it descends nearly to sea level in the more exposed and wetter situations in the north.

No satisfactory positive and negative collections are available for comparison, but Table 20 (e) of normal tips taken from the same general area as the samples in Table 20 (d) suggests that the two members of the genus *Tsuga* behave similarly.

Genus *Abies*

The *Abies* or true firs are widely distributed in the Pacific Northwest where three species—*Abies lasiocarpa*, *Abies amabilis*, and *Abies grandis*—are known.

By far the most important of the true firs is *Abies lasiocarpa*, and fortunately we have some data about this species. Critical data about the other two species are sadly lacking.

(a) *Abies lasiocarpa* (Hooker) Nuttall Alpine Fir

This species has many local names; it is variously known as mountain fir, white balsam, white fir, Western balsam fir, caribou fir, and Rocky Mountain fir. Except for a few areas in the vicinity of the southern coastal region this species is widespread throughout the whole of the Pacific Northwest especially at altitudes between 2000 and 7000 feet. Like many other species it thrives best on moist well-drained loam soils, but it can tolerate quite dry sites.

Tables 21 and 21 (a) illustrate some of the information we have collected dealing with this species. We have no comparable results for needles, but available data suggest that needles probably are a little lower in copper and in zinc than are the stems which bear them.

Possibly the following represents normal contents for this species:

	Copper	Zinc	Ash per cent	Cu: Zn
Needles..	5(180) ± 50%	30(1080) ± 30%	3.6	.17
Tips..... (Buds)	6(220) ± 50%	32(1200) ± 30%	3.6	.19
Stems.... (3 year's growth)	6(200) ± 50%	45(1500) ± 30%	3.3	.13

The discrepancy between the ash content of the above and that given in Tables 21 and 21 (a) results from our attempting to reconcile two sets of samples taken over 300 miles apart with widely differing climates. The trees have widely different ash contents. We have chosen the more southerly collection as more likely to represent the ash content of the area as a whole.

One feature which might prove useful is that the tips tend to show less variations from the normal than the tips of some other species, notably *Picea Engelmanni*. If this feature be substantiated by other workers it would be a most useful fact. Collecting tips of this species is a delightfully simple job compared with collecting stems of one year's growth!

We have no suites strongly positive of either zinc or copper mineralization. We do have a number of specimens from weakly mineralized copper areas shown in Table 21 (b).

Suites of tips from areas comparatively as well mineralized with zinc as those in Table 21 (b) were in copper gave no significant results. This may indicate that zinc is less easy to detect by means of this species. It may indicate, and this appears more probable, that we took our samples too close to the main water table of the

TABLE 21.—*ABIES*

ABIES LASIOCARPA (Rocky Mountain Fir, Western Balsam Fir)

Comparison between Stems of Different Ages

Sample No.	Copper		Zinc		Ash Per cent	
	1st Year	2nd Year	1st Yr.	2nd Yr.	1st Year	2nd Year
49.Y. 53	7 (170)	7 (200)	69 (1700)	76 (2300)	3.9	3.3
49.Y. 56	5 (150)	4 (130)	52 (1400)	58 (1800)	3.6	3.3
49.Y. 58	4 (120)	8 (190)	67 (2000)	77 (1900)	3.3	4.1
49.Y. 78	5 (140)	5 (130)	30 (830)	31 (820)	3.6	3.7
49.Y. 95	6 (150)	5 (140)	43 (1100)	45 (1300)	3.9	3.5
49.Y. 117	8 (240)	8 (260)	55 (1600)	49 (1600)	3.4	3.1
49.Y. 120	6 (140)	7 (180)	32 (830)	51 (1400)	3.9	3.7
49.Y. 123	10 (250)	8 (210)	50 (1200)	48 (1300)	4.2	3.8
49.Y. 170	4 (120)	6 (190)	47 (1400)	55 (1700)	3.4	3.1
49.Y. 183	10 (280)	6 (190)	42 (1100)	35 (1000)	3.7	3.5
Average.....	6 (180)	6 (180)	49 (1320)	52 (1510)	3.7	3.5

(a). ABIES LASIOCARPA (Alpine Fir)

Comparison between Tips (Buds) and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Tips	Stems	Tips	Stems	Tips	Stems
49.81	6 (250)	7 (220)	35 (1300)	48 (1400)	2.6	3.3
49.92	4 (170)	4 (170)	21 (890)	32 (1200)	2.3	2.7
49.94	4 (170)	6 (250)	30 (1300)	41 (1600)	2.4	2.6
49.96	6 (210)	8 (280)	32 (1100)	47 (1700)	2.8	2.8
49.97	9 (300)	Lost	39 (1300)	46 (1600)	3.0	2.9
Average.....	6 (220)	6 (230)	31 (1200)	43 (1500)	2.6	2.9

(b). ABIES LASIOCARPA (Alpine Fir)
Copper, Zinc, and Ash Content of some Positive (Copper) Tips

Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
49.45	10 (340)	36 (1200)	3.1	0.29
49.71	10 (370)	27 (950)	2.8	0.39
49.72	13 (430)	40 (1300)	3.1	0.34
49.73	9 (310)	30 (1100)	2.9	0.30
49.80	8 (310)	35 (1400)	2.5	0.23
Average.....	10 (350)	34 (1200)	2.9	0.31

(c). ABIES AMABILIS (Balsam fir)
Comparison between Tips (Buds) and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Tips	Stems	Tips	Stems	Tips	Stems
49.70	8 (310)	6 (280)	21 (810)	31 (1400)	2.6	2.3
49.76	6 (240)	6 (260)	27 (1000)	38 (1500)	2.7	2.5
49.78	5 (250)	5 (220)	21 (1100)	32 (1400)	2.0	2.3
49.81	4 (200)	4 (180)	20 (890)	29 (1200)	2.2	2.5
49.92	5 (190)	6 (210)	26 (730)	31 (1100)	2.6	2.7
Average.....	6 (240)	5 (230)	23 (910)	32 (1300)	2.4	2.5

(d). ABIES AMABILIS (Balsam fir)
Copper, Zinc, and Ash Content of some Positive (Copper) Tips

Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
49. 43	8 (260)	34 (1000)	3.3	0.25
49. 61	6 (380)	14 (880)	1.6	0.43
49. 65	8 (260)	17 (570)	3.0	0.46
49. 71	9 (310)	25 (810)	3.1	0.38
49. 72	13 (430)	31 (1000)	3.0	0.42
49. 80	8 (310)	35 (1400)	2.5	0.23
49.106	10 (360)	34 (1200)	2.7	0.29
Average.....	9 (330)	27 (980)	2.7	0.35

large lake near where many of the samples were taken.

(b) *Abies amabilis* (Douglas) Forbes
Amabilis Fir

Abies amabilis is also known as Red fir, lovely fir, alpine fir, larch, silver fir, white fir, balsam fir, and Cascade fir. This species extends from Oregon to southern Alaska in the coastal region. It is mostly confined to the western slope of the Coast Range and Vancouver Island but is found on the eastern margin of the Coast Range. *Abies amabilis* favors lower slopes and benches and grows on well-drained, gravelly sand, or sandy loams.

We know little about this species except that wherever *Abies amabilis* and *Abies lasiocarpa* are found in the same vicinity analyses have revealed a great similarity in the mineral content of the two species.

Table 21 (c) compares tips and stems bearing them. Table 21 (d) gives the ash contents of some weakly positive copper tips. The similarity

between the results appearing in these tables and those given in Tables 21 (a) and (b) is so striking that it seems safe to assume that these two species can be used interchangeably in biogeochemistry.

TABLE 22.—*SALIX* SP. (WILLOW)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
48. 1	15 (160)	17 (300)	200 (1800)	164 (2800)	9.2	5.7
48. 8	16 (250)	13 (160)	90 (1300)	92 (1100)	6.6	8.3
48.10	11 (150)	4 (80)	145 (2000)	115 (2300)	7.3	4.8
48.13	10 (150)	15 (310)	84 (1200)	102 (2100)	6.8	4.8
49. 1	11 (190)	14 (180)	37 (630)	45 (590)	5.9	7.6
49. 2	12 (210)	12 (200)	74 (1300)	108 (1800)	5.7	6.1
49. 3	11 (160)	9 (110)	54 (790)	38 (430)	6.8	8.8
49.10	11 (130)	19 (400)	224 (2600)	156 (3300)	8.6	4.7
49.11	14 (170)	11 (140)	110 (1300)	68 (820)	8.2	8.3
49.17	13 (130)	17 (220)	80 (800)	114 (1500)	10.0	7.6
49.19	13 (190)	14 (270)	256 (3600)	174 (3300)	7.2	5.2
48.U.501	7 (230)	6 (310)	103 (4100)	200 (10000)	3.2	1.9
48.U.502	7 (160)	7 (250)	120 (2900)	140 (5400)	4.1	2.6
Average.....	12 (180)	12 (230)	120 (1870)	120 (2730)	6.9	5.9

In our previous publications we have suggested that the Cu:Zn ratio always be noted. In Tables 21 (b) and (d) the Cu:Zn ratio in many cases suggests anomalous conditions even where the absolute amount of copper does not.

(c) *Abies grandis* (Douglas) Lindley
Grand Fir

Abies grandis has a host of names including Lowland fir, white fir, western balsam, silver fir, larch, giant fir, lowland white fir. It is more limited in its range than the two species described above and is found only in the coastal and interior wet belts of southern British Columbia, Washington, and Oregon, and in the wet belts of Idaho and western Montana. It prefers deep, moist, but well-drained alluvial soils and may be found chiefly along streams and lower slopes.

No systematic collections of this species have been made, but the few analyses we have suggested that it conforms with the general pattern exhibited by *Abies lasiocarpa* and *Abies amabilis*.

B. THE ANGIOSPERMAE

General Considerations

We have not been able to investigate the Angiospermae as thoroughly as we have the Gymnospermae, largely because fewer positive suites were available for collection during our preliminary investigations.

One of the most important problems facing one wishing to use the Angiospermae in biogeochemical work is the difficulty of identifying the species of tree at the time of sampling. Several dozen varieties of *Salix* exist in the Pacific Northwest, and it is virtually impossible to identify them unless fruit is available, which is during a relatively short period each year. Further work may show that species identification will not be of vital importance, or perhaps important only in the case of genera possessing a wide variety of species such as the willows.

If such genera as *Salix* be excluded, different species within each genus appear to have similar response to mineralization. Such is not the case with the different families of Angiospermae any more than it is with the families of the Gymnospermae. The genus *Betula* and the genus *Alnus*, although both Betulaceae, appear to have little in common with respect to their selective absorption of trace elements.

Recent work by Lakin and Stevens (1949)

TABLE 23.—*SALIX* SP. (WILLOW)
Comparison between Stems of Different Ages

Sample No.	Copper			Zinc			Ash Per cent		
	1st year	2nd year	3rd year	1st year	2nd year	3rd year	1st Yr.	2nd Yr.	3rd Yr.
48.14	16 (260)	10 (380)		70 (1100)	48 (1800)		6.1	2.6	
48.15	9 (210)	12 (470)		60 (1400)	74 (2900)		4.2	2.5	
48.16	8 (150)	5 (140)		72 (1300)	50 (1400)		5.4	3.4	
48.17	8 (240)	7 (260)		109 (3300)	108 (4500)		3.3	2.7	
48.19	10 (250)	6 (220)		66 (1600)	50 (1800)		4.0	2.7	
49. 1(a)	14 (180)	10 (300)		45 (590)	33 (930)		7.6	3.5	
49. 1(b)	9 (96)	5 (210)		33 (350)	40 (1500)		9.4	2.6	
49. 2	12 (200)	7 (250)	6 (220)	108 (1800)	75 (2500)	72 (2600)	6.1	3.0	2.8
49. 3	9 (110)	5 (190)	3 (140)	38 (430)	33 (1200)	26 (1200)	8.8	2.6	2.2
49. 4	10 (260)	7 (260)	5 (250)	28 (740)	24 (890)	22 (1100)	3.8	2.7	2.0
49. 5	7 (180)	5 (230)		43 (1100)	41 (1700)		3.8	2.4	
49. 6	20 (480)	8 (380)		130 (3100)	69 (3300)		4.2	2.1	
49. 7	4 (67)	8 (390)		148 (2500)	80 (3900)		6.0	2.0	
49. 8	13 (210)	5 (210)		118 (1900)	98 (3800)		6.3	2.6	
49. 9	6 (120)	5 (220)		144 (2900)	112 (4900)		5.0	2.3	
49.10	19 (400)	7 (340)		156 (3300)	109 (5400)		4.7	2.0	
49.11	11 (140)	8 (230)		68 (820)	48 (1300)		8.3	3.6	
49.17	17 (220)	9 (240)		114 (1500)	88 (2200)		7.6	4.0	
49.19	14 (270)	8 (320)		174 (3300)	144 (5800)		5.2	2.5	
49.20	12 (280)	6 (300)		114 (2500)	80 (3700))		4.5	2.1	
49.27	8 (240)	7 (290)		78 (2200)	105 (4300)		3.5	2.4	
48.U.501	6 (310)	7 (420)		200 (10000)	190 (12000)		1.9	1.5	
48.U.502	7 (250)	6 (330)		140 (5400)	130 (6600)		2.6	1.9	
Average.....	11 (220)	7 (290)	5 (200)	98 (2310)	80 (3400)	40 (1630)	5.3	2.6	2.3

(a). SALIX SP. (Willow)

Comparison between the Copper, Zinc, and Ash Contents of Willow Leaves, Sullivan Area, B. C.

Sample No.	Copper	Zinc	Ash %	Cu:Zn
46.2	12 (200)	300 (4800)	6.2	0.040
46.14 Group 3	12 (280)	460 (11000)	4.3	0.027
46.19	2 (30)	460 (7600)	6.0	0.004
46.21	8 (140)	640 (10500)	6.1	0.013
Average.....	8 (160)	460 (8500)	5.6	0.020
46.9	8 (250)	800 (24000)	3.4	0.01
46.11	9 (190)	900 (18000)	4.9	0.01
46.13	11 (170)	370 (6000)	6.2	0.03
46.17	7 (110)	160 (2600)	6.1	0.04
46.23 Group 4(a)	6 (120)	380 (7400)	5.2	0.02
46.24	2 (30)	1100 (16000)	6.2	0.02
46.27	9 (150)	740 (12000)	3.1	0.01
46.29	8 (100)	860 (11000)	7.6	0.01
46.42	5 (110)	170 (3600)	4.9	0.03
Average.....	7 (140)	610 (11200)	5.3	0.02
46.31	6 (100)	1000 (18000)	5.6	0.01
46.32	8 (140)	860 (15000)	5.6	0.01
46.36 Group 4(b)	9 (160)	800 (13000)	6.0	0.01
46.38	9 (170)	800 (8800)	5.6	0.02
46.40	7 (130)	500 (9000)	5.6	0.01
Average.....	8 (140)	730 (12800)	5.7	0.01

(b). SALIX SP. (Willow)

Comparison between Copper, Zinc, and Ash Contents of young Willow Stems, Tweedsmuir Park Area, B.C.

Sample No.	Copper	Zinc	Ash %	Cu:Zn
49.21*	21 (460)	104 (2300)	4.6	0.20
49.22	6 (170)	33 (960)	3.5	0.18
49.23	15 (450)	65 (2000)	3.3	0.23
49.29*	26 (460)	110 (1900)	5.7	0.24
49.30*	28 (620)	204 (4400)	4.6	0.14
49.45	17 (370)	80 (1700)	4.7	0.22
49.46*	14 (310)	168 (3600)	4.7	0.08
49.61	17 (390)	56 (1300)	4.4	0.30
49.72	24 (590)	66 (1600)	4.1	0.36
49.98*	15 (480)	140 (4400)	3.2	0.11
Average....	18 (430)	103 (2400)	4.3	0.20

* This sample or the suite from this area may be zinc positive.

has promoted interest in any attempt to assess the relative reliability of stems and leaves for biogeochemical work. If stems are more, or even as, reliable as leaves the possibility of collecting and carrying out biogeochemical investigations on frozen and snow-covered ground will be greatly enhanced.

Tables 22–48 deal chiefly with comparisons between the copper, zinc, and ash content of leaves and stems bearing them, and of stems of different ages.

Family SALICACEAE

Genus *Salix*

Although *Salix amygdaloides* and *Salix sitchensis* have probably provided us with more samples than any other species we have had to lump all species together in a general presentation of this genus.

In Table 22 the copper, zinc, and ash contents of a collection of negative willows are compared. Wide and at first sight disheartening variations are immediately apparent. Similarly in Table 23 we find many deviations from any general rule. However, the following general relationships appear to justify their assumption if only as working hypotheses:

In general the copper, zinc, and particularly the ash contents of twigs decrease markedly during the first 3 years. Resulting in part from the decreased ash content the amount of both copper and zinc in ash increases in the second year over the first year. The copper and zinc do not vary proportionately, and thus we may infer that the two elements act independently.

There is no marked difference between the copper and the zinc content of dry plant material, but the stems tend to carry more copper and more zinc in their ash than do the leaves. Leaves average higher in ash than stems.

On the assumption that 1 full year's growth is more apt to reflect the mineral content of the soil than is the growth of a shorter period we now suggest that young leaves or stems are less likely to be accurate guides than stems of 1 full year's growth.

As a working hypothesis we suggest the following as normal for *Salix* sp.:

	Copper	Zinc	Ash per cent	Cu: Zn
Leaves...	12(180) ± 50%	120(1800) ± 30%	6.8	.10
Young stems..	12(220) ± 50%	120(2200) ± 30%	5.6	.10
Stems.... (1 year's growth)	7(270) ± 50%	80(3100) ± 30%	2.6	.09

We have three groups of zinc-positive willow leaves, all from the Sullivan area (Table 23 (a)). The details concerning these samples have already been given (Warren and Delavault, 1949).

