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A REACTION OF THE ADRENALS TO VITAMIN D

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SUMMARY

When rats were given an intravenous injection of a water-miscible preparation of vitamin D_3 there was an increase in the concentration of an unidentified substance in the adrenal glands. This substance appeared as an ultraviolet-absorbing spot on a paper chromatogram after passage of the non-saponifiable extracts of the glands through MgO-kieselguhr columns. It was at its maximum concentration in the glands 30 min after injection of the vitamin, had different chromatographic and spectroscopic properties from vitamin D and had no anti-rachitic activity.

INTRODUCTION

There have recently been several reports on the relationship between the adrenal glands and vitamin D metabolism. RAOUL AND GOUNELLE[1] found that 35 μg of an intravenously injected dose of 40 μg of vitamin D_3 was accumulated in the adrenals in 30 min. The substance found was identified as vitamin D by its ultraviolet absorption and by its ability to induce healing in rachitic chicks. The recovery of this proportion of the injected dose implies a very great flow of blood through the adrenal glands. Other workers, however, using labelled vitamin D have found only a small percentage of the dose in the adrenals a few hours after administration but the concentration, compared with other tissues, was nevertheless high. Thus KODICEK, CRUICKSHANK AND ASHAY[2] found that 20 min after an intracardial injection of 50 μg ^{14}C-labelled vitamin D_2, rat adrenals contained 1.3% of the dose whereas the liver had 54.3% and the blood 10.7%. BLUMBERG, ABBI, HURNI AND SCHÖNHOLZER[3] gave rhesus monkeys an oral dose of 1.18 mg ^{14}C-labelled vitamin D_2 in oil and found 24 h later that the activity in the adrenals was 13.38 counts/min per mg dry tissue whereas that in the liver was 25.10 and in the blood 5.52. NORMAN AND DELUCA[4] used tritiated vitamin D_3. 20 min after their rats had received 500 I.U. of the labelled vitamin the activity in the adrenals was 11000 counts/min per mg dry tissue and in the liver was 12300, which means that as a percentage of the dose, the amount of the administered activity was 0.11 and 44.4 respectively for the two organs.

All these workers point to the importance of the adrenals in vitamin D metabolism by demonstrating a high concentration of the vitamin in the glands though RAOUL AND GOUNELLE's[1] observations differ considerably from the others in the degree of concentration. SALLIS AND HOLDSWORTH[5] have evidence of another sort. They found that some adrenal steroids and synthetic steroids enhanced the anti-

hitic effect of vitamin D when they were given to chicks along with the vitamin even though these steroids had no antirachitic effect themselves. When, however, the steroids were given daily for 3 days before the vitamin D was given, the anti-rachitic effect of the vitamin was reduced. They suggest that the vitamin may be converted to an active form by the adrenals and the steroids either stimulate the glands if given with the vitamin or cause some decrease in activity of the glands if given over a period of time before the vitamin is given.

This paper presents some observations on the production of an unidentified substance by adrenal glands in response to an injection of vitamin D.

EXPERIMENTAL

Injection of vitamin D

Pure vitamin D_2 or D_3 was dissolved in 0.05 ml ethanol per mg, mixed with an equal volume of Tween 80 (polyoxyethylene sorbitan mono-oleate) and diluted with water. This solution was injected into the tail vein of rats, the ear vein of rabbits or the jugular vein of larger animals. Sham injections were made with the same solution but without vitamin D. Mature hooded Norwegian rats were used in all the rat experiments.

Analysis of adrenal glands

Rats and rabbits were killed by a blow on the head and larger animals with a humane killer. The glands were removed immediately and kept at 0° or below, saponified at once by heating at 70–80° for 10 min with 2 ml 10% KOH in ethanol–water (1:1, v/v) or with 2 ml of the same solution per g tissue if the weight of tissue was more than 1 g. About 2 mg pyrogallol were added before saponification. After heating the mixture was diluted once with water and extracted three times with two volumes of ether. The ether was removed by distillation and the remaining solution, which contained water and ethanol, was evaporated *in vacuo*. The residue was made into a slurry with light petroleum (b.p. 40–60°) containing 0.5% (v/v) acetone and applied to a column of MgO–kieselguhr (50:50) as described by KODICEK AND ASHBY[6]. Their procedure for the subsequent chromatography of the eluate on paraffin-impregnated paper was followed except that the intermediate precipitation with digitonin was latterly omitted because this did not influence the results. Their Solvent B, isopropanol–methanol–water (15:82:3, v/v), gave the best definition and separation of ultraviolet-absorbing spots and was used in all the work described. The papers were prepared by dipping 25 cm square sheets of Whatman No. 2 chromatography paper through 5% (v/v) liquid paraffin in light petroleum, once in the direction of flow of the developing solvent and once in the opposite direction, drying for 15 min in air after each dipping.

For the estimation of substances on the paper chromatograms the following procedure was adopted. Where possible all the samples from one experiment were run on the same sheet of chromatography paper. Ten points, 2.5 cm apart were made on the starting line of each sheet of paper. Nothing was put on two of the points, on four were spotted known amounts of vitamin D, usually about 15, 30, 45 and 60 μg in ethanol and on the remaining four the adrenal extracts were applied. The papers were allowed to equilibrate in the atmosphere of the developing solvent for at least

5 h and usually overnight before developing which usually took about 5 h. The papers were dried in air for 30 min and then *in vacuo*. Spots which could be seen in ultraviolet light were outlined in pencil, cut out of the paper as a rectangle of a standard size and extracted with 5 ml ethanol. The blanks were necessary because the absorption in the ultraviolet due to residues from the solvents was appreciable and varied from sheet to sheet.

The curative, radiographic assay with rats of vitamin D activity in the non-saponifiable extract of rat adrenals was made using the method described by DALGARNO, HILL AND MCDONALD[7].

RESULTS

Rats were given 100 μg vitamin D_3 by injection into the tail vein and 30 min later were killed and their adrenals removed and analysed for vitamin D_3 by paper chromatography. No vitamin D was detected in the adrenals of rats which were given vitamin D_2 or D_3 or those which were sham-injected. In the animals given vitamin D_2 or D_3 an ultraviolet-absorbing substance was found with a mean R_F of 0.40 compared with a mean R_F of 0.51 for the vitamin. This material had an absorption maximum at 275 mμ and a minimum at 262 mμ. The absorption curve is given in Fig. 1 with that of vitamin D for comparison.

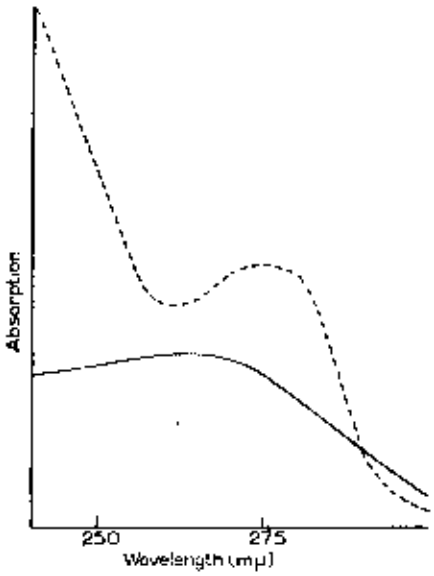

Fig. 1. Absorption spectra of vitamin D (— · —) and the unidentified substance isolated from adrenals (- - - -).

The experiment was repeated with a sheep given 15 mg of the vitamin and the adrenals were dissected into medulla and cortex before saponification. The substance was found to be entirely in the cortex and not at all in the medulla. In the sheep and the goat the level of the substance was found to be much greater in one gland, usually but not always, the left. This was not observed in the rat and other animals were not examined. The extracts from rat adrenals had no anti-rachitic activity for rats. The

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ultraviolet-absorbing substance was detected in liver and ileum in small quantities 30 min after an intravenous injection of vitamin D but not in spleen, kidney, duodenum or jejunum.

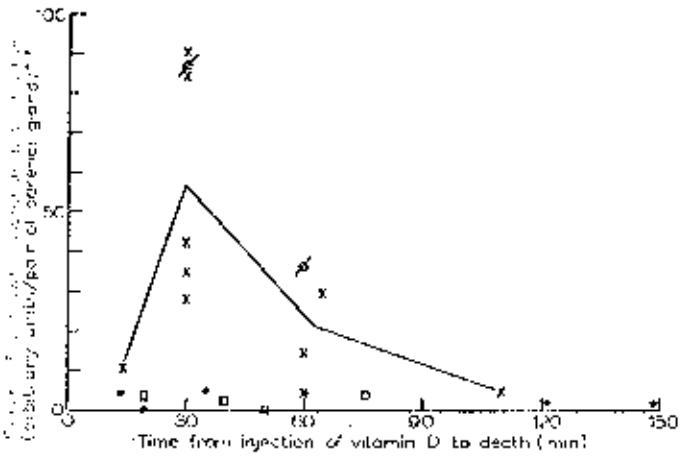

Fig. 2. The concentration of the unidentified substance in adrenals in rats (×), sheep (●), goats (▲), a rabbit (⊛) and a pig (○), also of vitamin D in rats found by RAOUL AND GOUNELLE (β)* (ref. 1). The line shows the mean changes in the rat.

* For these data the ordinate is percentage recovery of vitamin D.
** For the calculation of this figure see the text.

The concentration of this unidentified substance in the adrenals varied with the time between injection and death of the animal and also with the species of animal. Fig. 2 shows results from experiments with 27 rats, 7 sheep, 4 goats, 3 rabbits and 2 pigs. In the rats there was a rapid increase in concentration which reached a maximum 30 min after injection followed by a rapid decrease. In the figure the absorbancy of the unknown substance is assumed to be the same as that of vitamin D so that the ordinate is the concentration of the substance as a percentage of the injected vitamin D. These results can then be compared directly with those of RAOUL AND GOUNELLE[1] who claimed a high recovery of the vitamin from the adrenals. With a larger injection than 100 μg of vitamin D the concentration of the substance in the adrenals was not increased. The substance appeared on the chromatograms of extracts from 5 out of 10 rats which had had a sham injection or no injection at all but the spot produced was then of low intensity, having a mean value of 6.8 ± 3.9 units on the ordinate of Fig. 2. The value for the control in each experiment was subtracted from the concentration of the substance in the adrenals of rats which had received an injection of vitamin D. Similarly values of 2 and 3 units were obtained from two uninjected sheep and were subtracted from the values obtained from experimental animals.

With the sheep there was a maximum between 35 and 80 min after injection of a dose of 15 mg of vitamin D. The goats were given the same dose and showed similar changes while the rabbit was given 2.5 mg and the pig 13 mg. The concentrations observed in all these animals were very small compared with those from the rats. The actual concentration of the substance was about 10 times as high at its maximum

in the rat than in any of the other animals. In the larger animals only one level of injection (about 0.4 mg per kg live wt.) was used for each species.

A few colour reactions were observed with this substance on chromatography paper. The substance appeared dark in ultraviolet light, gave a similar but darker reaction than vitamin D with the $SbCl_3$ reagent and a fainter reaction than the vitamin with I_2 in light petroleum. There was a strong reaction with Schiff base, DNP-hydrazine and tetrazolium reagent, a faint reaction with the PORTER–SILBER reagent[6] and none with pH indicators. Thus the substance has reducing properties, probably a reactive carboxyl group and a neutral reaction.

DISCUSSION

The changes in concentration of the unidentified substance produced in response to an injection of vitamin D have been studied in the adrenal gland. The substance has been found in liver, kidney and ileum as well as adrenal gland but was not studied in these larger organs because of the difficulty of purification and the poor quality of the chromatograms of extracts from these organs. There were indications that similar changes occurred in these organs as occurred in the adrenals. SALIS AND HOLDSWORTH[5] have suggested that the adrenals or their secretions have a function in controlling the conversion of vitamin D to an active form but this effect need not, of course, be confined to the glands themselves.