We have no samples of willow from strongly copper-positive areas. Some results recorded in Table 23 are from areas which contain copper minerals but which show nothing approaching ore. However, we do have some young twigs taken during an exploration survey. These positive areas do not contain any mineralization which may be considered as ore now (1950), but they contain enough mineralization for future consideration. Table 23 (b) gives these results.

If Tables 22 and 23 are examined it will probably be agreed that willows leaves or stems may be useful in biogeochemical work. They have greater ranges for their normal content than many other trees, but in the future when the reasons for these variations are better understood we expect that willows will be widely used by biogeochemists. Its relatively rapid growth, its widespread root systems, the ease with which samples can be collected, and its relatively high mineral content all are points in its favor. Willow is one of the genera which should repay further and more exhaustive studies.

Genus *Populus*

Two species of this genus are widespread in the Pacific Northwest—*Populus tremuloides* and *Populus trichocarpa*. We have examined many samples of both, and *Populus tremuloides* in particular shows promise of being of use to biogeochemists.

(a) *Populus tremuloides* Michaux Trembling Aspen, Poplar

This species also is known as asp, quaking asp, aspen poplar, white poplar, popple, and

smooth-barked poplar. Except for the coastal areas of British Columbia this species is common in most parts of the Pacific Northwest that provide reasonable growing conditions. It grows best on well-drained loam but it does grow on a wide variety of soils. However, it is intolerant of dense shade. It multiplies quickly and easily by means of root suckers and it may come in rapidly on new burns.

Populus tremuloides deserves extended study. It is well known, it is widespread, and it can be recognized and collected in winter almost as readily as in summer. Furthermore except for the willows and birches it is apparently the species most susceptible to zinc. Boron and zinc deficiencies are known to occur in the same area, and it may be that the rotten and evil-smelling cores of many individual poplars are caused by a deficiency of one or both of these elements.

Until recently we have considered this species to possess one great handicap for the biogeochemist: it is, in some areas, much slower growing than many of the species associated with it. Fortunately, as can be seen by Table 25, there appears to be much less difference in the copper, zinc, and ash content of first- and second-year stems of poplar than there is in the first- and second-year stems of most of the other common species. If this be established as a fact then poplar stems of 2 years' growth may be collected as general practice. Because the annular rings marking growth are conspicuous it should be easy to collect uniform samples. Furthermore, poplar ash may carry close to 1 p.p.m. of gold, an amount exceeded only by willow ash. We have no collections of poplar taken over strongly positive areas of either copper or zinc. However, occasional specimens taken from weakly positive areas suggest that poplar may respond to either element.

Tables 24 and 25 indicate the mineral and ash content of leaves and variously aged stems of this species. The decreasing copper and zinc content of dry-plant material and the increasing ash content of copper and zinc is to be noted.

TABLE 24.—*POPULUS TREMULOIDES* (TREMBLING ASPEN, POPLAR)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
48.10	21 (330)	7 (140)	64 (1000)	57 (1100)	6.5	5.1
49. 2	18 (270)	11 (200)	130 (1900)	128 (2200)	6.8	5.8
49. 3	14 (180)	10 (160)	168 (2100)	133 (2200)	7.8	6.1
49.11	11 (160)	11 (190)	141 (2000)	97 (1700)	7.0	5.7
49.13	17 (190)	16 (210)	99 (1100)	87 (1100)	9.1	7.7
49.17	9 (150)	12 (270)	121 (2000)	112 (2500)	6.1	4.5
Average.....	15 (210)	11 (195)	120 (1680)	102 (1800)	7.2	5.8

As a working hypothesis we suggest the following as normals:

	Copper	Zinc	Ash per cent	Cu: Zn
Leaves.....	15(230) ± 50%	120(1800) ± 30%	6.5 ± 50%	.12
Stems (1st Yr.)....	12(220) ± 50%	94(1700) ± 30%	5.5 ± 50%	.13
Stems (2nd Yr.)....	9(250) ± 50%	60(1700) ± 30%	3.6 ± 50%	.15

(b) *Populus trichocarpa* Torrey and Gray
Black Cottonwood

Also known as balsam cottonwood, balm cottonwood, western balm, western balsam, and western balsam poplar, this species is found throughout the Pacific Northwest in river-bottom lands. It is very intolerant of shade and normally can survive in mixed stands only if its more rapid growth enables it to keep ahead of other species. The authors have found it growing at the top of a 7000-foot ridge in southern British Columbia.

TABLE 25.—*POPULUS TREMULOIDES* (TREMBLING ASPEN, POPULAR)

Comparison between Stems of Different Ages

Sample No.	Copper			Zinc			Ash Per cent		
	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.
48.16	11 (230)	6 (180)		55 (1100)	49 (1300)		4.8	3.7	
48.18	7 (130)	7 (180)		88 (1700)	74 (1900)		5.3	3.8	
48.19	11 (220)	11 (370)		49 (990)	51 (1700)		5.0	3.0	
49. 1	14 (210)	13 (240)	14 (220)	61 (910)	45 (820)	46 (720)	6.7	5.5	6.4
49. 2	11 (200)	7 (210)	6 (170)	128 (2200)	80 (2400)	83 (1700)	5.8	3.3	3.9
49. 3	10 (160)	12 (290)	12 (320)	133 (2200)	82 (1900)	76 (1900)	6.1	4.2	3.9
49. 5	12 (250)	11 (400)		71 (1400)	57 (2100)		5.1	2.7	
49.11	11 (190)	9 (240)		97 (1700)	55 (1500)		5.7	3.7	
49.13	16 (210)	7 (220)	8 (250)	87 (1100)	44 (1300)	47 (1400)	7.7	3.4	
49.14	16 (300)	13 (400)		93 (1700)	57 (1700)		5.5	3.4	3.4
49.17	12 (270)	8 (230)		112 (2500)	59 (1700)		4.5	3.4	
49.27	8 (270)	6 (230)		62 (1900)	51 (1900)		3.2	2.6	
Average.....	12 (220)	9 (270)	10 (240)	86 (1620)	59 (1680)	63 (1430)	5.4	3.6	4.4

The data in Tables 26 and 27 are far too skimpy to permit any satisfactory generalizations, and as we have no samples of satisfactory copper or zinc anomalous material we hesitate to draw any conclusions about normals. However, a much greater number of first-year stems were collected during the summer of 1949, and on the basis of the results obtained from these samples it is suggested that the copper and zinc contents of some of the samples in Tables 26 and 27 are lower than average. The normal copper and zinc content of *Populus trichocarpa*, therefore, probably more closely approximates that of *Populus tremuloides* than the tables indicate.

All the specimens numbered U come from Ungava where they were collected by Dr. J. Rousseau.

TABLE 26.—*POPULUS TRICHOCARPA* (COTTONWOOD)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
48.13	12 (110)	6 (80)	160 (1300)	65 (910)	12.2	7.1
49.11	10 (120)	11 (130)	108 (1300)	86 (1000)	8.1	8.6
Average.....	11 (115)	8 (105)	130 (1300)	75 (950)	10.1	7.8

TABLE 27.—*POPULUS TRICHOCARPA* (COTTONWOOD)
Comparison between Stems of Different Ages

Sample No.	Copper		Zinc		Ash Per cent	
	1st Yr.	2nd Yr.	1st Yr.	2nd Yr.	1st Yr.	2nd Yr.
48.17	11 (190)	9 (210)	69 (1200)	68 (1600)	5.5	4.2
49. 4	6 (95)	4 (80)	31 (480)	40 (710)	6.4	5.6
49.11	11 (130)	3 (70)	86 (1000)	68 (1600)	8.6	4.2
49.20	7 (130)	4 (170)	120 (2100)	90 (3800)	5.8	2.4
49.54	18 (260)	7 (240)	110 (1600)	63 (2000)	7.0	3.1
Average.....	11 (160)	5 (150)	80 (1280)	66 (1940)	6.7	3.9

Family BETULACEAE

Genus *Betula*

Rather than omit this important genus we present the available information in the belief that the results will be of value, especially when we can say that all our other results substantiate the general conclusions arrived at in Tables 28 to 33.

(a) *Betula glandulosa* (Michaux) Scrub Birch

This species, which incidentally is found in suitable habitats all across Canada, is widespread throughout the Pacific Northwest especially in subalpine meadows where it is in many instances the only deciduous species of shrub found.

Usually it is of slow growth and normally would not be favored by a biogeochemist. However, this species seems to be peculiarly susceptible to zinc, and as such it may prove to be very useful in exploration biogeochemistry.

Tables 28 and 29 indicate clearly the most interesting features of this species. All the samples are from areas believed to be copper and zinc negative, consequently it would be unwise to hazard a guess as to what might be expected in anomalous samples. It seems hard to believe that sample 48.U 918 has a normal zinc content and possibly that area should be investigated!

If the ash content be considered it may be noted that for both copper and zinc the stems are richer than the leaves they bear, and the second-year stems are richer than the first-year stems.

The two samples presented in Tables 32 and 33 would not be worth mentioning were it not that we had more data which indicate the tendency of this species to be high in zinc. These data are not yet available for release.

TABLE 28.—*BETULA GLANDULOSA* (SCRUB BIRCH)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
49.10	10 (210)	9 (330)	216 (4600)	165 (5700)	4.7	2.9
49.19	12 (240)	11 (430)	240 (4600)	155 (5700)	5.2	2.7
48.U. 499	7 (270)	7 (440)	65 (2500)	85 (5300)	2.6	1.6
48.U. 918	8 (260)	6 (370)	216 (7000)	230 (14000)	3.1	1.6
48.U.1077	7 (190)	7 (460)	140 (3800)	100 (7100)	3.7	1.4
48.U.1457	16 (570)	9 (640)	160 (5900)	36 (2600)	2.7	1.4
48.U.1565	9 (270)	7 (390)	158 (5100)	148 (8200)	3.1	1.8
Average.....	10 (290)	8 (440)	170 (4800)	130 (6940)	3.6	1.9

(b) *Betula occidentalis* Hooker Red Birch

This species is widespread in the Pacific Northwest east of the Coast Range Mountains and south of headwaters of the Fraser and Peace rivers.

It is also called mountain birch, black birch, and water birch and has also gone by the scientific name of *Betula fontinalis* (Sargent).

Betula occidentalis is chiefly found on moist soils along streams and around springs. It may be found in pure stands, but it is more often mixed with alders, cottonwoods, and willows.

The scanty evidence presented in Tables 30 and 31 indicate that *Betula occidentalis* like *Betula glandulosa* has a liking for zinc and tends to have a normal zinc content higher than that found in species of any other genus.

(c) *Betula papyrifera* (Marshall) Western White Birch

This species is also known as birch, silver birch, western birch, British Columbia birch, black birch, and white birch. The name "Western White" is misleading as the tree is also found in Eastern Canada. In Canada it occurs only in southern British Columbia but extends southward into the neighboring States.

Genus *Alnus*

In the Pacific Northwest four species of this important genus are of interest to biogeochemists—*Alnus sinuata*, *Alnus rubra*, *Alnus rhombifolia*, and *Alnus tenuifolia*. We have concentrated on *Alnus sinuata*. However, we have collected enough data to enable us to conclude that the other alders have the same biogeochemical characteristics as *Alnus sinuata*.

(a) *Alnus sinuata* (Regel) Rydberg Sitka Alder

This species is also known as green alder, northern alder, and mountain alder; furthermore, it has been described by the scientific name of *Alnus sitchensis* (Regel) Sargent.

Probably this will prove to be one of our more useful trees from the point of view of the biogeochemist. It extends from the Arctic Circle to California and from the Pacific Coast to the Rocky Mountains. It favors moist bottoms and marshy flats and in places forms a formidable obstacle to those who attempt to penetrate slide areas in the Coast Range Mountains.

Tables 34 and 35 represent all the information available to use on leaves and twigs of various species.

TABLE 29.—*BETULA GLANDULOSA* (SCRUB BIRCH)

Comparison between Stems of Various Ages

Sample No.	Copper			Zinc			Ash Per cent		
	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.
49. 6	10 (350)	5 (320)		160 (5300)	139 (8600)		3.0	1.5	
49. 9	8 (250)	13 (760)		188 (5600)	180 (11000)		3.3	1.7	
49.10	9 (330)	5 (330)		165 (5700)	158 (11000)		2.9	1.5	
49.16	4 (120)	6 (400)		162 (5200)	110 (7300)		3.1	1.5	
49.17	12 (410)	7 (440)		116 (3900)	88 (5200)		3.0	1.7	
49.19	11 (430)	7 (520)	6 (480)	155 (5700)	144 (10000)	155 (12000)	2.7	1.4	1.2
48.U. 499	7 (440)	8 (530)		85 (5300)	103 (6900)		1.6	1.5	
48.U. 918	6 (370)	5 (440)		230 (14000)	390 (23000)		1.6	1.2	
48.U.1077	7 (460)	5 (500)		100 (7100)	130 (13000)		1.4	1.0	
48.U.1457	9 (640)	5 (550)		36 (2600)	140 (14000)		1.4	1.0	
48.U.1565	7 (390)	6 (480)		148 (8200)	164 (12100)		1.8	1.3	
Average.....	8 (380)	7 (480)	6 (480)	140 (6240)	160 (11100)	155 (12000)	2.3	1.4	1.2

The main conclusions we believe tenable are: alders are readily susceptible to copper—witness sample 48.19—and somewhat less susceptible to zinc than the genera *Populus*, *Salix*, or *Betula*. In a previous paper (Warren and Delavault, 1948) a comparison was made between the zinc content of twigs of *Alnus rubra* and *Salix* sp. growing on identical ground. In all cases the zinc content of *Alnus rubra* was well below that of the willow.

During the 1949 field season nearly 100 samples of tips of young stems of *Alnus sitchensis* were collected, and the average for these young tips was as follows:

Copper	Zinc	Ash per cent	Cu:Zn
~~1st yr tips 18(c)± 5%~~	~~30(zinc)± 5%~~	~~2%~~	~~.30~~

We stated (Warren, Delavault, and Irish, 1949) that on the basis of the evidence available for *Alnus rubra* a copper content of more than 10 p.p.m. dry-plant material and a copper to zinc ratio of greater than .40 might be considered anomalous. All this indicates that in general alders are relatively more responsive to copper than they are to zinc. Originally we considered that this zinc deficiency might be caused not by the tree but by the soil from which the tree was feeding. However, the fact that willows growing in close proximity to the alders consistently carried greater quantities of zinc suggests that the alders do not favor zinc.

During the 1949 field season a large number of young stems of *Alnus sitchensis* were collected, and a few samples were from weakly positive zinc and copper sites. A few of these results

TABLE 30.—*BETULA OCCIDENTALIS* (MOUNTAIN, RED, OR WATER BIRCH)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
48.2	15 (270)	4 (85)	34 (610)	23 (490)	5.6	4.7
49.2	8 (110)	8 (120)	120 (1600)	130 (2000)	7.6	6.6
49.3	11 (150)	15 (170)	127 (1800)	168 (2000)	7.1	8.6
Average....	11 (180)	9 (125)	94 (1340)	110 (1500)	6.8	6.6

TABLE 31.—*BETULA OCCIDENTALIS* (MOUNTAIN, RED, OR WATER BIRCH)
Comparison between Stems of Different Ages

Sample No.	Copper			Zinc			Ash, Per cent		
	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.
49.2	8 (120)	6 (210)		130 (2000)	124 (4200)		6.6	2.9	
49.3	15 (170)	6 (310)	2 (150)	168 (2000)	75 (3600)	46 (2700)	8.6	2.1	1.7
Average...	11 (145)	6 (260)	2 (150)	150 (2000)	100 (3900)	46 (2700)	7.6	2.5	1.7

TABLE 32.—*BETULA PAPYRIFERA* (SILVER OR WESTERN WHITE BIRCH)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
48.10	12 (170)	6 (140)	213 (3000)	137 (3300)	7.0	4.2

are given in tables 35(a), (b), and (c).

TABLE 33.—*BETULA PAPYRIFERA* (SILVER OR WESTERN WHITE BIRCH)

Comparison between Stems of Different Ages

Sample No.	Copper		Zinc		Ash Per cent	
	1st Yr.	2nd Yr.	1st Yr.	2nd Yr.	1st Yr.	2nd Yr.
49.2	22 (260)	11 (410)	104 (1200)	65 (2500)	8.4	2.8

TABLE 34.—*ALNUS SINUATA* (GREEN, MOUNTAIN, OR SITKA ALDER)

Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
49.11	13 (180)	12 (250)	34 (470)	36 (700)	7.3	5.1

TABLE 35.—*ALNUS SINUATA* (GREEN, MOUNTAIN, OR SITKA ALDER)

Comparison between Stems of Different Ages

Sample No.	Copper		Zinc		Ash Per cent	
	1st Yr.	2nd Yr.	1st Yr.	2nd Yr.	1st Yr.	2nd Yr.
48.18	7 (200)	5 (200)	27 (700)	28 (1100)	3.5	2.4
48.19*	10 (300)	9 (500)	23 (690)	23 (1300)	3.3	1.8
49.11	15 (250)	5 (200)	36 (700)	18 (670)	5.1	2.7
49.20	8 (200)	7 (260)	24 (570)	38 (1400)	4.2	2.7
49.27	5 (160)	5 (190)	24 (700)	24 (910)	3.4	2.6
49.54	7 (190)	5 (200)	30 (770)	17 (690)	3.9	2.4
Average.....	9 (220)	6 (260)	27 (690)	25 (1010)	3.9	2.4

(a). ALNUS SINUATA (Sitka Alder)

Copper, Zinc and Ash Content of some Normal Young Stems

Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
49.20	8 (200)	24 (570)	4.2	0.35
49.24	10 (370)	21 (780)	2.7	0.48
49.25	13 (400)	30 (870)	3.4	0.46
49.26	3 (100)	29 (940)	3.1	0.10
49.27	5 (160)	24 (700)	3.4	0.23
49.31	14 (370)	32 (810)	3.9	0.45
49.32	8 (230)	28 (800)	3.5	0.29
49.33	9 (300)	36 (870)	3.0	0.35
49.60	9 (300)	25 (780)	3.2	0.38
49.75	7 (210)	20 (570)	3.5	0.38
Average.....	9 (260)	27 (770)	3.4	0.35

* Sample known to be copper positive.

(b). ALNUS SINUATA (Sitka Alder)

Copper, Zinc, and Ash Content of some Weakly Copper Anomalous Young Stems

Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
49.22	10 (380)	31 (1100)	2.7	0.33
49.23	11 (500)	33 (1500)	2.2	0.33
49.36	13 (330)	27 (680)	4.0	0.48
49.37	13 (340)	32 (840)	3.8	0.41
49.39	12 (460)	34 (1300)	2.7	0.37
49.40	24 (630)	37 (950)	3.9	0.66
49.43	10 (300)	36 (1200)	3.3	0.26
49.45	12 (390)	30 (940)	3.2	0.42
49.61	10 (580)	33 (1800)	1.8	0.32
49.62	15 (520)	44 (1500)	3.0	0.35
Average.....	13 (440)	34 (1200)	3.1	0.39

(c). ALNUS SINUATA (Sitka Alder)

Content, Zinc, and Ash Content of some Weakly Zinc Anomalous Young Stems

Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
49.56	10 (440)	37 (1600)	2.3	0.27
49.64	12 (420)	41 (1400)	3.0	0.31
49.82	13 (580)	36 (1600)	2.2	0.36
49.86	11 (430)	37 (1400)	2.7	0.31
49.88	7 (300)	39 (1700)	2.3	0.18
49.100	16 (530)	42 (1400)	3.1	0.39
49.101	4 (160)	35 (1400)	2.5	0.11
49.103	6 (280)	39 (3000)	2.3	0.09
49.105	10 (390)	32 (1300)	2.5	0.31
49.112	11 (390)	40 (1400)	2.9	0.29
Average.....	10 (390)	38 (1600)	2.6	0.26

These results are not inspiring to a beginner, but when taken as a part of numerous suites they begin to fit into a pattern. These results illustrate the type of anomaly which may be encountered in exploration work. It would be impossible to miss a Sullivan or a Britannia if one got within a few hundred feet of them. Without careful study it would be useless to try to interpret the results given in Tables 35 (a), (b), and (c). In considering these three tables it is well to remember that in most areas in British Columbia it is usual to find small amounts of copper with zinc, and small amounts of zinc with copper. The localities from which these suites were collected represent no exception to this rule.

Family SAXIFRAGACEAE

We have collected data only on a single species belonging to this family—namely *Philadelphus Lewisii.*

Genus *Philadelphus*

Philadelphus Lewisii Pursh Syringa

The species extends all across the southern portion of British Columbia from the coast to the Rocky Mountains and carries on into the adjoining American States. It is also known as Mock Orange.

In spite of a woefully small number of available analyses and no anomalous samples it seems clear that this species has some distinc-

ive qualities. Its leaves have the highest ash content of any tree or lesser plant examined—horsetails be excluded. Furthermore, in spite of its high ash content its leaves carry in their ash less copper and zinc than any other species examined. Furthermore, its stems are also high in ash and low in copper and zinc. This might well have been caused by regional deficiencies in the soil, but this possibility, although not ruled out as a contributory factor, is not the sole cause because other plants growing near by show neither such high ash contents nor such low copper and zinc in their ash (Tables 36, 37).

Solely as a working hypothesis we suggest the following as possible normals for leaves and stems bearing them:

	Copper	Zinc	Ash per cent	Cu: Zn
Leaves.....	8(60) ± 50%	23(170) ± 30%	13.9	.35
Stems bearing them..	7(100) ± 50%	34(180) ± 30%	7.5	.20
Second year stems.....	5(140) ± 50%	28(800) ± 30%	3.5	.18

TABLE 36.—*PHILADELPHUS LEWISII* (SYRINGA OR MOCK ORANGE)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
48. 6	10 (65)	7 (90)	22 (140)	25 (330)	15.2	7.6
48. 7	11 (60)	10 (110)	25 (140)	36 (420)	18.0	8.6
48. 9	5 (45)	5 (130)	22 (200)	26 (660)	10.8	4.0
48.10	8 (50)	6 (100)	23 (140)	34 (650)	16.3	6.0
48.11	8 (60)	9 (140)	18 (130)	28 (440)	13.5	6.3
48.12	5 (50)	5 (60)	15 (140)	31 (350)	11.0	8.9
49. 2	9 (73)	10 (90)	33 (270)	56 (510)	12.3	11.0
Average....	8 (60)	7 (100)	23 (170)	34 (480)	13.9	7.5

TABLE 37.—*PHILADELPHUS LEWISII* (SYRINGA OR MOCK ORANGE)
Comparison between Stems of Different Ages

Sample No.	Copper			Zinc			Ash, Per cent		
	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.
49.2	9 (70)	5 (150)	7 (200)	38 (280)	30 (900)	19 (510)	11.0	3.3	3.7

Family ROSACEAE

GENUS *Prunus*

Prunus demissa Nuttall Choke Cherry

This species is widespread throughout the whole Pacific Northwest. However, it is relatively intolerant of shade and prefers rich moist soils in open spaces. *Prunus demissa* may be found in small thickets along streams, highways, forest borders, and in small clearings.

Tables 38 and 39 represent pretty well the present state of our knowledge of this species. As a working hypothesis the averages given in Table 38 may be taken as normal.

Genus *Amelanchier*

Amelanchier alnifolia Nuttall Saskatoon

This species has a range paralleling that of *Prunus demissa*. In general it has a lower ash content and a higher copper and zinc content in its ash than *Prunus demissa*, and Tables 40 and 41 summarize our information about this species.

TABLE 38.—*PRUNUS DEMISSA* (CHOKE CHERRY)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
48. 4	8 (80)	9 (130)	22 (220)	46 (680)	10.0	6.7
48. 5	9 (180)	8 (160)	30 (610)	27 (550)	4.9	3.6
48. 6	12 (110)	8 (160)	17 (150)	17 (330)	11.0	5.2
48. 7	15 (190)	3 (100)	49 (610)	18 (570)	8.1	3.1
48. 8	9 (90)	5 (70)	23 (240)	36 (470)	9.6	7.6
48. 9	9 (80)	9 (200)	18 (160)	32 (720)	11.0	5.0
48.11	10 (70)	5 (90)	26 (190)	38 (670)	14.0	5.6
48.12	6 (60)	6 (90)	16 (150)	68 (1100)	10.4	6.3
49. 2	8 (110)	9 (190)	23 (300)	32 (690)	7.5	4.7
49. 3	14 (220)	12 (230)	34 (520)	48 (870)	6.5	5.4
Average....	10 (120)	7 (140)	26 (315)	36 (665)	9.3	5.3

TABLE 39.—*PRUNUS DEMISSA* (CHOKE CHERRY)
Comparison between Stems of Different Ages

Sample No.	Copper			Zinc			Ash, Per cent		
	1st Yr.	2nd Yr.	3rd Yr.	1st Yr	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.
49.2	9 (190)	7 (140)	6 (160)	32 (690)	16 (300)	11 (310)	4.7	5.4	3.7
49.3	12 (230)	8 (290)	6 (300)	48 (870)	18 (620)	13 (650)	5.4	2.9	2.0
Average...	10 (210)	7 (215)	6 (230)	40 (780)	17 (460)	12 (480)	5.0	4.1	2.8

TABLE 40.—*AMELANCHIER ALNIFOLIA* (SASKATOON)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
48. 2	10 (130)	4 (90)	20 (270)	17 (370)	7.5	4.6
48. 3	10 (200)	7 (290)	36 (730)	38 (1600)	4.9	2.4
48. 4	19 (210)	7 (160)	25 (270)	20 (470)	9.2	4.2
48. 5	12 (160)	5 (150)	17 (220)	19 (560)	7.7	3.4
48. 6	12 (150)	3 (110)	17 (220)	16 (580)	7.9	2.7
48. 7	16 (190)	5 (140)	40 (600)	25 (720)	7.4	3.4
48. 8	11 (140)	5 (110)	35 (440)	36 (760)	8.0	4.7
48. 9	7 (110)	4 (130)	35 (530)	40 (1300)	6.6	3.0
48.10	14 (160)	8 (300)	48 (510)	37 (1300)	9.0	2.7
48.11	10 (160)	5 (150)	26 (430)	26 (720)	6.3	3.6
48.12	10 (160)	3 (110)	25 (410)	20 (740)	6.1	2.7
49. 2	8 (83)	10 (190)	49 (500)	52 (960)	9.7	5.4
49. 3	7 (84)	16 (240)	23 (260)	37 (540)	8.9	6.9
49.11	14 (160)	13 (220)	41 (460)	40 (690)	8.9	5.8
Average.....	11 (150)	7 (170)	31 (420)	30 (810)	7.7	4.0

We have one zinc-positive sample of *Amelanchier alnifolia*, as follows:

	Copper	Zinc	Ash per cent	Cu:Zn
Leaves.....	5(60)	30 (350)	8.6	.17
Stems bearing them..	Not available	71((2000)	3.6	Not available

Alaska southward along the Pacific Coast. Below about Lat. 51° N. it extends eastward to Alberta and southward throughout the area under consideration. It grows on thin usually gravelly soil along gulches, mountain streams, cliffs, and canyon sides. As these are just the localities where prospecting may be difficult

TABLE 41.—*AMELANCHIER ALNIFOLIA* (SASKATOON)
Comparison between Stems of Different Ages

Sample No.	Copper			Zinc			Ash, Per cent		
	1st Yr.	2nd Yr.	3rd Yr	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.
49.1	16 (250)	4 (140)	3 (160)	39 (590)	15 (520)	12 (560)	6.5	2.8	2.2
49.2	10 (190)	5 (200)	3 (170)	52 (960)	50 (1800)	31 (1500)	5.4	2.8	2.1
49.3	16 (240)	8 (330)	7 (350)	37 (540)	33 (1400)	28 (1400)	6.9	2.4	2.0
49.4	10 (240)	6 (180)	3 (83)	41 (1000)	33 (1000)	30 (830)	4.1	3.2	3.6
49.11	13 (220)	8 (250)		40 (690)	21 (670)		5.8	3.1	
49.27	5 (160)	4 (240)		24 (760)	15 (910)		3.1	1.6	
Average...	12 (220)	6 (220)	4 (190)	39 (760)	28 (1050)	25 (1070)	5.3	2.6	2.5

TABLE 42.—*ACER GLABRUM* (MOUNTAIN MAPLE)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
48. 1	11 (150)	8 (170)	45 (600)	28 (600)	7.5	4.7
48. 2	13 (190)	2 (40)	17 (250)	16 (310)	6.9	5.2
48. 8	11 (160)	4 (110)	35 (510)	14 (390)	6.9	3.6
48.11	10 (150)	2 (50)	24 (360)	26 (630)	6.7	4.1
49. 2	16 (180)	12 (110)	25 (290)	29 (260)	8.6	11.0
49. 3	16 (180)	11 (100)	42 (470)	31 (290)	8.8	10.6
Average.....	13 (170)	6 (100)	31 (410)	24 (410)	7.6	6.5

This suggests that stems may be better indicators than leaves, but we suggest that any such conclusions be withheld pending more data.

Family ACERACEAE

Genus *Acer*

Acer glabrum Torrey
Rocky Mountain Maple

This tree variously known as dwarf maple, red maple, and mountain maple is found from

owing to lack of outcrops further work on this species is needed because we have no examples of either zinc or copper anomalous samples.

Tables 42 and 43 present the information on this species. Some of the evidence may appear contradictory, but in general probably *Acer glabrum* represents a fair medium of the species which we have studied, in that it is neither strikingly high nor low in its ash, copper, or zinc contents, or in the copper to zinc ratio.

Family Araliacea

Genus *Echinopanax*

Echinopanax horridus Devil's Club

Formerly classed as *Fatsia horrida* (Bentham and Hooker) this species has long been the bane of all who had to traverse areas in which it was conspicuous. Apparently it can thrive where there is abundant moisture and not too great cold; it attains its most luxuriant growth on the western slopes of the Coast Range Mountains.

It seemed to us that if this ill-favored plant could be put to any use our labors would not have been in vain. It carries more than 1 per cent of copper in the ash of its leaves in a copper-positive area and a bare 300 p.p.m. in a near-by negative area.

Table 43.—*ACER GLABRUM* (Mountain Maple)
Comparison between Stems of Different Ages

Sample No.	Copper			Zinc			Ash, Per cent		
	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.
49.2	10 (110)	3 (140)	4 (250)	29 (260)	10 (490)	11 (610)	11.0	2.1	1.8
49.3	11 (100)	6 (230)	3 (150)	31 (290)	20 (680)	24 (980)	10.6	2.8	2.4
Average...	10 (105)	4 (185)	3 (200)	30 (275)	15 (585)	17 (795)	10.8	2.4	2.1

Table 44 tends to confirm the biogeochemical potentialities of this unpleasant species.

Family Caprifoliaceae

Genus *Symphoricarpos*

Symphoricarpos racemosus Michaux Wax Berry

This species is general throughout the Pacific Northwest and is also known as snow berry and broom bush.

Its ash content is relatively high, and its copper and zinc content relatively low: like all the Angiospermae we have studied, the leaves have a higher ash content than the stems which bear them. The ash of stems is slightly higher in zinc than the ash of the leaves they bear.

Tables 45 and 46 illustrate the available results. No anomalous samples are available to us, so we have no idea what variations may be expected. The following normals are suggested:

	Copper	Zinc	Ash per cent	Cu:Zn
Leaves....	9(90) ± 30%	24(240) ± 30%	10.0	.37
1st Year stems...	9(130) ± 30%	30(430) ± 30%	7.0	.30
2nd Year stems...	6(170) ± 30%	18(410) ± 30%	3.5	.33

Family Compositae

Genus *Artemisia*

(a) *Artemisia tridentata* Nuttall Sage brush

This species and the closely associated *Artemisia trifida* are widespread throughout the dry interior of the Pacific Northwest. The obvious value of these bushes, if they could be used by a biogeochemist, prompted us to take a few samples. Not much may be assumed from the few available analyses. However, it seems clear that copper:zinc ratio is relatively high and that unless the copper:zinc ratio is greater than 1 the specimen need not be considered copper anomalous. The behavior of sage brush in zinc-anomalous ground is as yet unknown.

Table 47 presents some typical analyses of this species.

(b) *Artemisia trifida* Nuttall

The two analyses presented in Table 48 illustrate that this species of sage brush is apparently as sensitive to copper as is *Artemisia tridentata*.

In view of recent investigations in South Australia where desert conditions have been

TABLE 44.—*ECHINOPANAX HORRIDUS* (DEVIL'S CLUB)
Copper, Zinc, and Ash Content of some Normal 1st Year Stems

Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
49. 31	13 (160)	42 (520)	8.1	0.30
49. 32	12 (150)	35 (450)	7.8	0.34
49. 44	15 (200)	48 (650)	7.4	0.31
49. 48	15 (170)	58 (630)	9.1	0.27
49. 49	13 (220)	58 (940)	6.2	0.23
49. 50	11 (240)	14 (290)	4.8	0.82
49. 51	10 (110)	20 (210)	9.3	0.51
49. 75	11 (160)	38 (520)	7.2	0.31
49. 78	8 (140)	22 (350)	6.3	0.39
49. 79	18 (280)	46 (720)	6.4	0.39
Average	13 (180)	38 (530)	7.3	0.39

(a). ECHINOPANAX HORRIDUS (Devil's Club)
Copper, Zinc, and Ash Content of some Weakly Copper Anomalous 1st Year Stems

Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
49. 36	18 (220)	34 (400)	8.4	0.54
49. 37	15 (270)	38 (650)	5.8	0.41
49. 65	22 (190)	60 (520)	11.6	0.36
49. 80	18 (240)	53 (700)	7.6	0.35
49.106	16 (220)	51 (710)	7.2	0.31
Average	18 (230)	47 (600)	8.1	0.39

(b). ECHINOPANAX HORRIDUS (Devil's Club)
Copper, Zinc, and Ash Content of some Weakly Zinc Anomalous 1st Year Stems

Sample No.	Copper	Zinc	Ash Per cent	Cu:Zn
49. 56	14 (230)	53 (880)	6.0	0.26
49. 64	10 (150)	47 (690)	6.8	0.21
49. 86	11 (190)	57 (970)	5.9	0.19
49.100	10 (130)	70 (940)	7.4	0.14
49.101	12 (180)	57 (860)	6.6	0.21
49.105	11 (190)	50 (880)	5.6	0.22
49.112	10 (130)	66 (880)	7.5	0.15
Average	11 (170)	57 (870)	6.5	0.20

shown to have been caused in part by zinc and copper deficiencies, and remembering that zinc and copper deficiencies are highly probable in many areas of the Pacific Northwest it seems relevant to suggest that further studies of these sage brushes may prove very useful not only to prospectors but also to agriculturists.