The large amounts of vitamin D found in the liver, kidney and intestine could be involved in processes such as storage, degradation to inactive products and excretion as well as activation. KODICEK[9] has shown that in the liver 50% of the radioactivity derived from an oral dose of labelled vitamin D is not biologically active and is presumably in the form of breakdown products and the types of breakdown products are different from those which appear in the intestine. NORMAN AND DELUCA[4] found about 1% and KODICEK[9] about 2% of the radioactive label in the urine after 24 h but the latter showed that none of this was biologically active vitamin D. KODICEK *et al.*[3] have shown that 2.5% of injected vitamin D can be found in the gut contents 5 h after the injection. This may, in part, be due to desquamation of the intestinal epithelium immediately after death (BADAWY *et al.*[10]).

This paper describes the formation, in response to vitamin D dosing, of a substance which is not vitamin D. There is no evidence whether or not it is derived from vitamin D but comparisons with the data of other workers who used similar dosage levels show some similarities in the way the concentration changes with time. In the adrenals there is a very close correspondence in time and concentration (as estimated by ultraviolet absorption) with RAOUL AND GOUNELLE's[1] data (Fig. 2) although there are differences in the spectral and biological properties of the substances concerned. Using intracardial doses of the vitamin, KODICEK *et al.*[3] found maximum levels of radioactivity in the adrenals at 20 min after the dose while NORMAN AND DELUCA[4] found the highest levels at 20 and 60 min.

It is well known that there is a time lag of from 3 to 8 h between the oral administration of vitamin D and the improvement of Ca absorption by the small intestine of a rachitic animal. This is a longer time than is taken by the adrenal to show its maximum concentration of vitamin D or of the unidentified substance after an injection of the vitamin. The difference in time is probably due to the difference in the

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route of administration. NORMAN AND DELUCA[4] have shown the maximum levels of radioactivity in liver and intestine occur at a longer interval after an oral dose of the vitamin, namely at about 4 h.

It is possible, therefore, in view of all the similarities in behaviour, there is some relationship between the unidentified substance in the adrenals described in this paper and the recognised physiological functions of vitamin D.

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MODE OF ACTION OF VITAMIN D: SYNTHESIS OF CITRATE IN EXPERIMENTAL RICKETS AND IN HEALING INDUCED BY VITAMIN D.

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INTRODUCTION.

RAMALINGASWAMI *et al.* (1954) had suggested on the basis of their histochemical studies on rachitic and healing cartilage of the rat that the glycogen metabolism in the rachitic cartilage probably suffered a breakdown at the stage of the formation of citric acid. Soon after the above suggestion was made, Tulpule and Patwardhan (1954) reported that the tibial epiphyseal cartilage of rachitic rats could not oxidise pyruvate as efficiently as that from the vitamin D treated rats. Dikshit *et al.* (1956) later found a reduction in the citrogenase activity of the rat cartilage in rickets and Joshi *et al.* (1957) noted an essentially similar phenomenon in the rachitic cartilage of dogs. Meyer *et al.* (1959) have recently confirmed the observations of Dikshit *et al.* (*loc. cit.*).

The term citrogenase, however, denotes a group of enzymes, rather than a single enzyme (Dixon and Perkins, 1952). When pyruvate is used as one of the substrates, it undergoes several reactions leading ultimately to the formation of citric acid. The various steps in the biosynthesis of citric acid from oxaloacetate and pyruvate or acetate occur according to Gunsalus (1954) as shown in the Text-figure.

Dikshit *et al.* (*loc. cit.*) had observed that the addition of diphosphothiamine (DPT) had no effect on the citrogenase activity of the rachitic rat cartilage indicating that the initial decarboxylation of pyruvate may not be effected in rickets. The observations of Dikshit *et al.* (*loc. cit.*) and Joshi *et al.* (*loc. cit.*) on the decrease in citrogenase activity in rickets were not sufficient to locate definitely the metabolic defect in vitamin D deficiency. Experiments were, therefore, undertaken to study the rate of formation of (i) acetyl lipoate from pyruvate, and (ii) acetyl coenzyme A from acetate by the rachitic rat cartilage before and after the administration of vitamin D. Carboxylic acid anhydrides, acid chlorides, acyl phosphates, thiolesters and N-acyl imidazoles react chemically with hydroxylamine to form hydroxamic acids. Ordinary esters, aldehydes, ketones and amides do not interfere even at relatively high concentrations (Stadtman, 1957).

The hydroxamic acids thus formed yield essentially equivalent colour values when estimated according to the method of Lipmann and Tuttle (1945).

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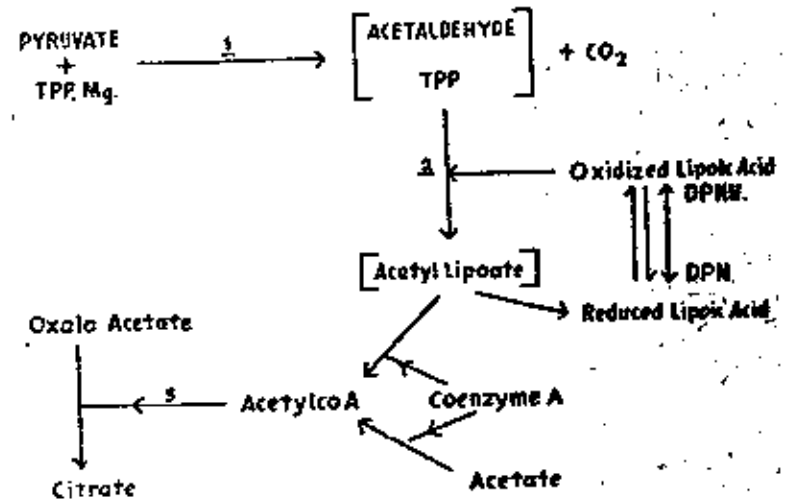
In the experiments described below the formation of acethydroxamic acid with hydroxylamine can only be due to the formation of acetyl lipoate and acetyl coenzyme A, both of which are thiol esters, and not due to any of the other intermediates or substances present in the incubation mixtures.

Since the locus of action of lipoic acid lies between the acyl generating step and the acyl donor step to coenzyme A (Gunsalus, *loc. cit.*, Text-figure), the formation of acethydroxamic acid, when pyruvate formed the substrate, would be due to the formation of acetyl lipoate, while, when acetate formed the substrate in the study of aceto-CoA-kinase activity, the acethydroxamic acid formed would be due to the formation of acetyl coenzyme A.

TEXT-FIGURE.



Enzymatic steps in citrate synthesis.

EXPERIMENTAL.

I. *Formation of acetyl lipoate from pyruvate.*—Sixty-three young albino rats, 28 days old, were fed a low phosphorus rachitogenic diet described by Dikshit and Patwardhan (1947). After 14 days on this diet, rats were divided into three groups taking litter and sex distribution into account. Each rat in group 1 received a single dose of 4,000 I.U. of vitamin D_2 in oil, orally, on the 14th day and those in group 2 on the 16th day. The animals were sacrificed on the 18th day on rachitogenic diet including those in group 3 which received no vitamin D and which served as the rachitic control. The tibial epiphyseal cartilage was removed in cold as thin slices as described by Krishna Rao and Patwardhan (1960). Cartilage slices from 3 rats in the same group were pooled, weighed and incubated for 20 minutes at 37°C. in a medium (pH 8·0) containing pyruvate and hydroxylamine. The composition of the incubation mixture is given in Table I. The acethydroxamic acid formed in the reaction mixture was estimated according to the method described by Lipmann and Tuttle (*loc. cit.*).

TABLE I.

Compositions of the incubation media.

Number.	Name of solution.	Pyruvate oxidation, c.c.	Acetate activation, c.c.
1	Tris (hydroxymethyl) amino methane Buffer : 1·0 M, pH 8·2	1·0	1·0
2	Thiamine pyrophosphate	0·1	..
3	Diphosphopyridine nucleotide : 0·0027 M	1·0	..
4	Cysteine hydrochloride : 0·20 M, pH 4·5	1·0	1·0
5	Adenosine triphosphate : 0·10 M, pH 7·6	..	1·0
6	Magnesium chloride : 0·20 M	0·5	0·5
7	Potassium fluoride : 1·0 M	..	0·5
8	Hydroxyl amine hydrochloride : 2·0 M, pH 7·4	1·0	1·0
9	Sodium pyruvate : 0·2 M	1·0	..
10	Potassium acetate : 0·2 M	..	1·0
11	Distilled water	4·4	4·0
	Total volume	10·0	10·0
	pH	8·0	8·0
	Volume dispensed	1·0	1·0

The results of the experiment are given in Table II.

TABLE II.

Influence of vitamin D on acetyl lipoate.

(Formation from pyruvate by rachitic rat cartilage Unit : μg. acethydroxamic acid/100 mg. fresh tissue/hr. Incubation at 37·0°C. for 20 min.)

Observation number.	Rachitic controls (No vitamin D).	RATS TREATED WITH VITAMIN D_2 (4,000 I.U./RAT) :	
		48 hours after treatment.	96 hours after treatment.
1	157	201	225
2	134	127	132
3	167	236	260
4	163	311	391
5	142	323	365
6	159	240	259
7	161	245	274
Mean	153·4	240·7	272·3
S.E.	±4·45	±25·00	±32·74
't'	..	3·439	3·600
p		<0·01	<0·01

1. Each observation on pooled cartilage from 3 rats.
2. Statistical comparison made with controls.

2. *Formation of acetyl coenzyme A from acetate.*—Eighty-one young albino rats, 4 weeks old, were made rachitic as described above, some of them were treated with vitamin D and sacrificed according to the procedure described in the previous experiment. The composition of the incubation medium (pH 8·0), however, differed from the previous one only in that it contained acetate instead of pyruvate as the substrate. The medium was constituted according to the method

of Jones and Lipmann (1955) with slight modifications as shown in Table I. The addition of coenzyme A was found to be unnecessary since preliminary experiments revealed no difference in the rate of formation of acethydroxamic acid when CoA was added or omitted. It is possible that the cartilage contained sufficient amount of CoA for recycling purposes. The acethydroxamic acid formed from acetyl coenzyme-A and hydroxylamine was estimated according to the method of Lipmann and Tuttle (*loc. cit.*).

The results of the experiment are given in Table III :—

TABLE III.

Influence of vitamin D on aceto-coA-kinase activity of the epiphyseal cartilage of the rachitic rat.

(Unit : μg. acethydroxamic acid/100 mg. fresh tissue/hr. incubation at 37·0°C. for 20 minutes.)

Observations number.	Rachitic cotrols (No vitamin D.)	RATS TREATED WITH VITAMIN D_2 (4,000 I.U./RAT.)	
		48 hours after treatment.	96 hours after treatment.
1	161	211	216
2	86	167	142
3	83	152	136
4	97	128	187
5	70	163	168
6	136	182	227
7	93	165	164
8	97	161	175
9	92	167	163
Mean	102·7	165·2	175·2
S.E.	±9·05	±7·43	±10·16
't'	..	5·329	5·323
p	..	<0·001	<0·001

RESULTS.

From Tables II and III it is evident that the rate of formation of acetyl lipoate from pyruvate and acetyl coenzyme A from acetate were both reduced in the rachitic cartilage and that administration of vitamin D increased the activity of the respective enzymes.

The activities of the two enzymes at 48 and 96 hours after vitamin D administration were significantly higher than the corresponding activities in the rachitic state. The difference between the activities at 48 and 96 hours, however, was not statistically significant.

DISCUSSION.

It will be seen from the Text-figure that the first step in the oxidation of pyruvate is a non-oxidative decarboxylation for which diphosphopyridine nucleotide (DPN) is not required (Gunsalus, *loc. cit.*). The oxidative reaction step seems to commence from the acyl generation stage. The subsequent acyl transfer leaves lipoic acid in a reduced state whether the acyl acceptor is coenzyme A as under the conditions obtaining *in vivo* or hydroxylamine *in vitro*, as in the present investigations (Gunsalus, *loc. cit.*). The reduction in the rate of formation of acethydroxamic acid

observed in the present investigations could be due to either (i) a reduced rate of acyl generation owing to a reduction in the activity of the acyl generating enzyme or (ii) a reduction in the rate of reoxidation of reduced lipoic acid which would then result in a gradual loss of the oxidized form of lipoic acid required for the oxidation of pyruvate molecules. The latter possibility could have been subjected to experimental verification by the addition of lipoic acid to the incubation medium but owing to certain technical difficulties it could not be done.