SELECTION OF PLANT ORGANS

In selecting the organ of a plant to be used for a biogeochemical survey three main questions must be considered:

(1) Which organ can be most readily collected?

TABLE 45.—*SYMPHORICARPOS RACEMOSUS* (BROOM BUSH, SNOW OR WAX BERRY)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
48. 4	14 (160)	17 (230)	19 (220)	25 (360)	8.8	6.9
48. 5	9 (70)	7 (80)	24 (190)	22 (260)	12.8	8.6
48. 6	10 (80)	6 (80)	26 (190)	22 (300)	13.1	7.4
48. 7	10 (110)	8 (110)	25 (270)	25 (360)	9.4	7.0
48. 8	8 (86)	7 (100)	31 (310)	61 (870)	9.9	7.0
48. 9	6 (50)	6 (120)	20 (170)	32 (620)	12.0	5.2
48.11	7 (83)	4 (90)	30 (350)	52 (1200)	8.6	4.3
48.12	5 (51)	3 (60)	18 (180)	21 (420)	9.7	5.0
49. 3	11 (100)	12 (110)	34 (290)	35 (320)	11.7	10.8
49.11	9 (120)	10 (160)	17 (220)	20 (340)	7.6	6.1
Average.....	9 (90)	8 (110)	24 (240)	31 (500)	10.4	6.8

TABLE 46.—*SYMPHORICARPOS RACEMOSUS* (BROWN BUSH, SNOW OR WAX BERRY)
Comparison between Stems of Different Ages

Sample No.	Copper			Zinc			Ash, Per cent		
	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.	1st Yr.	2nd Yr.	3rd Yr.
49.2	10 (140)	7 (150)	4 (120)	30 (420)	24 (510)	16 (450)	7.2	4.7	3.6
49.3	12 (110)	6 (150)	5 (180)	35 (320)	20 (510)	16 (600)	10.8	3.9	2.7
49.5	11 (210)	5 (190)		27 (490)	16 (540)		5.5	2.9	
49.11	10 (160)	6 (220)		20 (340)	10 (380)		6.1	2.6	
Average...	11 (155)	6 (180)	4 (150)	28 (390)	17 (485)	16 (525)	7.4	3.5	3.1

TABLE 47.—*ARTEMISIA TRIDENTATA* (SAGEBRUSH)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
48. 3	10 (140)	8 (170)	19 (280)	17 (360)	6.9	4.7
48. 4	35 (330)	21 (250)	35 (330)	21 (250)	10.7	8.5
48. 5	10 (140)	17 (270)	37 (510)	20 (320)	7.3	6.3
48. 6	12 (90)	11 (90)	22 (170)	22 (180)	13.3	12.1
Average.....	17 (175)	14 (195)	28 (320)	20 (280)	9.5	7.9

(2) Which organ can be most easily analysed?

(3) Which organ responds most readily to anomalous conditions?

We do not yet have sufficient data to answer these questions with any degree of finality. We have, however, considerable experience on which to make some tentative suggestions, and we shall discuss briefly each question.

(1) Which organ can be most readily collected?

We have already discussed at some length why we do not consider roots, bark, wood, or cones to be satisfactory in spite of their easy availability (Warren and Delavault, 1948; 1949).

Leaves have much to commend them and

TABLE 48.—*ARTEMISIA TRIFIDA* (SAGEBRUSH)
Comparison between Leaves and Stems Bearing Them

Sample No.	Copper		Zinc		Ash Per cent	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
48.12	12 (210)	8 (200)	36 (620)	25 (610)	5.8	4.1
48.50	30 (350)	19 (510)	35 (410)	29 (780)	8.5	3.7
Average.....	21 (280)	13 (355)	35 (515)	27 (695)	7.1	3.9

undoubtedly may be used successfully under favorable circumstances, such as when ashing is to be done shortly after collecting. Lakin and Reichen (1949) have shown how leaves may be used to advantage.

On the other hand leaves can be collected only during a portion of the season, and our experience suggests that the copper and the zinc content of leaves may vary considerably with the time of the season at which the sample is taken.

Needles, although they are available at all times of the year, are not easy to collect, and indeed in the case of the spruces and junipers they are bothersome if they are not dried thoroughly before they are plucked from the stem bearing them.

Young tips and buds of the evergreens are the easiest organs of all to collect, except in the early part of the growing season, when collecting a satisfactory sample may be wearying. In the case of some slow-growing species such as *Tsuga Mertensiana* prospectors loathe collecting this species and are apt to collect second-year material, accidentally, if not by design.

Young stems are often easy to collect, particularly late in the growing season.

With suitable collecting tools we have found that stems of one full season's growth are easy to gather, after a little practice, and have the advantage of being easier to deprive of their leaves or needles. These stems are easily prepared for shipping and do not mould as leaves may.

In short, although any organ can be collected readily, and tips or buds of evergreens perhaps most readily of all, stems of one full season's growth are probably the best organ on which to base any new investigation.

(2) Which organ can be most easily analysed?

Second-year stems have a higher copper and zinc content in their ash, a lower overall ash content, and they ash more readily than any other organ we have been discussing. For these reasons we favor stems of one full season's growth. The numerous tables would seem overwhelmingly to favor this conclusion.

(3) Which organ responds most readily to anomalous conditions?

Much more statistical evidence must be collected before this question can be answered. However we do have some data on this question.

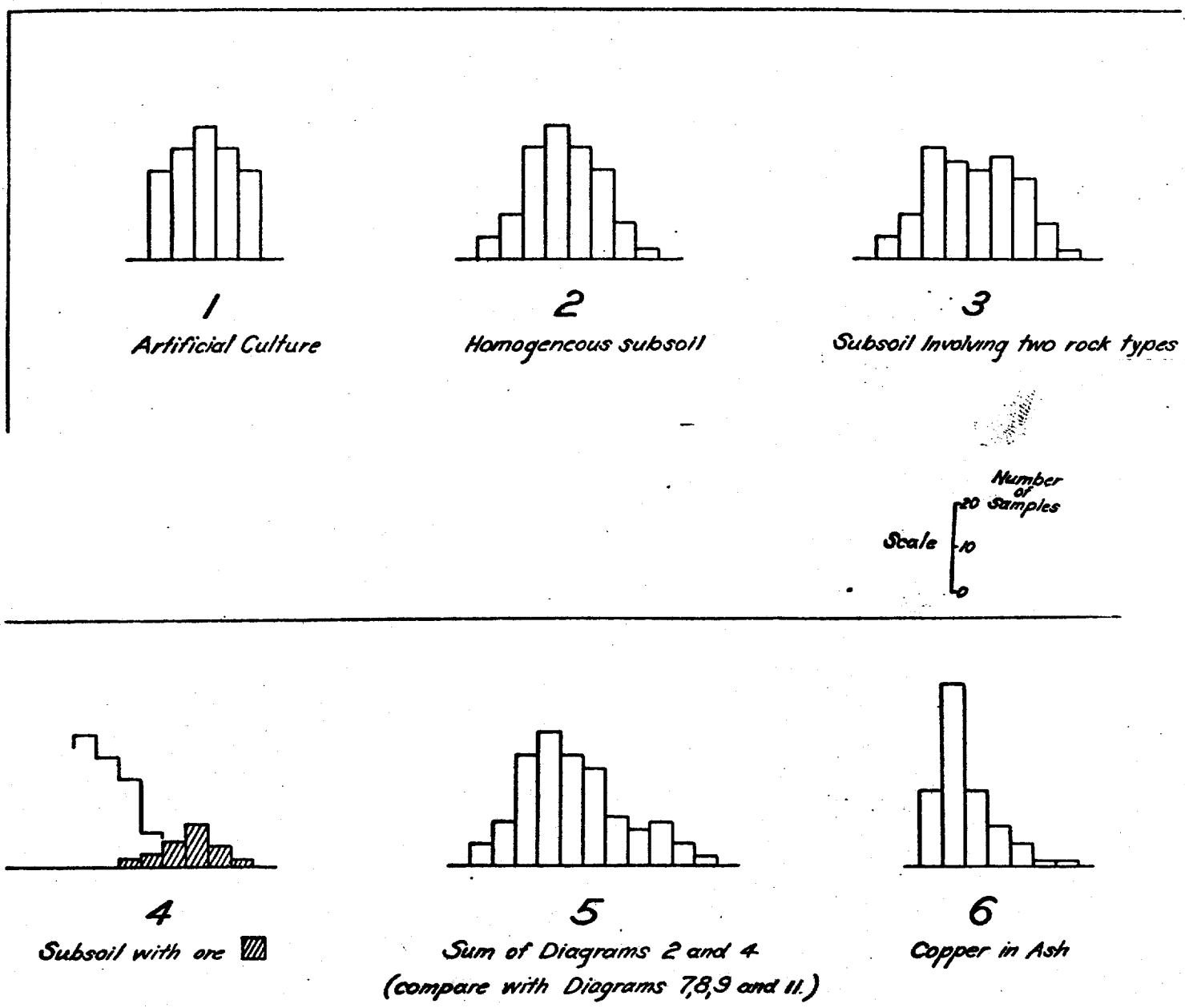
Obviously some very young tips of spruce are unreliable, and indeed the very young growth of any species seems most erratic, insofar as copper is concerned, although the pines possibly may not be so erratic as some of the other genera.

Some tips, notably those of *Thuja plicata*, appear to show very little response to abnormal zinc conditions.

On the other hand, provided that the tips or young buds have been well developed, we have found in some species, such as *Pseudotsuga taxifolia* and *Abies lasiocarpa*, that they are satisfactory, at least for reconnaissance work.

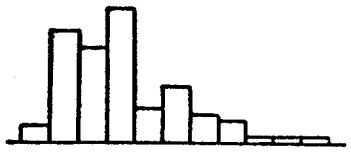
In short, until an area has been studied, tips are apt to be deceiving, but under some conditions in a known area tips may be most useful. Stems of one full season's growth, although they may not always show the spectacular, and sometimes puzzling, variations of leaves and other young organs, are probably the most reliable indicators of zinc and copper anomalies. Further statistical work should be presented before this premise is generally accepted.

Thus, in spite of some factors favoring younger organs we are now firmly convinced that, for most species of trees, stems of one full season's growth are the best organs for any preliminary biogeochemical investigation.



TYPICAL HISTOGRAMS

Abscissae show metal content at various scales. All results in p.p.m. dry plant or ash as indicated. Ordinates indicate number of samples.
Diagrams Nos. 1 - 5 are typical histograms obtained from a large number of samples.
Nos. 6 - 14 are histograms resulting from actual field work from relatively small numbers of samples (about 100).



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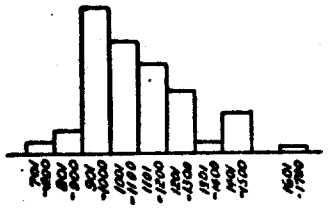
Copper in dry plant.



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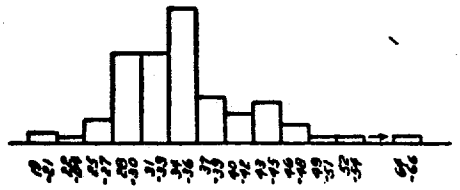
Zinc in Ash



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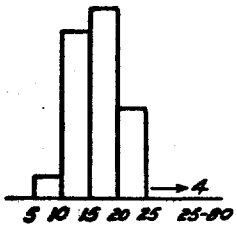
Zinc in dry plant



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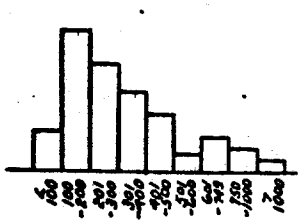
Iron in dry plant



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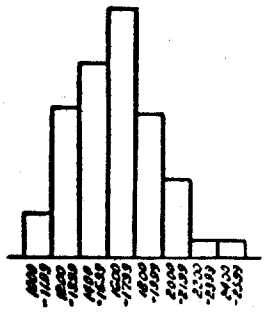
Manganese in dry plant



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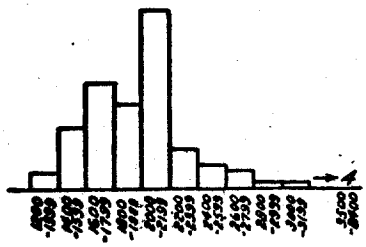
Zinc in Ash



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Zinc in Ash

14

Copper in Ash

Notes on the Use of Biogeochemical Data

The present paper is largely concerned with fundamental information, which is needed before biogeochemical prospecting can be usefully initiated, and deals mostly with the copper and zinc contents of different young parts of many plant species. Most of the samples come from widely separated localities. Hampered as we have been by lack of funds, we have been quite unable to carry out any systematic field work such as would naturally have been most desirable. However, thanks to the support of various private companies, and particularly New Jersey Zinc Explorations, Limited, and the British Columbia International Engineering Company, Limited, extended field work has been made possible, and on the basis largely of this field work we can illustrate how the tables may be utilized in achieving the ultimate goal of all these biogeochemical researches—namely, the detection of ore through the systematic investigation of the metal content of vegetation.

We have described (Warren and Delavault, 1949) the metal content of trees from three richly mineralized areas. In such cases a glance at the analytical results was sufficient to ascertain which samples came from rich areas and which ones from barren places. In practice results are not so clear cut. We found that the normal metal contents ranged considerably from area to area largely because of variations in the climate, physiography, and underlying rock types.

At this point a biogeochemist searching for mineralization is faced with a dilemma. He must choose between two possible methods of attack. On the one hand he may take samples, as many as practical, and hope to stumble on one or two, or perhaps even a few, very rich samples which will directly indicate the location of some mineralization. On the other hand he may attempt to interpret every sample taken, and thereby to obtain a general pattern of the metal distribution in the area he is investigating. We do not wish too much to deprecate the first system, which perhaps accords best with the prospector's traditional gambling spirit.

Unfortunately, this first method of attack has little chance of success in exploration unless an inordinately large number of samples are taken, and the cost of doing this may be prohibitive. Furthermore, we have not yet perfected any simple field method whereby results can be obtained in a few minutes on the spot: water testing has a great advantage in this respect. Independent of this technical difficulty, we have found that it is possible to get disappointing results from a single sample taken almost on top of a lode. In short we do not favor looking for mineralization by looking for samples with very high metal contents. The odds would seem too much against success. It would be like panning at random without taking the general geological background into consideration in an unknown area in the hope of finding a buried vein.

The second system which may be thought of as one in which a search is made for anomalous areas is undoubtedly slower and less spectacular, but we believe it capable of finding mineralization under unfavorable circumstances. Furthermore the biogeochemical data obtained by mapping anomalies can sometimes be of assistance in tracing geological formations in heavily drift-covered areas as well as drawing attention to any indications of mineralization.

If 50 or more samples of one species have been collected in an area it is possible to treat the analytical results independently of any previous data. This may best be done by plotting the results to a suitable scale on squared paper, giving what is termed a frequency diagram or histogram. By this diagram it is possible visually to estimate the normal copper, zinc, or copper: zinc ratio of the suite in question. In general, percentage variations from the normal are the significant items. Again speaking in general terms we have found that variations of more than 50 per cent and less than 100 per cent above normal may safely be termed "possibly anomalous" in the case of copper, and more than 100 per cent above normal "probably anomalous." In dealing with zinc somewhat smaller percentage variations appear significant: variations of more than 30 per cent and less than 50 per cent above normal are usually taken by us as being "possibly anomalous," and those of more than 50 per cent above normal as "probably anomalous."

To illustrate, we might cite results obtained in prospecting in a portion of southern British

Columbia. We found that using twigs of one full season's growth 6 p.p.m. of copper and 42 p.p.m. of zinc could be considered a normal content of dry-plant material of this particular species. In this instance a copper content greater than 9 p.p.m., or zinc greater than 56 p.p.m., was considered as possibly anomalous, and 12 or more p.p.m. of copper and 63 or more p.p.m. of zinc as probably anomalous. In using copper:zinc ratios we have found it necessary to eliminate any samples which carry either copper or zinc in amounts significantly below normal for the area being investigated.

In selecting scales to be used in compiling a frequency chart, different units will have to be chosen to suit the particular circumstances under consideration: the number of analyses available, the species of tree being considered, and the anomalous element being sought are all factors which determine the selection of a suitable scale.

In most natural-history problems variations from a normal can best be shown on a logarithmic scale. However, in this particular case the higher contents are much more interesting than the lower ones, and consequently an ordinary scale may draw attention to anomalous specimens more strikingly than a logarithmic one, even though the latter would be technically more correct.

There will always be something arbitrary in deciding what is normal and what is not in any given area. Nevertheless during several years of investigations on trace metals we have noticed that for a given organ of determined age of any one species variations owing to different growing conditions on the same subsoil are usually of the same order of magnitude as variations due to different subsoils, always provided that there was no metal present as ore or a a notable addition to the country rock. Some limestones are abnormally high in copper.

In diagrams from areas of little or no mineralization most data will appear in a chart in a few brackets forming a compact pattern which we now consider a "normal" pattern (Pl. 1, Diagram 2). An ideal frequency diagram would be obtained from a lot of equally healthy plants growing on homogeneous soil without competition of any sort (Pl. 1, Diagram 1).

Actually in most areas many species are competing for existence, and geological and physiographical variations are inevitable. Thus in the absence of specific mineralization most diagrams look like Diagram 2. In some areas two widely differing geological formations will result in a "two-bumped" diagram such as Diagram 3.