In either case a diminution in the oxidation of pyruvic acid should result in the formation of smaller amounts of DPNH in the rachitic cartilage which consequently would utilize less oxygen. This conclusion seems to be justified in the light of the observations of Tulpule and Patwardhan (*loc. cit.*) who found a marked reduction in the uptake of oxygen by the rachitic cartilage during the oxidation of pyruvate.

The formation of acetyl coenzyme A from acetate and CoA requires the presence of ATP, and is catalysed by the enzyme aceto-CoA-kinase (Jones and Lipmann, *loc. cit.*). This enzyme seems to have suffered a reduction in its activity in rickets as shown by the results presented in Table III. Cartilage contains an ATP-ase (Cartier, 1952). This enzyme, as also pyrophosphatase, are known to be inhibited by fluoride (Kornberg and Pricer, 1953). Hence, the addition of fluoride to the incubation mixture (Table I) ensured the availability of added ATP to aceto-CoA-kinase. Under conditions obtaining *in vitro* where fluoride inhibition of ATP-ase and pyrophosphatase is absent the formation of acetyl coenzyme A could be assumed to be still less in the rachitic cartilage than revealed in the present experiments.

Hence, these results lead to the conclusion that the decrease in the citrogenase activity observed in the rachitic cartilage by Dikshit *et al.* (*loc. cit.*) and Joshi *et al.* (*loc. cit.*) may be partly due to a breakdown in the processes leading to the formation of acetyl lipoate and acetyl coenzyme A from pyruvate and acetate respectively.

Summary.

1. The formation of acetyl lipoate from pyruvate and acetyl coenzyme A from ATP and acetate were studied in the rat cartilage in low phosphorus rickets and during healing induced by vitamin D.

2. A reduction in the formation of acetyl lipoate and acetyl coenzyme A was observed in rickets. Treatment with vitamin D increased the rate of formation of these compounds significantly by 48 hours. Further increase in the rates at 96 hours was not significant.

3. The results of the investigations are discussed in relation to the decrease in pyruvate oxidation and citrogenase activity, observed in the rachitic cartilage by previous workers.

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Sci American, Oct 1970

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CALCITONIN

This recently discovered thyroid hormone plays an important role in metabolism: it inhibits the breakdown of bone and thus keeps the calcium in the blood from reaching an excessively high level

by Howard Rasmussen and Maurice M. Pechet

The endocrinology of mammals has been investigated so intensively and with such a wealth of discoveries over three-quarters of a century that one might suppose the subject by this time would have lost some of its novelty and excitement. In actuality, however, the story of the endocrine glands and their hormones retains an unending fascination. New mammalian hormones are currently being discovered at a high rate, as in the early rush of exploration during the first quarter of this century. The new hormone we shall discuss in this article turns out to be of great importance in the regulation of a crucial phase of human metabolism. Ironically, this hormone escaped attention for nearly 80 years although it was more or less constantly on stage throughout that time. It is a product of the thyroid gland, which over the years has been studied more intensively than any other endocrine organ. Discovered less than a decade ago, the long overlooked hormone, calcitonin, has evoked such interest among physiologists, biochemists and physicians that it has already been isolated in pure form, synthesized completely in the laboratory, investigated in detail as to its functions and used therapeutically in human disease.

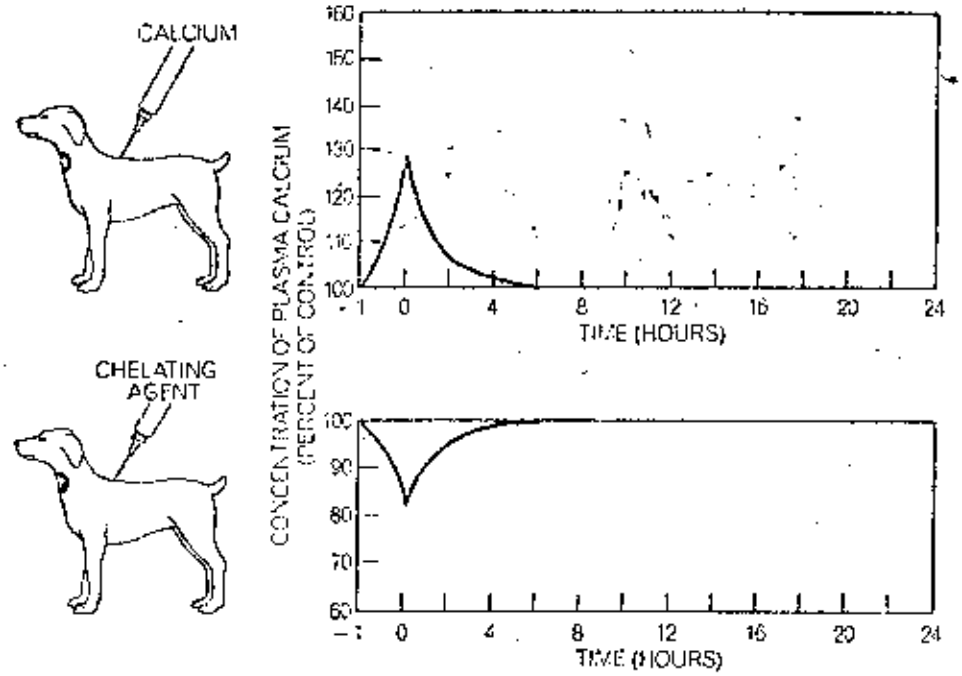
The discovery of calcitonin grew out of a puzzling question regarding the regulation of the calcium level in the blood. A constant supply of calcium (and phosphate as well) is required in the circulating blood for the building of bone and the control of certain functions of cells. If the concentration of calcium ions in the blood plasma falls below normal, the nerve and muscle cells, for instance, begin to discharge spontaneously and the voluntary muscles go into continuous contraction—the condition known as tetany. It was known that two agents, vitamin D and a hormone of the parathyroid gland, were active in maintaining the plasma's calcium supply: vitamin D by assisting the uptake of calcium from food by the intestinal cells, the parathyroid hormone by causing the release of calcium to the blood from bone and by inducing the kidney tubules to capture, for return to the circulating blood, calcium that would otherwise be lost in the urine.