In a mineralized area, such as a porphyry copper, occurring as a part of a much larger area, a small diagram, indicated on Diagram 4, will be superimposed on Diagram 2, giving a diagram such as Diagram 5.

Obviously there will be overlapping on some diagrams: the lowest copper content of organs from a mineralized area will overlap with the highest copper contents of some "normal" areas. This is unavoidable, but the greater the number of samples taken the less troublesome is the problem of overlapping. Equally obvious is the conclusion that no deductions should be drawn, except in conjunction with all available geological and physiographical data.

We have found it advisable when moving into new country to take, where convenient, samples of several species from the same place. Not all the samples are likely to be high by some physiological freak.

Diagrams 6–14 illustrate examples from our own experience. The weak copper anomaly indicated by Diagrams 6 and 7 has now been related to copper mineralization. The well-marked anomaly indicated on Diagrams 8 and 9 has been correlated with significant zinc mineralization.

Diagrams 12–14 come from an area now known to contain zinc with some copper, but with the copper being of no commercial importance, and indeed being only of mineralogical and biogeochemical significance.

We submit that, although Diagram 12 might well be said to have missed the zinc, now known to be present, the four samples beyond the normal right-hand scale of Diagram 13 could not fail to point to this zinc-anomalous area.

Having decided that some samples are anomalous there remains to decide on the best method of plotting the results. Ordinarily a map showing such geology and topography as is practical is prepared. Then a set of contour lines is superimposed indicating either the absolute amount of metal or the variation from the normal of the metal content of the plants within the area under consideration. White (1950) has illustrated this technique. As this

varies in no significant manner from geophysical practices it will not be discussed further here.

at all but one of the places indicated as anomalous. The commercial significance of this mineralization remains to be determined.

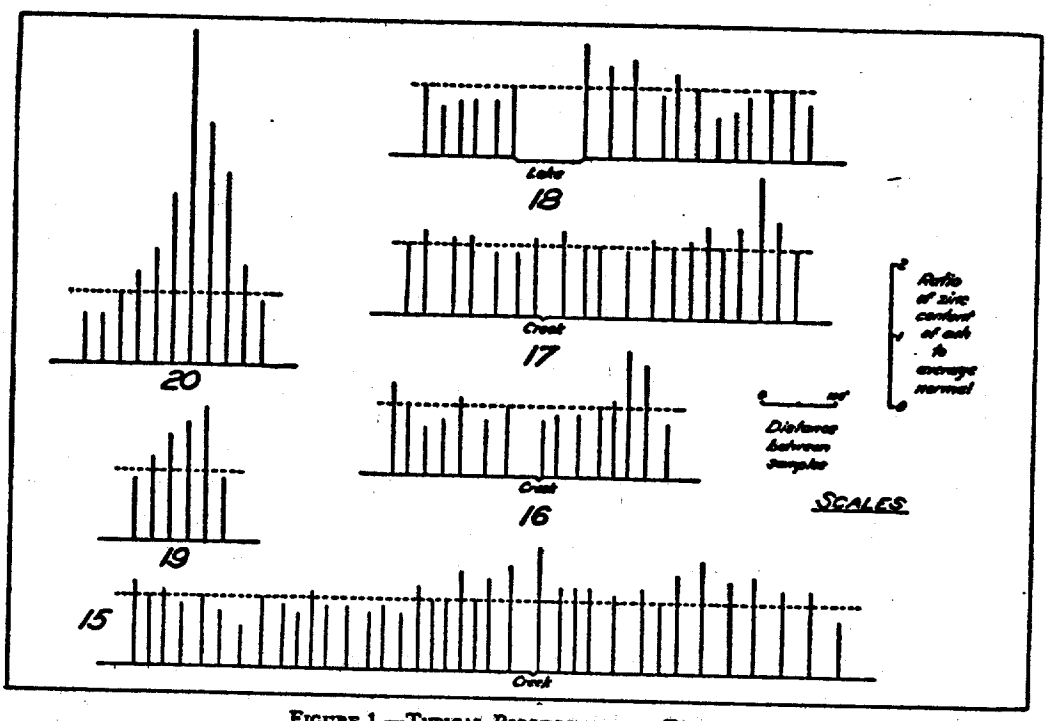

FIGURE 1.—TYPICAL BIOGEOCHEMICAL PROFILES

Each vertical line represents one sample. The length of the line is proportional to the ratio of the zinc contents to the normal for the species. The horizontal dotted line indicates this normal. The slope of the ground is not indicated because no survey was available when we made these studies.

Diagrams 15–20 (Fig. 1), inclusive, indicate how results obtained from a suite of samples taken across a valley of varying width may be plotted in the form of a profile to indicate where mineralization may occur. In these diagrams each sample is represented by a vertical line, the height of which expresses the ratio of the metal content to the normal metal content of the species, considering similar organs of the same age. The intervals between the vertical lines represent to scale the distance between the trees. The horizontal dotted line represents the normal metal content and is superimposed on the diagram in order to indicate more clearly the variations from the normal.

In all these diagrams significant variations can readily be seen, even though six species of trees were involved in this survey, and each species had its own normal zinc content. At the time of writing, mineralization had been found

COMMON ERRORS

We have discussed errors connected with collecting and now indicate some of the other pitfalls into which we have fallen.

(1) *Collecting samples from trees growing where there is too much dilution.* This seems a very obvious mistake, but it is a very natural mistake and one apt to be made by beginners.

Willow and alders frequently grow along river banks and lake shores. These rivers and lakes may contain exceptionally pure water, and we have found that these species, particularly where sampled too close to large bodies of water, may fail to indicate anomalies, whereas samples taken near by from less thirsty species may show anomalies. The same may apply to some species of spruce which are tolerant of extremely damp conditions.

It is desirable, therefore, where possible to

select samples removed from too ready access to large bodies of pure water.

Perhaps we can illustrate this point best by giving an example encountered in actual prospecting in 1949. A narrow zinc vein crossed a creek. A short distance below the outcrop the zinc content of the water in the creek was some 40 parts per thousand million. Nearly half a mile lower down the creek the water content was determined as 2 parts per thousand million, but this was sufficient to make the trees near the creek weakly anomalous. Just down-stream another creek entered, and further dilution took place. The zinc content of the water fell below 1 part per thousand million, and no anomaly could be detected in a carefully selected suite of samples.

(2) *Collecting samples from mine dumps and vein outcrops in order to discover what anomalies may be expected in an area.* It has been our experience that samples collected from the vicinity of mine dumps and conspicuous outcrops containing copper and zinc are most erratic in their content of these metals. In our early experiments we hopefully looked for spectacular results from samples taken in such auspicious localities. We were rudely surprised when we more often than not obtained "normal" results.

We are by no means certain that we have the answer to this puzzle. However, it may lie in the fact that trees obtain their metal content by means of base exchange, and this involves the presence of suitable particles of clay or claylike minerals.

(3) *Neglecting to take pH variations into account when these variations are significant.* We have no usable statistical information on this point, but we have collected some data on variations on the iron:manganese ratio of some species under varying pH conditions. From these and preliminary copper and zinc data collected at the same time we are convinced that the uptake and hence both the relative and absolute amounts of copper and zinc in plants are affected materially by the pH of the soil at the time of growth.

(4) *Neglecting to take into account the fact that there are biogeochemical provinces in much the same way that there are metallogenetic provinces.* We have studied only a few species in sufficient detail to offer supporting evidence of this statement. However, the general, similar series of metals [illegible] reasonable that probably no further elaboration is necessary. In general it seems safe to assume that in any one geographic area the absolute and possibly the relative amounts of copper and/or zinc may vary markedly from those found in another. In staring work in a new area it is therefore necessary to determine as soon as possible what are to be considered normal contents. The safest way to do this is to collect suites from sterile outcrops. Normal contents usually appear from statistical curves which have been described above.

Conclusions

Although detailed work may enable one to obtain unusually high copper and zinc contents in trees and lesser plants, it seems clear that, in exploration work, biogeochemistry will prove its worth in the search for buried ore bodies through its ability to detect relatively unspectacular but nonetheless important anomalies.

We have concluded that for field work a stem of one season's growth is the most practical organ to use, except in a few special cases when leaves and stems cannot be separated easily.

Attempts to get results by using biogeochemistry without considering the relevant geology, climate, and physiography may result in much misdirected effort. Only rarely is it wise to use biogeochemistry alone. It is another tool which, when used under appropriate conditions, may play a useful part not only in the search for buried ore bodies but also in deciphering the distribution of some geological formations covered with overburden.

It is hoped that the many tables and diagrams given in this paper will aid those who attempt to apply biogeochemistry to their various problems.

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Manuscript Received by the Secretary of the Society, June 1, 1950

Project Grant 499/46/S47

THE VETERINARY RECORD

FOUNDED BY WILLIAM HUNTING, F.R.C.V.S., 1888

No. 8. FEBRUARY 21, 1948 VOL. 60

A Note on the Toxicity of Zinc Chloride

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INTRODUCTION

As a consequence of illness and deaths amongst cattle and sheep in Scotland believed to be caused by "smoke" from standard Service hexachlorethane type smoke generators (of which zinc chloride is an important end-product) it was decided to carry out a series of experiments to obtain further evidence concerning the toxicity of zinc chloride for large and small animals.

The toxicity of zinc salts may be considered under two headings, *viz., acute* (due to local corrosive action on the alimentary tract) and *chronic*. Most of the previous experimental work on the chronic form has been carried out with zinc oxide, which is variously believed to be converted into the chloride, lactate or albuminate in the alimentary tract (Grimett, McIntosh *et al.*, 1937) to pass through the intestine largely unchanged (Heller, 1927) or to be toxic on account of such impurities as lead, cadmium or arsenic.

Mallory & Parker (1925) caused acute glomerulonephritis in rabbits by injecting 0·5 to 1 gramme of powdered metallic zinc under the skin.

Heller & Burke (1927) fed rats with zinc salts in amounts equivalent to 0·25 and 0·5 zinc in the total diet. Toxic effects were not observed over several generations and from analyses it did not appear that the zinc content of the organs had increased.

Drinker, Thompson & Marsh (1927) fed cats and dogs with daily doses of from 0·175 to 1·0 gramme of zinc for periods varying from three to 53 weeks. Each of the three cats on the highest dose (1 gramme per day), which is greatly in excess of any likely to occur under practical conditions, was found on *post-mortem* examination to have a hardened, nodular and fibrous pancreas, with partial obliteration of the islets of Langerhans. All animals on the lower rates of dosage remained healthy and showed no lesions on *post-mortem* examination. Only a small fraction of the zinc was assimilated.

Engel (1931) considered that zinc has a toxicity, if any, similar to that of copper—which in rabbits, according to Hall & McKay (1931) and Mallory & Parker (1931), may cause cirrhosis and pigmentation (mostly due to haemofuscin stored in the Kupffer cells) of the liver. Engel quoted the work of Kunkel (1899), who had reported that 28 mg. of zinc albuminate per kg. body weight proved fatal when given by mouth to a rabbit, and of Macht (1931) who had found that in dogs and cats 0·5 gramme of zinc oxide per kg. body weight, caused repeated vomiting, wasting and shivering and that in rabbits a single dose of 1 gramme of zinc oxide was fatal and one of 20 mg. caused renal damage.

Graham *et al.* (1940), failed to induce symptoms of illness in pregnant mares and suckling foals by feeding single doses of 35 grammes of zinc lactate.

Johansson (1942) and Hoffman (1943) report cases of zinc poisoning occurring in cattle kept in byres in which galvanised iron piping was being welded. Symptoms included fever, dyspnoea, anorexia, suppression of milk yield and subcutaneous emphysema. This type of poisoning is due to the inhalation of zinc oxide vapour which is formed as a white smoke during the welding process. The action is a kind of anaphylactic shock caused by metallic particles damaging the lung tissues. It is equivalent to the disease in humans known as brass founder's ague.

Hegsted *et al.* (1945) stated that soluble zinc compounds might cause chronic gastritis in man and that caustic compounds of zinc might occasion skin irritation. He believed that in dermatitis due to zinc chromate it was the chromate portion of the molecule which was responsible.

Gross & Heller (1946) maintained that for white rats and mature mice the maximum non-toxic level in the feed is 1 per cent.

A natural outbreak of chronic zinc poisoning was reported by Grini (1938) to have occurred in four herds of cattle in Norway, shortly after the inclusion in the daily ration of whey that had been transported in iron drums and was later found to contain zinc. The symptoms, which included diarrhoea, followed later by constipation, with flatulence, and muscular weakness, lasted some three weeks and ended in death or slaughter of the affected animals.

EXPERIMENTAL

Both large farm and small laboratory animals were used.

(a) The Large Animals

These consisted of: One Ayrshire cow, two sheep and two goats, which were kept in a paddock measuring 75 yards by 10 yards, and of one calf, which was kept in a pen.

The paddock (75 yards by 10 yards) was fenced round with wire and was provided with two gates. Hay and green food were laid out for the animals on the pasture and the concentrates (cattle cakes) fed from movable troughs: water was ever-present and always available.

All animals were subjected to a clinical examination before being placed in the paddock, and found to be healthy and normal. Daily examinations were carried out thereafter until the end of the experiment.

(i) SPRAYING EXPERIMENTS (3.3.46 to 16.4.46)

The whole paddock, including the hay laid out, the water and the coats of the animals themselves, was sprayed with a solution of zinc chloride three times a week.

A knapsack sprayer was used; this conveniently accounted for one-third of the paddock at a fill. The required amount of zinc chloride was made into a concentrated solution and divided into three equal parts. Each third was added to the sprayer and diluted by adding water to fill the container, which had a capacity of two and a half gallons.

Up to March 26th, 1946, any hay left uneaten between sprayings was removed and burned. After this date any uneaten hay was left in the paddock; new hay was added and the whole lot subjected to zinc chloride spray. The gruel and concentrates fed to the calf were sprayed in a similar manner.

Spraying with zinc chloride solution was discontinued after six weeks. (All foodstuffs were henceforth subjected to the action of "smoke" in the gas chamber before being fed to the animals.)

In the *zinc chloride spraying*, from March 3rd to April 16th, 1946, the amounts and dosage were as follows:—

Date	Weight of zinc chloride	Dosage of zinc chloride
March 7th, 1946	750 grammes	1 gramme per square yard
" 11th, "	750 "	1 " "
" 14th, "	1,875 "	2·5 grammes "
" 19th, "	1,875 "	2·5 " "
" 23rd, "	3,750 "	5·0 " "
" 26th, "	3,060 "	4·0 " "
" 29th, "	3,375 "	4·5 " "
April 2nd, "	3,750 "	5 " "
" 8th, "	3,750 "	5 " "
" 12th, "	3,750 "	5 " "
Total	27,288 "	36·5 " "

It should be noted (1) that all the zinc chloride solution was made from commercial stick or fuzed pure zinc chloride except on March 29th and April 12th when it was made from zinc oxide and commercial hydrochloric acid: and (2) that one gallon of zinc chloride solution containing 375 grammes was used for spraying the concentrated food on and after March 29th.

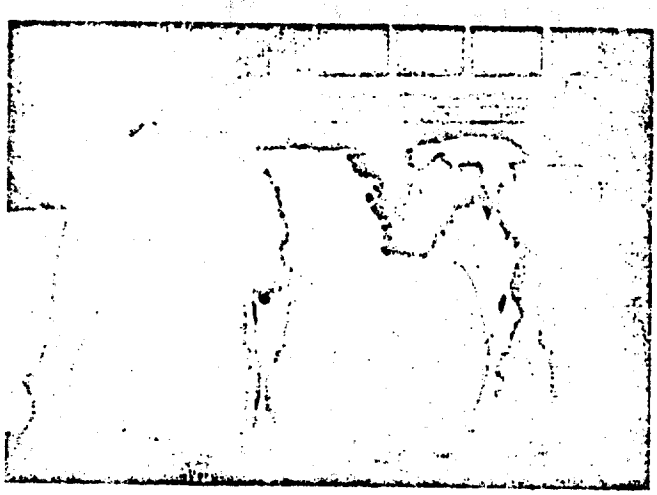
The following amounts of food were used per week: hay, 330 lb.; cake, 182 lb.; roots, 168 lb.; green food, 70 lb. The average proportion of food eaten by the one cow to the four sheep/goats was approximately 10:4.

The approximate weight of zinc chloride ingested between March 7th and April 12th, 1946, was computed to be:—

Cow: 19·492 kg. (average daily intake, 527 grammes).
Each sheep and goat: 1·949 kg. (average daily intake, 53 grammes).

Observations.—The Ayrshire cow showed slight loosening of the faeces on the fourth and eighth day after the beginning of the experiment, but returned to normal within 24 hours after its appearance. The milk supply went down by 3 lb. on the 20th day, but was back to normal by the 25th.

The sheep, goats and the calf remained healthy and normal throughout. To begin with the sprayed hay was only nibbled at by all animals. As they became hungry more was eaten, and soon it was being consumed quite avidly.



CALF
Feeding Expt. Zn Cl_2 Spray: six weeks. Smoke contaminated forage: 15 weeks.

AYRSHIRE COW
End of Feeding Expt. Zn Cl_2 Spray: six weeks. "Smoke" contaminated forage: 15 weeks.

(ii) SMOKING OF FOODSTUFFS (April 17th to July 31st)

Foodstuffs sufficient for one week were placed in the gas chamber (47 cubic metres) and 400 grammes hexachlorethane smoke composition (from the standard Service smoke generator) burned in the chamber. The smoke was allowed to settle for half to three-quarters of an hour and the chamber then opened. The foodstuffs were covered with a greyish-black deposit as a consequence of this treatment. The experimental animals were fed exclusively on this smoke-contaminated forage.