Experiments had established that the activity of the parathyroid was governed by a feedback system. When the calcium in the plasma dropped below the normal level, the gland increased its secretion of the hormone; when the calcium rose above normal, secretion of the hormone stopped. The question arose: Was this strictly negative control—the shutoff of the parathyroid hormone—sufficient to protect the animal against a dangerous rise in the blood's calcium concentration? Peter Sanderson and his associates at the Peter Bent Brigham Hospital in Boston and D. Harold Copp at the University of British Columbia began to look into the possibility that there was also some mechanism that exerted a positive control on the accumulation of calcium in the blood. They soon found evidence that such a mechanism was indeed at work.

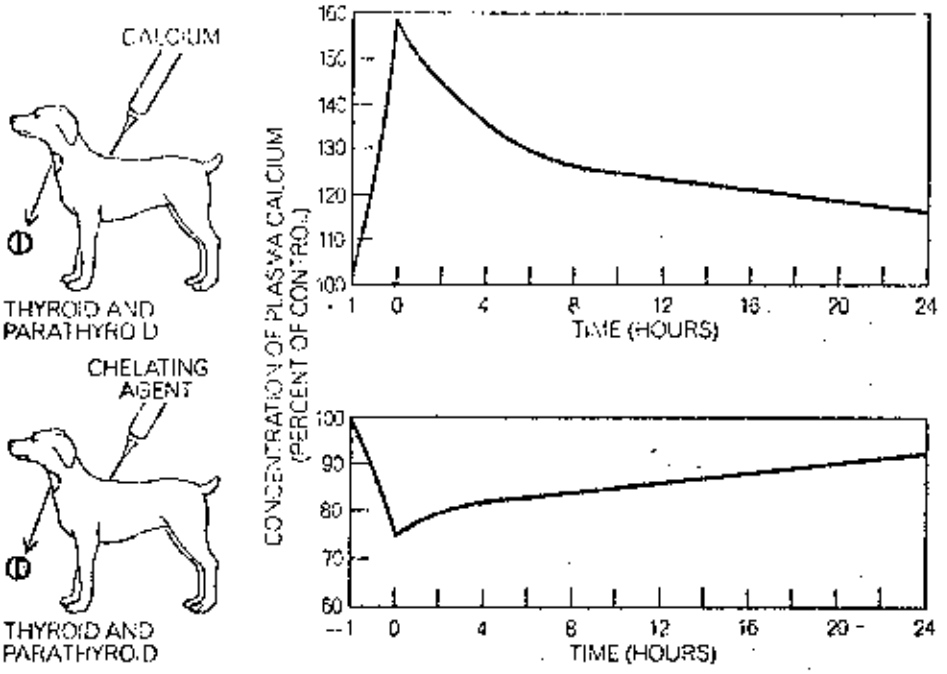
Sanderson's group performed an elegant series of experiments involving both the thyroid and the parathyroid glands. They first tested the reactions of dogs, with the glands intact, to abrupt experimental changes in the plasma calcium level; they raised the level by injecting calcium salts or lowered it by injecting a chelating agent that bound and inactivated the calcium ions in the plasma. They found that in either case the animals' plasma calcium quickly returned to the normal level after the infusions were stopped. They then removed the thyroid and parathyroid glands surgically from the same animals and retested them with the injection treatments. This time there was no rapid return to normal; the calcium content of the plasma remained elevated or subnormal for as long as 36 hours after the injection.

The failure of calcium to rise rapidly to normal in the cases where the level had been depressed could be explained by the absence of the stimulating parathyroid hormone (the source of the hormone having been removed). Obviously, however, lack of the hormone could not account for the fact that the calcium level remained high in the cases where the level had been elevated. It was clear that some other corrective agent must be missing in the animals with the thyroid and parathyroid glands removed—some positive agent that could cut back the delivery of calcium to the blood or speed up its removal. Copp, on the basis of experiments similar to Sanderson's, concluded that the agent must be a hormone that he named calcitonin, signifying that it participated in regulating the tone, or concentration, of calcium in the blood. Two groups of investigators, Philip F. Hirsch and Paul L. Munson at the Harvard Dental School and Iain MacIntyre

"C" CELLS that produce calcitonin are visible as luminous bodies in the micrograph on the opposite page. The tissue is from the thyroid gland of a dog. The C cells were made luminous by the injection of a fluorescent antibody that combines with calcitonin. The large dark areas are follicles that contain the principal thyroid hormone, thyroxin. Thyroxin is produced by cells forming grayish perimeter of each follicle. Small dark areas in some of the C cells are nuclei. Micrograph was made by A. G. E. Pearse of the Postgraduate Medical School of London.



FIRST PHASE OF DOG EXPERIMENT conducted by Peter Sanderson and his colleagues at the Peter Bent Brigham Hospital indicates that there is a positive mechanism preventing excessively high levels of calcium in the blood. At top dog is injected with calcium. Graph at right shows that one hour before injection calcium concentration in the blood is normal. At time of injection concentration rises. About six hours later, however, concentration is normal. Therefore some agent is probably reducing the flow of calcium from the bone into the blood. At bottom same dog is injected with a chelating agent that captures calcium in blood and renders it inactive. The graph shows that one hour before the injection the calcium concentration is normal. After the injection it is depressed. About six hours after the injection the concentration is normal again, as is expected because a hormone secreted by the parathyroid gland is known to elevate the calcium concentration in the blood.



SECOND PHASE OF DOG EXPERIMENT demonstrates that there definitely is a controlling agent. At top a dog with thyroid and parathyroid glands removed is injected with calcium. The graph indicates that the calcium concentration, which was normal one hour before the injection, quickly rises. More than 24 hours later the concentration is still high, indicating that some agent that normally reduces the concentration is no longer present because the glands are missing. At bottom a chelating agent is administered. The graph at right indicates that the calcium concentration, normal one hour earlier, is depressed. More than 24 hours later the concentration is still low, because the parathyroid hormone, which keeps the concentration high, has been eliminated by removal of the gland. D. Harold Copp of the University of British Columbia demonstrated the significance of these experiments.

and his colleagues at Hammersmith Hospital in London, soon located the source of the hormone. They found it not in the parathyroid, as Copp had suggested, but in the thyroid gland.

A. G. E. Pearse of the Postgraduate Medical School of London went on to discover the specific site where calcitonin is synthesized. It had been known for many years that the thyroid gland contains two types of cell: the type that produces the gland's classic hormone, thyroxine, and another type called C cells, which stain differently. Although the C cells were described by Jose F. Nonidez of Cornell University in 1931, their function was unknown until Pearse showed that they produced calcitonin. These cells have had an interesting evolutionary history. In mammals the C cells arise in ultimobranchial glands that lose their distinct identity during development and merge with the thyroid. In fishes, amphibians, reptiles and birds however, the ultimobranchial glands remain separate and persist as distinct organs in the adult. Stuart Tauber of the University of Texas Southwestern Medical School and Copp established that in these animals the ultimobranchial gland contains a high concentration of calcitonin but that the hormone does not show up at all in the thyroid gland.

Thus by 1967 the source of calcitonin in mammals was well established, and investigations of various aspects of its physiological activity were under way. One of the first aspects to be studied was the system that calls forth the secretion of calcitonin and its counterpart, the parathyroid hormone. Sanderson's and Copp's earlier studies had indicated, as we have seen, that the basic controlling factor was the calcium concentration in the blood. Direct measurements of the levels of calcium, calcitonin and the parathyroid hormone in the circulating blood of experimental animals now gave a quantitative picture of the control system. These measurements were made possible by newly developed assay techniques derived from the fundamental work of Solomon A. Berson and Rosalyn S. Yalow of the Veterans Administration Hospital in the Bronx. These investigators, working with John T. Potts, Jr., and Gerald D. Aurbach of the National Institutes of Health, had devised a radioimmunoassay for measuring the concentration of the parathyroid hormone; later Potts, and independently Claude Arnaud of the Mayo Clinic, had worked out a similar assay for calcitonin.

Using these assays, Potts and Arnaud found that in pigs infused with measured

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SECTIONS OF SHEEP BONE shown in these microradiographs demonstrate effects of the parathyroid hormone. The microradiographs were made by interposing a thin section of bone between a source of alpha radiation and a photographic plate. In microradiograph at left bright areas are calcified bone. Large dark holes are channels for blood vessels; small dark holes are occupied by osteocytes. The microradiograph at right was made after parathyroid hormone had caused osteocytes to resorb bone tissue. Resorption of bone tissue produces large blurred areas. The osteocyte spaces are enlarged and some of them have merged with one another.

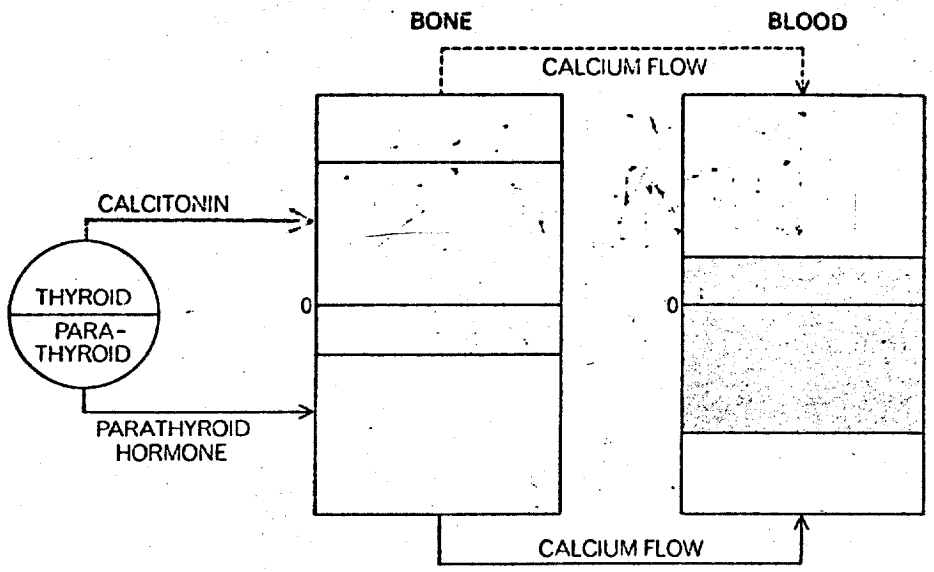
amounts of calcium the secretion of calcitonin increased in direct proportion to the rise in the blood's calcium content, and the secretion of the parathyroid hormone decreased in proportion to the calcium rise. Thus the results showed that the physiological control system responsible for keeping the blood's calcium supply at a stable level consists of two feedback loops: the parathyroid hormone operating to sustain the supply, calcitonin operating to prevent calcium from rising above the required level.

After the discoveries of calcitonin's role and the source that produced the hormone, the next challenge was to decipher its chemical nature, as a preliminary to learning how the hormone brings about its striking effect. A number of investigators set out to isolate the hormone, using various assay methods based on measurements of the potency of their preparations. Six laboratories reported late in 1967 and early in 1968 that they had isolated the hormone in pure form from thyroid glands. It turned out to be a polypeptide consisting of a single chain of 32 amino acids. Three laboratories soon determined the sequence of the amino acids in the molecule's structure, and shortly thereafter the Lederle Laboratories in Pearl River, N.Y., and the Ciba and Sandoz laboratories in Switzerland announced the synthesis of the hormone from its amino acids. The Ciba group, headed by R. Neher, also succeeded in isolating the human version of calcitonin (from tissue obtained from a patient with a carcinoma of the thyroid gland) and analyzed its amino acid sequence. This proved to be somewhat different from the structure of the pig hormone, indicating that the activity of calcitonin in curtailing the calcium content of blood depends on certain critical features of the amino acid sequence (common to both the pig and the human forms of the molecule) rather than on the structure as a whole.