Smoking of foodstuffs took place on the following dates (1946):—

April	May	June	July	May	June	July
17	3	3	4	17	17	19
23	6	4	8	20	21	22
30	8	7	10	22	24	24
	10	9	12	24	26	26
	13	12	15	27	28	29
	15	14	17	31		31

Zinc determinations showed that *hay* had 6,430 parts per million zinc after treatment (normal was 51 parts per million); grass had 943 parts per million zinc after treatment (normal was 35 parts per million); [illegible] cake had 162 parts per million zinc after treatment (normal was 25 parts per million).

Observations.—Smoke-contaminated foodstuffs were eaten with apparent relish.

The animals were in excellent condition throughout and no adverse clinical signs were shown. This is the more surprising when one considers that they had been confined in a tiny paddock for five whole months and that some slight falling away in condition might have been expected on this account alone.

Autopsy.—The cow, two sheep and two goats were slaughtered on August 7th, 1946. In all cases there were no lesions of pathological significance, the carcases were in excellent condition and were passed as normal and fit for human consumption. The calf was saved and to-day (January, 1948) is a normal healthy beast.

CONCLUSIONS

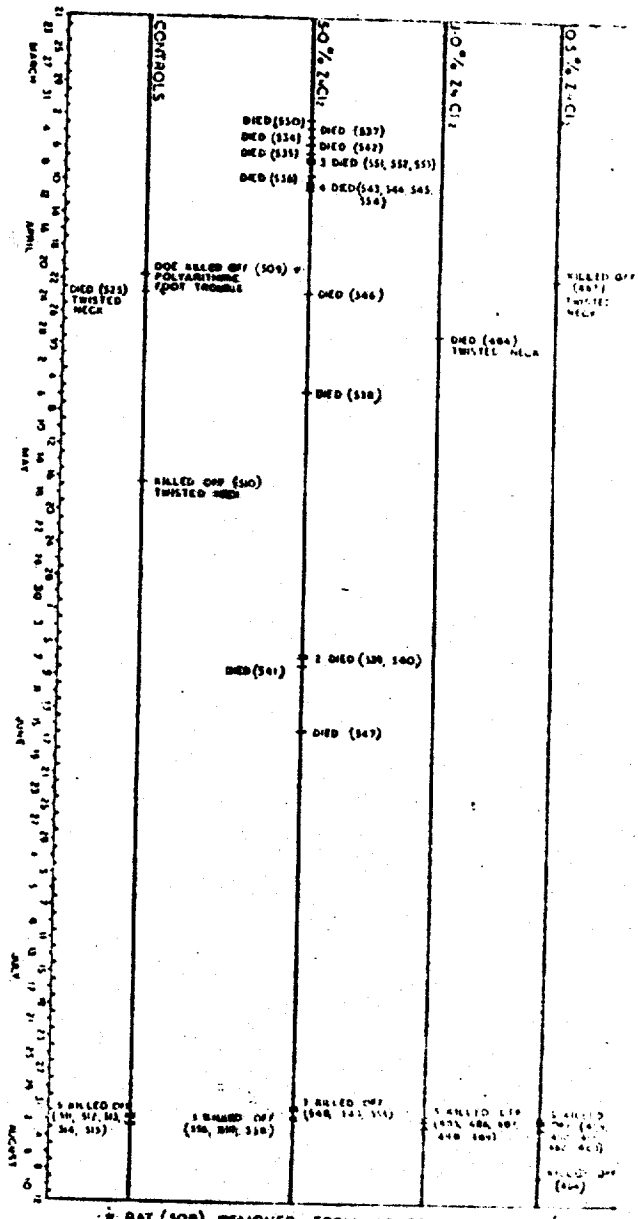
The prime object of the experiment was to induce, if possible, the chronic type of zinc poisoning. Large amounts of zinc chloride were used over a long period without any effect.

Smoke was then employed with equally negative results. Over 50 per cent. zinc chloride is produced on the burning of smoke generators (another product is zinc chromate) and it was considered that the exclusive feeding of foodstuffs contaminated by smoke over a period of months might induce a condition of chronic zinc poisoning. This did not prove to be the case.

(b) Small Laboratory Animals

One hundred Wistar Albino rats were used. These were housed in wire cages and divided into four batches of 25. Each batch of 25 rats was divided into three cages, each containing eight, eight and nine rats respectively.

GRAPH SHOWING THE DATE OF THE DEATHS OF THE RATS IN EACH GROUP



At first solutions of pure zinc chloride of 0·5 per cent., 1 per cent and 5 per cent. strength were made up. Water bottles were filled with these solutions:—

25 Group A rats had 0·5 per cent. solution in water bottles.
25 " B " " 1 " " " " " "
25 " C " " 5 " " " " " "
25 " D " " water and were used as Controls.

After a fair trial it was found that the rats would not drink the solutions. This idea was therefore abandoned.

The food was now dampened with solutions of zinc chloride. The rats did not mind this at all and ate the contaminated food. This procedure was adhered to consistently for the rest of the experiment. Water was used in dampening the food of the Control "batch" D.

The amount of food eaten and the amount of solutions used daily were carefully determined.

All rats were weighed every second day.

The following table demonstrates the amount of zinc chloride taken per rat per day:—

Group	No. of rats	Amount of food per group (grammes)	Amount of zinc chloride solution per group	Amount of food eaten (grammes) per group	Approximate amount zinc chloride taken per rat per day (mg.)
A	25	300	300 ml. of 0·5% solution	300	60
B	25	300	300 ml. of 1% solution	300	120
C	25	300	300 ml. of 5% solution	300	600
D	25	300	300 ml. water	300	—

The amount of zinc chloride used for dampening the food as deaths occurred had to be assessed proportionately, *e.g.*, when there were 15 survivors in Group C 180 grammes of food was dampened with 180 ml. of a 5 per cent. solution of zinc chloride. All of this was eaten which is equivalent to 600 mg. (approximately) of zinc chloride per rat per day.

Those which died were subject to careful *post-mortem* examinations and specimens were taken for chemical and histopathological examinations.

The experiment continued for six months. (Groups A, B and D were, however, kept for a further period of nine months and the feeding with foodstuffs treated with zinc chloride continued.)

Observations.—Group A (0·5 per cent. zinc chloride), Group B (1 per cent. zinc chloride) and Group D (Controls) were unaffected. Weights in these three groups were maintained during this period. There were no deaths. Two rats were destroyed suffering from twisted neck (torticollis) but nothing abnormal was detected on *post-mortem* examination in either case.

The rats were healthy throughout the experiment, fed consistently well and remained active and in good condition to the end.

In Group C (5 per cent. solution) there was a sudden reduction in weight during the first two weeks from an average of 210 grammes to one of 150 grammes per rat.

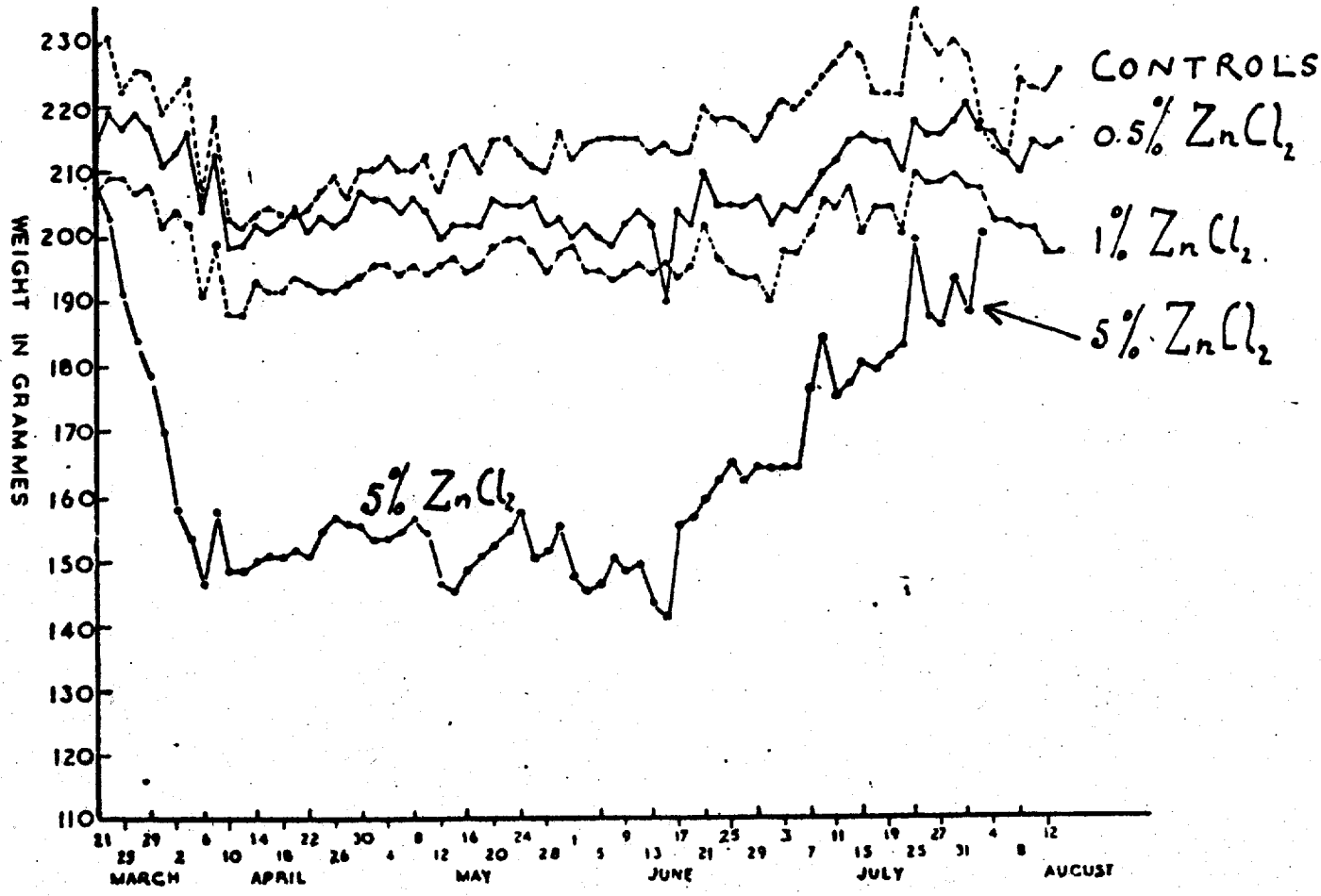
Deaths took place a fortnight after the commencement of the experiment. Thirteen rats died during the next ten days. A tolerance then seems to have been acquired and subsequent deaths occurred at approximately fortnightly to three-weekly or longer intervals. Six were still remaining when the experiment concluded. These six, along with equal numbers of the other groups, were destroyed at the end of six months and examined *post mortem*.

As a matter of interest the remaining animals of Groups A, B and D were kept for a further period of nine months (making a total of 15 months from the commencement of the experiment) and the observations continued. No clinical signs of illness whatsoever were shown.

The symptoms and clinical signs in Group C (see first of the rat Figs., overleaf) included general malaise, staring coat, huddled posture, and loss in weight, but feeding was unaffected throughout.

The *post-mortem* appearances were quite striking. On opening the abdominal cavity the black, slimy character of the intestines was very prominent. This appearance slowly changed, however, and in rats which died later on in the experiment the intestines showed only a grey, slimy appearance.

Graph Showing Average Weight of Rats in Each Group



RAT 535 (5 per cent. Zn Cl_2) 24 hours before death. Staring Coat: Huddled Posture.

NORMAL RAT

SUMMARY OF TYPICAL *Post-mortem* FINDINGS

ANATOMICAL:

Black discoloration of small intestines; haemorrhagic gastritis; generalised enteritis; slight degree of pulmonary congestion. Spleen, liver, kidney and heart showed no lesions.

HISTOLOGICAL:

Intestines: Multiple mucosal erosions with blood clots over them.

Kidney: Congestion. Much tubular degeneration of Henle's loops and collecting tubules, with much iron pigment in some tubules. Glomerular tufts swollen but not uniformly so.

Lungs: Congestion, haemorrhage and oedema with numerous foamy macrophages and some metaplasia of alveolar lining cells.

Spleen: Congestion. Large macrophages stuffed with pigment. Disappearance of lymphocytes. No necrosis apparent.

Heart: Showed small areas of oedema.

Liver and Adrenals: Showed congestion.

The Prussian Blue reaction for sections of the kidney, lung and spleen demonstrated the presence of iron.

CONCLUSION

Rats [illegible] food contaminated with various concentrations of zinc [illegible] could tolerate the daily intake of 12 ml. 0·5 per cent. zinc [illegible] (equivalent to 60 mg. zinc chloride per day) and 12 [illegible] per cent. zinc chloride (equivalent to 120 mg. zinc chloride per day) over a period of 15 months. Those rats taking [illegible] 12 [illegible] per cent. zinc chloride solution (equivalent to 600 mg. zinc chloride per day) succumbed comparatively quickly however. *Post-mortem* appearances were characteristic. Damage to the kidney, suggesting a cumulative action of zinc, was the most significant point. The corrosive action of zinc on the alimentary tract was shown.

RAT 535 (5 per cent. Zn Cl_2).
Black slimy character of intestines.

Summary

An unsuccessful attempt was made to induce a chronic type of zinc poisoning in two sheep, two goats, a calf, and a cow, by feeding them exclusively for a period of over five months on forage contaminated with zinc chloride solutions and "smoke." The "smoke" was produced by burning hexachlorethane smoke composition as used in standard Service smoke generators which produces over 50 per cent. zinc chloride as an end product.

No adverse clinical signs nor symptoms were manifested and no pathological lesions were shown on *post-mortem* examination. The carcases were normal and passed fit for human consumption. The calf is to-day (January, 1948) a normal healthy beast.

Rats could tolerate the daily intake of up to 120 mg. zinc chloride in their food over a period of 15 months. Those taking in 600 mg. zinc chloride daily, however, succumbed quickly showing characteristic symptoms and *post-mortem* lesions. The main effect seen was the corrosive action on the intestines and a characteristic damage to the kidneys due perhaps to the cumulative action of zinc.

Acknowledgments.—The writer wishes to thank the Chief Scientist, Ministry of Supply, for permission to publish this paper.

Thanks are also due to Captain K. R. Gabb, R.A.V.C. and members of the Veterinary and Technical Chemistry Sections, Experimental Station, Porton, for technical assistance.

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ANALYTICAL BIOCHEMISTRY 14, 191–204 (1966)

Zinc Analysis in Biological Specimens by X-Ray Fluorescence

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Received August 5, 1965

The nondispersive x-ray emission technique has been applied in our laboratory to the detection of selected elements in biological material (1, 2). In this method inner shell ionizations of the atoms of the sample are caused by x-rays and the energy distribution of the resultant fluorescence radiation is then determined by means of pulse-height analysis of the voltage pulses produced by a proportional counter. This method has an advantage of increased sensitivity over that of x-ray emission spectroscopy of the dispersive type (crystal monochromator) and in simplicity of sample preparation over some of the other analytical methods employed in the determination of trace and near-trace elements in biological material, e.g., atomic absorption, light emission spectroscopy. The fraction by weight of the element of interest can be obtained directly for certain trace and near trace elements in samples of (*a*) dried, microtomed tissue sections and (*b*) dried suspensions or solutions. The fraction is obtained without the requirement of accurate weighings or volumetric determinations (1, 2). With this technique we have obtained limits of detectibility of about 10^{-9} gm of bromine in 35-μg samples of deoxyribonucleic acid (2), and Hall (1) has obtained limits of between 10^{-9} and 10^{-10} gm of zinc in favorable biological tissue sections and fluids with 1- to 5-min integration times.

However, the poor resolution of this method limits its application to samples in which interferences from extraneous lines are minimum. This limitation can be overcome in some cases by the use of differential filtration using two proportional counter detectors (1), or by a special method of pulse-height analysis similar to that applied by Dolby (3). The differential filtration technique lacks general applicability and the method of Dolby involves quite complicated electronics.

We have in turn developed an analysis technique which uses x-ray dispersive analysis to determine the mass of an element present in relatively small samples or a combination dispersive-nondispersive technique

for the determination of the fraction by weight of the element of interest. The use of dispersive analysis results in a sensitivity which is somewhat lower than nondispersive (4) with adequate resolution to make negligible any spectral line interferences expected in most biological specimens. A comparison of resolution of the two methods is made graphically clear in Fig. 1, where a spectrum taken with a curved crystal spectrometer is compared to that of a proportional counter spectrometer. Both spectrometers are operated under what we consider approximately maximum

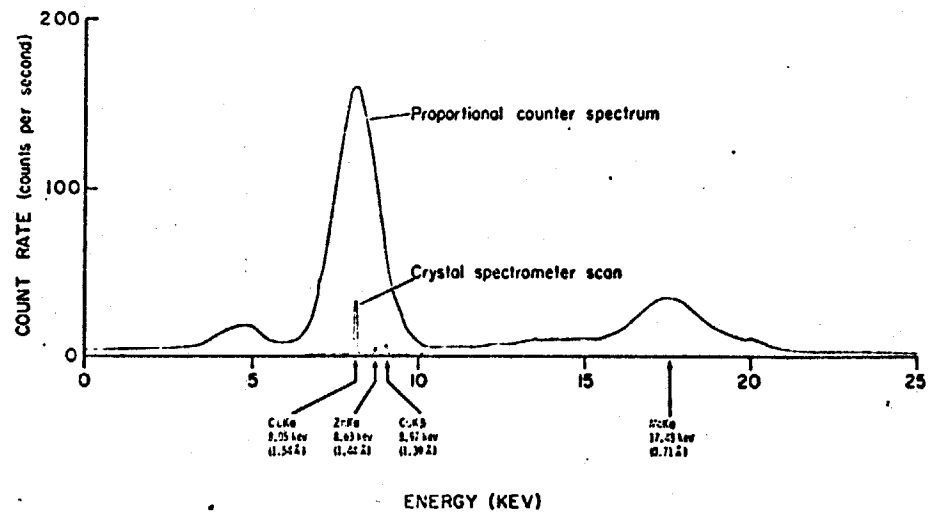

FIG. 1. Comparison of fluorescence spectra taken with a proportional counter spectrometer (nondispersive) and a crystal spectrometer (dispersive). The sample for both spectra is a 400-μg sucrose sample containing 1 μg copper and 0.1 μg zinc. The dispersive spectrum is obtained with the x-ray tube operating at 50 kv and 30 ma with no filter in the exciting x-ray beam, while the nondispersive spectrum was obtained at 50 kv and 4 ma with a 0.003" titanium filter in the exciting x-ray beam and no filter between the sample and proportional counter window. Under these operating conditions, the integral count rate is about 1000 cps.

sensitivity conditions for analysis. It should be noted that we are not using the maximum intensity available from the x-ray tube (50 kv, 30 ma) in the nondispersive spectrometer. The positive ion sheath effect in the proportional counter (5), which in our counter[1] is no longer negligible above an integral count rate of 1000 counts per second, requires that we use this relatively low value of tube current for accurate analytical results.

[1] This counter was built to our specifications by LND Corp. It has an aluminum cathode with an inside diameter of 1.960" and central wire diameter of 0.002". The filling is 90% argon and 10% methane at 1 atm. The window is 0.002" beryllium.

METHOD

The essential arrangement of the dispersive-nondispersive method is shown in Fig. 2. The important dimensions are given in the legend of this figure. The vacuum chamber was already in use in our laboratory for nondispersive analysis (2) and with slight modifications is used in the new dispersive-nondispersive apparatus. Because an available

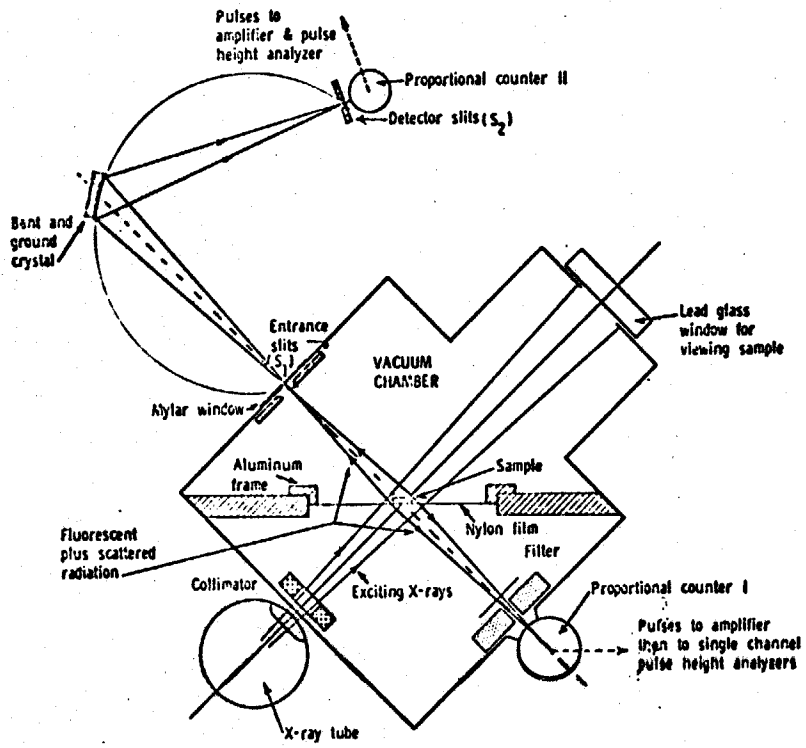

FIG. 2. Arrangement for dispersive-nondispersive x-ray fluorescence analysis. Distance from center of x-ray tube to sample and from sample to S_1 is 2¾". Distance from sample to collimator of proportional counter I is ⅞". The x-ray and proportional counter collimators are both titanium, ¼" thick with a ⅛" aperture. Filters employed vary depending on the analytical problem. A nickel filter from 0.0025" to 0.007" thick is used for zinc analysis in samples from the male reproductive tract.

vacuum chamber was used, the dimensions were not adjustable and those given in the legend of Fig. 2 are not necessarily dimensions for optimum sensitivity. The chamber head, containing the lead glass window, is vacuum sealed to the body of the chamber by means of an "O" ring. The aluminum frame (Fig. 3, lower right), and hence the sample, is changed by removing the chamber head. A lead shutter directly above

the x-ray tube allows for removal of the head with the x-ray tube maintained at full power (50 kv, 30 ma). Beryllium, 0.005" thick, is used as a vacuum window at the entrance port for the exciting x-radiation and 0.002" mylar is used at the exit port where slit S_1 is located. Proportional counter I is made vacuumtight by means of an "O" ring seal which is designed into the body of the counter.[1] The vacuum is produced by a forepump. It requires about 20 sec to reach operating pressure after being opened to the atmosphere.

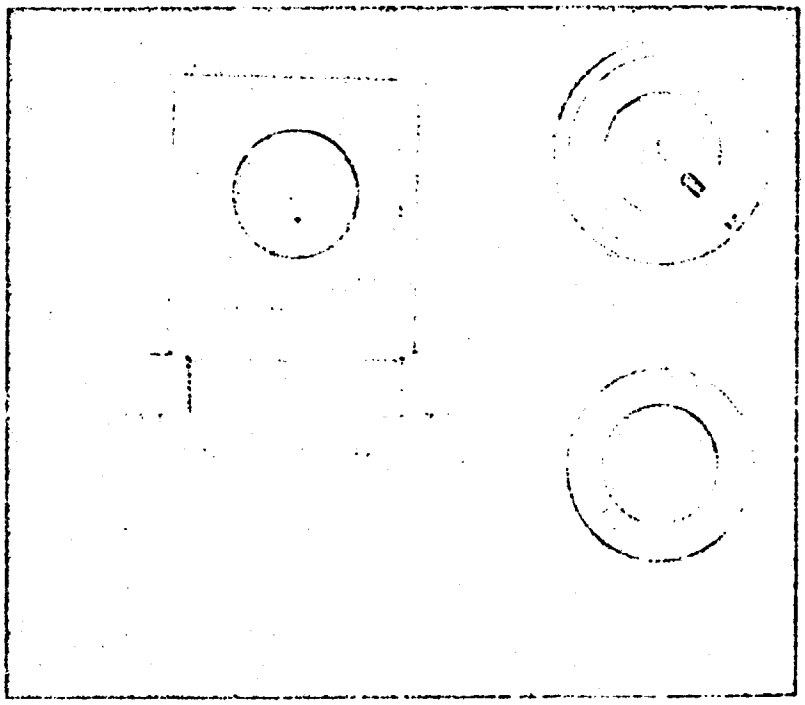

FIG. 3. Aluminum frame to hold nylon film (lower right), frame holder and frame as it goes into specimen chamber with a dried sample in the center (left), and aluminum frame with nylon film in centering template (upper right).

The amount of the element of interest is inferred from the intensity of the element line obtained with the crystal spectrometer. The weight of the sample is determined from the intensity of scattered x-ray lines in the exciting radiation, Mo-K lines when a molybdenum target x-ray tube is used, and is detected by proportional counter I in Fig. 2. The intensity of the scattered Mo-K radiation is very nearly proportional to the mass of the sample (1, 2), since most biological samples consist of a relatively small percentage of a heavy element in a light matrix and the matrix

elements scatter x-rays of these wavelengths with approximately the same intensity per unit mass.

A vacuum specimen chamber is required since the number of Mo-K photons scattered from the relatively small samples and entering the counter is manyfold less than the number entering due to scattering from air at atmospheric pressure (2). The vacuum chamber also serves to reduce background of the crystal spectrometer (4). The analyses are generally carried out at a chamber pressure of about 70 μ of Hg.

Our work to date with this instrument has involved the analysis of zinc in tissues and fluids. For this purpose a Machlett AEG 50S x-ray tube with a molybdenum target was used obtaining power from a constant-potential 50-kv generator (Norelco #12045) with milliamp and voltage stabilizers. A reflecting-type curved crystal spectrometer[2] uses the (200) Bragg reflection planes of lithium fluoride bent to a radius of 22″ and the surface ground to 11″ according to the method of Johansson (6). The motion of the spectrometer maintains focus at all wavelengths available to it and, at the same time, results in a crystal motion which allows the crystal to intercept approximately the same cone of radiation emitted from the source at all wavelengths (7). The detector used in the spectrometer is a side-windowed proportional counter or a scintillator counter.[3] The spectrometer slit widths are adjusted for maximum line square over background L^2/B as is the window width on the pulse-height analyzer. For the $ZnK\alpha_{1,2}$ line, the maximum L^2/B is obtained with $S_1 = S_2 \simeq 0.025''$ and with the window width on the pulse-height analyzer[4] set for 0.7 volt when the midpoint of the $ZnK\alpha_{1,2}$ line lies at 2 volts, with the proportional counter used as the detector.

The samples are supported by a single layer of nylon between 900 and 1300 A thick. The nylon support is prepared by floating a 10% solution of nylon in isobutyl alcohol on water and lifting the film off with an aluminum frame. Figure 3 shows the aluminum frame, sample and frame holder, and positioning template. This method of sample presentation allows us to reposition the center of different specimens to better them a few thousandths of an inch.

Samples of suspensions or solutions are prepared by placing them on the nylon support with a micropipet. The sample is then dried in a desiccator for about 1 hr and is then subjected to a pressure of 10^{-3} mm Hg for 1 hr.

Our work with assays for zinc in tissues and fluids of the male mam-

[2] This is an early Applied Research Laboratories continuous-motion spectrometer.

[3] LND Corp. proportional counter #451 with a 0.002″ beryllium window and a Hamner Electronics scintillation counter #SX10, Serial 60.

[4] Hamner Electronics #N-686.

malian reproductive tract[5] requires, in certain cases, only an absolute determination of zinc mass. This is so, for example, when a sperm count is available. A zinc assay then leads to zinc mass per sperm. When the mass of dried tissue, suspension, or solution can be obtained by other means, then a zinc assay will lead to per cent by weight of zinc in the sample of interest.

RESULTS

(*a*) *Calibration and Limits of Detectability.* For absolute determinations use is made of calibration curves based on known amounts of zinc in a simulated biological matrix, sucrose is presently used as this matrix (1, 2). Such a calibration curve is shown in Fig. 4. The data for this curve was obtained with samples whose original liquid volume was 35 μl. This

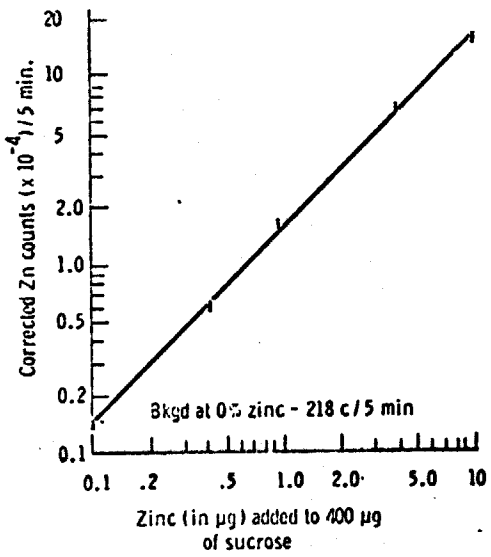

FIG. 4. Calibration curve for zinc in sucrose standards. Standards prepared by reacting accurately weighed amounts of zinc in nitric acid and adding this solution to known concentrations of sucrose. Samples are placed down in 35-μl volumes of solution. The indicated points and the levels of confidence for the 0.1-μg and 0.4-μg zinc levels are the means and standard deviations based on measurements of 6 separate 35-μl samples in each case. The points for the other zinc levels are based on single measurements and the relative standard deviations are assumed equal to that found at 0.4 μg.

[5] Publication of two papers are in preparation in collaboration with Drs. S. Saito, I. M. Bush, and W. F. Whitmore, Jr., of Urology Service, Memorial Hospital. Analyses on about 400 different specimens of sperm suspensions, supernatant solutions, and seminal plasma have been accomplished in connection with these studies in a four-month period.

curve suggests linearity over at least two decades. The calibration curve indicates a limit of detectability for zinc in biological specimens, whose mass is about 400 μg, of about 4×10^{-9} gm in a 5-min integration period. Loss of detectability is arbitrarily assigned to a line whose magnitude is less than four times the magnitude of the counting error in the background. The quoted limit of detectability should vary only slightly with sample mass, being slightly lower for smaller mass of sample. Determinations of bromine content have also been made with our spectrometer. The limit of detectability for bromine in a 35-μg sample of sucrose, with a scintillation counter as the detector, is found to be about 1.5×10^{-9} gm for a 5-min integration.

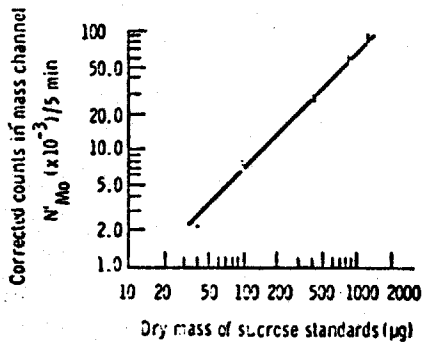

FIG. 5. Calibration curve for mass. Samples are dried residue of a solution of pure sucrose placed down in 35-μl volumes. No "precalibration" of nylon films is employed. The points and standard deviations are (*1*) determined on the basis of measurements of 6 separate 35-μl samples for the 400-μg mass, (*2*) estimated on the basis of measurements of 2 separate 35-μl samples for the 100-μg mass, (*3*) based on single measurements for the 800- and 1200-μg masses, with the relative standard deviations assumed equal to that found with 400 μg, and (*4*) based on a single measurement for the 10-μg mass, with no estimate of level of confidence given.

The calibration curve of Fig. 5, used to determine the mass of the sample, is obtained with sucrose standards. The error in determining the magnitude of the line N'_{Mo}, where $N'_{Mo} = N^{S}_{Mo}$(counts due to variously scattered Mo-K photons from sample, nylon film, and specimen chamber) $-N^{B}_{Mo}$ (counts due to scattered Mo-K photons from nylon film and chamber), becomes too large to make useful mass determinations on samples of less than 40 μg. It is evident from Fig. 5 that there is a considerable error in the determination of a mass of 40 μg. In obtaining the above data, the intensity of the background N^{B}_{Mo} is determined on only one nylon film and assumed to be the same for all other frames made from the same large piece of floated nylon. The thickness can vary considerably in one large nylon film and this could result in an error in

the estimate of the level of the background. With small samples, where the intensity of the signal is not much larger than the background intensity, small errors in background intensity will introduce large errors in mass determination.

It is found that the mass determinations for small samples can be considerablly improved over that shown in Fig. 5 by using smaller liquid volumes (10 μl), which results in a smaller spread in the dried samples, and by "precalibrating" the nylon films for each sample. Precalibration is accomplished by measuring N^{B}_{Mo} for each film before placing the sample on it. With this technique we have obtained the calibration curve shown in Fig. 6. With this more involved and tedious procedure we are able to measure masses down to at least 20 μg with accuracy of better than 10%.

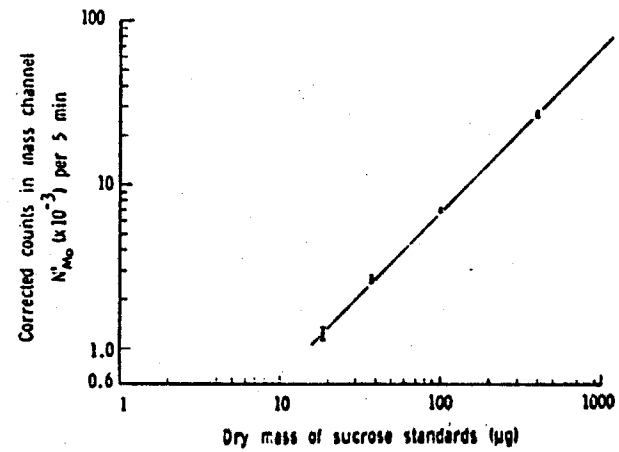

FIG. 6. Calibration curve for mass using "precalibrated" nylon films and 10-μl volumes of sucrose solution. The indicated points and standard deviations for the 18- and 38-μg masses are the mean and standard deviations based on measurements of 6 separate 10-μl samples in each case. The other two points are based on single measurements where the relative standard deviations are taken equal to that obtained with the 38-μg mass. The data are corrected for instrumental drift, which is relatively small.

(*b*) *Precision and Accuracy.* The sample dimensions must be kept below an experimentally determinable maximum size if the magnitude of the element line is to be a function only of the amount of element present and not sample size. The same is true for the magnitude of the line in proportional counter I (Fig. 2) for the determination of mass. From consideration of the data graphed in Fig. 7, one can conclude that sample size variation below ¼" will introduce an error of at most 2% in the ratio N'_{Zn}/N'_{Mo}, and 4% in the magnitude of the ZnKα line, N'_{Zn}.

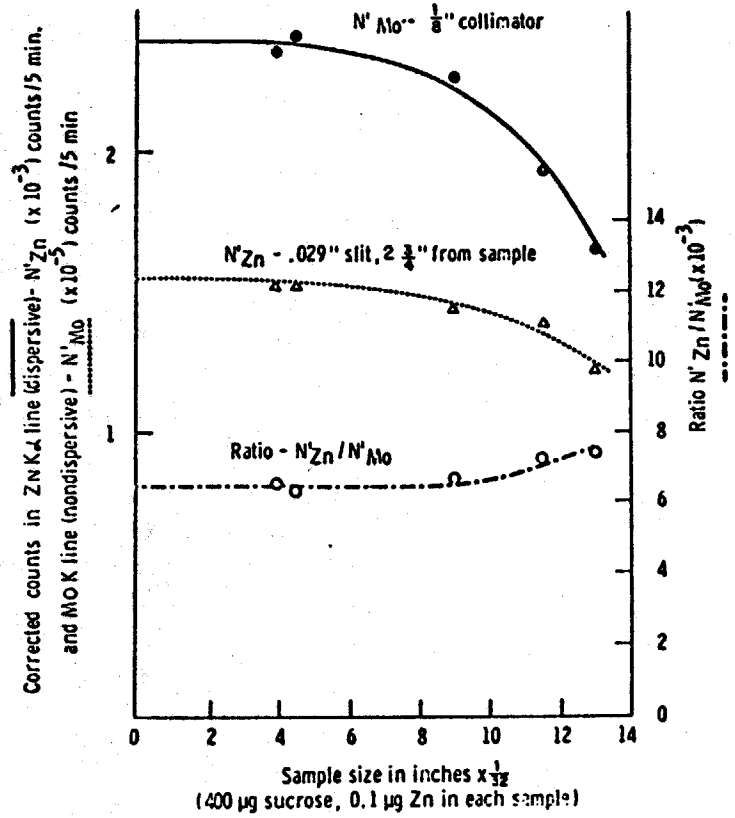

FIG. 7. Corrected counts in zinc channel N'_{Zn}, corrected counts in molybdenum channel N'_{Mo}, and their ratio N'_{Zn}/N'_{Mo} as a function of sample size. N'_{Zn} is obtained from the relation:

$$N'_{Zn} = N^{s}_{Zn} - \left(N^{n}_{Zn} + \frac{\Delta N^{m}_{Zn}}{\Delta M} \cdot M_s\right)$$

where N^{s}_{Zn} is the measured intensity of the ZnKα line for a 400-µg sucrose sample containing 0.1 µg zinc, N^{n}_{Zn} is measured intensity at the position of the ZnKα line for the nylon film, $\Delta N^{m}_{Zn}/\Delta M$ is the rate of change of ZnKα counts with mass of pure sucrose standards, and M_s the mass of the sample by an x-ray determination. N'_{Mo} is the difference in counts in the molybdenum channel with 400-µg sucrose sample and with no sample, nylon film only.

This would suggest an error due to size variation, where samples are maintained below ¼", of about 2% in the ratio, N'_{Zn}/N'_{Mo}, and 4% in the magnitude of the ZnKα line, N'_{Zn}. This would indicate an error due to size variation, where samples are maintained below ¼", of about 2% for determination of weight per cent of element and about 4% for absolute determination of zinc mass in a given sample. A log-log plot of the ratio of corrected counts in the element channel to corrected counts in the mass

channel versus actual mass of zinc to mass of sample in accurately prepared standards is given in Fig. 8. The fact that a straight line approximates all but one of the data points so accurately indicates that the dispersive-nondispersive method developed can be used to determine the ratio of the mass of the element of interest to that of the sample for biological specimens, i.e., specimens with a small percentage of a heavy element in a light matrix. The one point which lies appreciably off the line is for the sample whose mass is 40 µg. This results from the error in

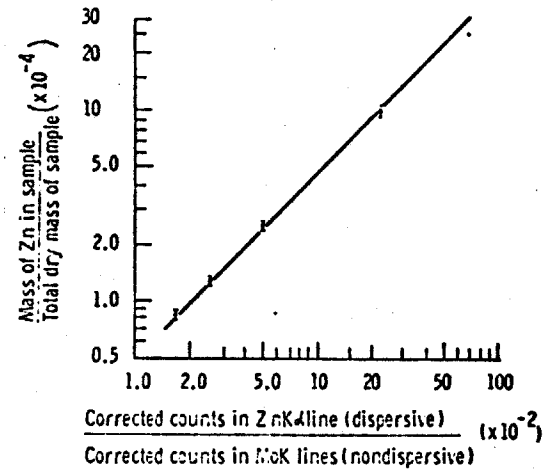

FIG. 8. Ratio N'_{Zn}/N'_{Mo} versus the ratio of mass of zinc to mass of sample in accurately prepared standards of zinc in sucrose. N'_{Zn} and N'_{Mo} are as defined in the legend of Fig. 7. All the standards contain 0.1 µg of zinc in varying masses of sucrose and all samples are placed down in 35-µl volumes. The points and standard deviations are (1) determined on the basis of measurements of 6 separate 0.1-µg zinc in 400-µg sucrose standards for the 2.5×10^{-4} ratio, (2) based on measurements of 2 separate 0.1 µg zinc in 100 µg sucrose for the 10^{-3} ratio, (3) based on single measurements of 0.1 µg zinc in 800 and 1200 µg sucrose for the 1.25 and 0.83×10^{-4} ratio samples, respectively, where the relative standard deviation is assumed equal to that found in the case of 0.1 µg zinc in 400 µg sucrose standards, and (4) based on a single measurement on the 0.1 µg zinc in 40 µg sucrose standard for the 25×10^{-4} ratio, with no estimate of the level of confidence given.

determination of this small a mass which has already been discussed in relation to Fig. 5. By use of the more involved procedure described just above, we should be able to improve the analysis for mass and, concomitantly, for fraction by weight with samples whose mass is 40 µg or less.

An experimental determination of precision was obtained by analyzing 6 separate 20-µl samples prepared from the same standard solution of zinc in sucrose. All the samples should have, within the errors involved in

pipetting, 0.1 μg of zinc in 400 μg of sucrose determined by a volumetric measure of the solution deposited. The actual relative standard deviations obtained for 5-min integration are 5% for N'_{Zn}, 4% for N'_{Mo}, and 6% for the ratio N'_{Zn}/N'_{Mo}, where N'_{Zn} and N'_{Mo} are as defined in the legend of Fig. 7. The values of relative standard counting errors expected on the basis of counting statistics are 3, 0.2, and 3%, respectively, where these values take into account the error added due to the subtraction of background from the signal. Evidence obtained from repositioning studies with the same sample indicates that the major portion of the increase in actual error over the counting error results from the inability to reproduce position exactly.

A similar precision analysis for 6 separate samples of 10 μl of seminal plasma of dog gave approximately 4, 3, and 2%, respectively, for the relative standard deviations of N'_{Zn}, N'_{Mo}, and N'_{Zn}/N'_{Mo}, where the relative counting error expected is 1.2, 0.8, and 1.4%.

TABLE 1
COMPARISON OF ZINC DETERMINATIONS BY X-RAY EMISSION AND ATOMIC ABSORPTION

Seminal plasma specimens	Dry mass of 20 μl of specimen, determination by x-ray measure, μg	Mass of zinc in 20 μl of plasma as determined by x-ray measure, μg	Volume in ml of distilled water added to 0.35 ml of plasma suspension	Expected concentration of zinc in diluted suspension, based on x-ray determinations of zinc mass, ppm	Actual determinations of zinc concentration in these diluted suspensions as measured with atomic absorption, ppm
Dog 694 6/15/65	601	0.01	8.8	1.17	1.23
Dog 694 6/16/65	1008	2.17	35.5	1.21	1.20
Dog 754 6/17/65	630	1.05	14.8	1.21	1.17

Determinations obtained with our analytical technique were compared with that given by atomic absorption spectrophotometry,[6] as a check on the accuracy of our method. The comparison given in Table 1 show that, in this very limited comparison, the two methods agree to 5% or better.

A study was carried out to see if changes in filtration and filling of the proportional counter used for mass determination (counter I) would have an effect on the analysis. Acceptable differences were found on the use of two different counter fillings and filtration as is shown in Table 2. However, from our work, we have found that the improper use of the combination of filtration and filling can lead to erroneous results.

[6] These analyses were kindly supplied by Dr. A. Cavhalo of the Institute for Muscle Disease, New York, New York. They were performed on a Perkin-Elmer model 303 atomic absorption spectrophotometer with a hollow cathode lamp.

TABLE 2
FILLING AND FILTRATION FOR PROPORTIONAL COUNTER I (Fig. 2)

Frame #	Specimen	90% neon, 10% methane at pressure of 350 mm Hg. 0.0035" nickel filter		90% argon, 10% methane at pressure of 700 mm Hg. .007" nickel filter	
		Dry mass of sample determined by x-ray measure, μg	Zinc content, parts per thousand dry weight	Dry mass of sample determined by x-ray measure, μg	Zinc content, parts per thousand dry weight
10	Supernatant[a]	432	<L.D.[b]	442	<L.D.
4	Supernatant	404	<L.D.	409	<L.D.
0	Sperm	1679	0.77	1567	0.80
48	Sperm	736	0.29	636	0.34
42	Sperm	483	0.12	393	0.14
11	Sperm	1137	0.29	1137	0.29
32	Sperm	1468	2.07	1455	2.04
47	Sperm	437	0.21	422	0.22
26	Sperm	519	0.51	518	0.50
30	Sperm	1793	0.27	1764	0.28
18	Sperm	1462	0.35	1461	0.35
12	Sperm	1831	0.21	1747	0.21
8	Sperm	2524	0.16	2433	0.15
41	Sperm	1744	0.43	1670	0.43
39	Seminal plasma	1319	2.20	1377	2.11

[a] Supernatant of sperm suspension.
[b] Indicates less than the limit of detectability which for a 400-μg sample would be about 0.01 part per thousand.

Recovery data on zinc content of dog epididymal sperm suspensions have been obtained. These experiments show that within expected experimental error the zinc content in the original sperm suspension equals the sum of the zinc contents in sperm sediment and supernatant plasma. This study will be discussed in detail in an article in preparation.[5]

DISCUSSION

The precision of the method for zinc in routine analysis is equivalent to an error of about 5% in the weight fraction of the element present at the level or 0.1 μg of zinc in samples of the order of several hundred micrograms. We feel that this precision is adequate for the purposes of the studies we have undertaken, which require assays for zinc on different specimens from the male, mammalian reproductive tract.[5] With very small samples (10 μl) and special care, we have been able to improve this to an error of 2% in the weight fraction of the element present (see preceding section). Accurate analyses of samples with masses in the

order of 20 μg requires the use of 10-μl samples and precalibration of films.

Lack of centering of samples in the frame holder has the same effect as making the diameter too large. For this reason accurate positioning (centering) is essential. Even with the special care taken to maintain accurate positioning, this factor remains the main source of error in reproducibility.

Theoretical considerations suggest that with the proper choice of crystals and detectors our spectrometer will give detectable limits close to those already found for zinc and bromine for elements from Ti (22) to Pb (82). Detectable limits of approximately these values were experimentally found by Barstad and Refsdal (4) for these elements. A discussion of detectable limits for elements below atomic number 22 is beyond the scope of this article. However, the factors governing detectability in ordinary emission x-ray spectroscopy (8) apply here as well and can be used to estimate sensitivity for the detection of the light elements. Detectability of some of the heavier elements, above about molybdenum (42), can be considerably improved by the use of an x-ray tube operated at 100 kv instead of the 50-kv tube we presently use or the 35-kv used by Barstad (4).

SUMMARY

An x-ray fluorescence analysis technique is described which allows for the direct determination of the weight fraction of zinc in biological specimens from the male reproductive tract of several mammalian species. It can also be used for absolute determinations of zinc content in small biological specimens with a limit of detectability of about 4×10^{-9} gm in a 5-min integration period. The same instrumentation, with modifications, can be applied to the determination of a number of elements above atomic number 11 found in biological material.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Research Grant HD01410 from the National Institutes of Health.

The authors wish to thank Dr. A Cavhalo for supplying the determinations of zinc content by atomic absorption spectrophotometry. We wish particularly to thank Dr. R. Rikmenspoel for his criticism of this manuscript.

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Proc. Soc. Exp'tl. Biol. Med. 51(2): 249-251 (1942)

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On the Adenosine Triphosphate-Myosin System.

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Recent data indicate that the chemical energy resulting from the breakdown of carbohydrate in muscle is converted to the mechanical energy required for muscle contraction by means of a reaction between adenosine triphosphate and myosin.[1] Engelhardt and Ljubimova have shown that this is an enzymatic reaction during which adenosine triphosphate is hydrolyzed to adenosine diphosphate and inorganic phosphate.[2] Needham *et al.* suggest that this reaction is associated with the stretching of the myosin fiber from the contracted state, to which it returns on nerve stimulation.[3] Since the adenosine triphosphate-myosin system is so intimately related to the contraction of the myosin fibrils,

1 Kalckar, H. M., *Chem. Rev.*, 1941, 28, 71.

2 Engelhardt, W. A., and Ljubimova, M. N., *Nature*, 1939, 144, 668.

3 Needham, J., *et al.*, *Nature*, 1941, 147, 766.

TABLE I.
Hydrolysis of Adenosine Triphosphate by Myosin in Presence of Added Substances.

No.	System*	Mg inhibitor added	Total P liberated in γ	γ P liberated enzymatically	% enzymatic hydrolysis
1	A.T.P.	—	9	—	0
2	A.T.P. + Myosin	—	17	8	14
3	A.T.P. + Ca + Myosin	—	27	18	31
4	A.T.P. + Ca + Myosin + Acetylcholine Bromide	0.1	27	18	31
5	A.T.P. + Ca + Myosin + Ethoxycholine Bromide	0.1	27	18	31
6	A.T.P. + Ca + Myosin + Curare†	0.1	28	19	33
7	A.T.P. + Ca + Myosin + Zinc Chloride	1.0	9	0	0
8	A.T.P. + Ca + Myosin + Copper Sulfate	1.0	10	1	1.7

*1.2 mg of the barium adenosine triphosphate were added in each experiment as the sodium salt. The time of reaction was 12 minutes.

† The curare, Squibb Intocostrin, was obtained in solution from E. R. Squibb and Sons, New York City. Its concentration is based upon physiological assay.

it was considered of interest to study the effects of chemical mediators of nerve stimulation, acetylcholine and epinephrine, which influence muscle contraction, on the velocity of this reaction with a view toward elucidating the site of action of these substances.

It was observed that the liberation of inorganic phosphate was entirely uninfluenced by any of the following substances: acetylcholine, ethoxycholine, epinephrine, eserine, atropine, curare, nicotine, cysteine, heparin, fluoride, thiocyanate, and cyanide. Only when copper* and zinc salts were added was the reaction inhibited. It would appear, therefore, that the point of action of such muscle stimulators as acetylcholine, epinephrine, curare, etc. is not the adenosine-triphosphate system, at least as it was studied *in vitro*.

Myosin was prepared by the method of Greenstein and Edsall.[5, †] The monobarium salt of adenosine triphosphate was prepared according to Kerr.[7] It had a total phosphorus content of 13.1%, an easily hydrolyzable phosphorus content of 8.6%, and was free of inorganic phosphate. Before each experiment it was converted to the sodium salt by treatment with sodium sulfate. The runs were carried out in borate buffer of pH 8.6. To 0.2 cc of a myosin solution containing 2.8 mg N per cc, 1.8 cc of borate buffer, 0.1 cc of a solution of the substance being tested, 0.05 cc of a 0.1 molar calcium chloride solution, and 0.01 cc of a solution containing the equivalent of 1 mg of the barium salt of adenosine triphosphate were added. This system was incubated at 37°. After a specific interval which varied from 12 to 25 minutes in different experiments, 2 cc of a 10% trichloracetic acid solution were added and the protein precipitate was removed by centrifugation. Inorganic phosphate was determined in the supernatant solution by the method of Fiske and Subbarow.[8] In some experiments the calcium chloride was omitted. Appropriate control experiments were carried out without myosin and without adenosine triphosphate.

In Table I are given results obtained in a typical experiment. It is apparent that the organic substances tested were inactive. When studied in the same manner, epinephrine

* The inhibiting effect of copper salts has recently been reported by Bailey.[4]

[4] Bailey, K., *Biochem. J.*, 1942, 36, 121.

[5] Greenstein, J. P., and Edsall, J. T., *J. Biol. Chem.*, 1940, 133, 397.

† It was considered desirable for the purpose of the present experiments to avoid more than one precipitation of the myosin. It may be expected, for this reason, that it contained significant amounts of adenosine diphosphatase.[4,6]

[6] Ljubimova, M. N., and Pevsner, D., *Biochimia*, 1941, 6, 178.

[7] Kerr, S. E., *J. Biol. Chem.*, 1941, 139, 121.

[8] Fiske, C. H., and Subbarrow, Y., *J. Biol. Chem.*, 1925, 66, 375.

hydrochloride, eserine sulfate, atropine sulfate, nicotine and sodium fluoride, in 100 γ quantities, and cysteine hydrochloride, potassium thiocyanate, potassium cyanide, and the sodium salt of heparin‡ in 1 mg quantities were also inactive.

Summary. Acetylcholine, epinephrine, a series of alkaloids, and several other substances were without influence on the hydrolysis of adenosine triphosphate. Copper and zinc salts inhibited the reaction.

‡ This was obtained from Hoffmann-La Roche and Co., Nutley, N.J.