The way was now open to investigate calcitonin's mode of action. How does the hormone function to lower the concentration of calcium in the blood plasma? Experiments with preparations of purified calcitonin in many laboratories in the U.S. and Europe soon showed that the hormone produces its effect by decreasing the release of calcium to the blood from bone. Calcitonin was found to act on the metabolism of bone in a way that inhibits bone resorption.

Bone is by far the most refractory and durable of all biological tissues, as is evident in the survival of fossil bones that have been buried for hundreds of thousands of years. Because of the apparent obdurability of bone it was supposed until very recently that once the skeleton of a vertebrate has been formed it ceases to partake of metabolism or to have any appreciable breakdown. Definitive evidence that this is by no means the case did not come until the availability of radioactive isotopes made it possible to examine the events actually occurring in the bone of living animals.

Tracer experiments with labeled isotopes of the main elements that go into the makeup of this tissue (calcium, phosphorus and the carbon of amino acids) showed that even in fully mature bone there is a constant turnover of these materials. It then became evident that metabolic activity in the skeleton is a vital necessity for at least two reasons. For one thing, such activity allows remodeling of the skeleton to enable it to deal with the developing mechanical



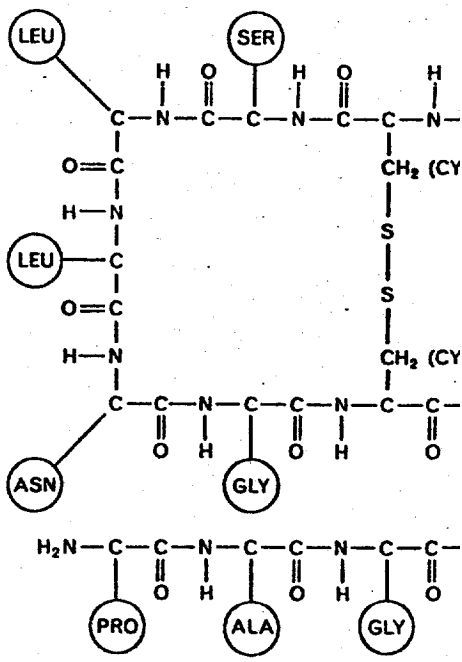
CONTROL LOOP regulating balance of calcium in bone and blood is established by calcitonin and the parathyroid hormone. Thyroid (*left*) secretes calcitonin (*colored arrow*) when blood calcium is high. Calcitonin prevents resorption, so that bone calcium level (*color in upper part of box in middle*) remains high and flow of calcium into blood is reduced (*broken arrow at top*). Therefore calcium level in the blood (*shading in upper part of box at right*) is low. When calcium level gets too low, parathyroid gland (*left*) increases it by secreting hormone (*black arrow*) so that more calcium enters blood (*solid arrow at bottom*).

stresses on the body. It is as if each bone were an elaborate Gothic structure in which a resident engineer, in response to changes in stresses, continually directs the replacement of supporting arches with new ones providing a slightly different center of thrust. The other vital function of metabolic activity in bone is that, by allowing an exchange of minerals (calcium and phosphate ions) with the blood, it provides a storehouse of these materials to help meet the body's general requirements. And in fact normally there is an overall balance of supply and demand for the bone minerals, new bone being laid down in some parts of the skeleton and old bone being resorbed elsewhere at the same rate.

To understand the operations of calcitonin and the parathyroid hormone in this system we need to look into the events in the formation and the resorption, or destruction, of bone. We are concerned here with what goes on at the surface of existing bone as new tissue is formed or old bone is dissolved away. The building on of new bone is initiated by a surface layer of osteoblasts, the cells that synthesize and extrude molecules of the fibrous protein collagen. The collagen molecules form thin, insoluble fibrils that pack themselves closely in a definite geometric array constituting a matrix, firmly anchored in the extracellular spaces of the bone. The matrix, which takes several days to form, then acts as a template for the deposit of crystals of mineral in an ordered arrangement. Studies by Melvin J. Glimcher and Stephen M. Krane of the Massachusetts General Hospital indicate that the buildup of the minerals begins with the attachment of phosphate groups on specific sites in the matrix. Once the first mineral crystals form, the depositing of phosphate and calcium proceeds rapidly, and within a few days the new bone contains 70 percent or more of all the mineral it will ever possess. As mineral is deposited, it displaces water and eventually occupies most of the available space in the matrix. It is then locked in so that (in an adult animal) most of the mineral is not free to take part in exchanges of mineral between the bone and the blood. This, of course, is an essential condition, because unrestricted removal of mineral from the bone could deprive it of its rigidity and mechanical strength.

Exactly where in the matrix is the mineral deposited? Is it encrusted around the collagen fibrils? If this were so, one would expect the mineral to play a large role in maintaining the structural form (shape and size) of the bone. Actually experiments have shown that when the mineral is extracted (by artificial means), the bone is not changed in shape or size but merely softens. A more plausible picture of the location of the mineral crystals, suggested by John A. Petruska and Alan J. Hodge of the California Institute of Technology and fitting the known facts, is that they are lodged in spaces between the ends of the fibrils, which are supposedly arranged in an overlapping array. This model implies that the mechanical properties of a bone are determined by the location of mineral in the matrix, and the bone's shape and size are determined by the three-dimensional configuration of the matrix, which is fixed by strong chemical bonds tying the fibrils together.

We come now to the opposite of the bone-building process: the resorption of bone that makes possible the continuous exchange of phosphate and calcium ions between bone and the blood. It turns out that the bone-forming cells play a role in resorption as well. As the osteoblasts complete their function of producing collagen, they become entrapped in these fibrils and are continually overlain by new osteoblasts growing on the surface. The buried osteoblasts are now converted into osteocytes and put out thin extensions, forming an extensive cellular network throughout the bone. When the bone has been fully formed, it is covered by a film of fluid topped by a thin layer of "resting" osteoblasts, which are no longer making collagen. These cells, together with the osteocytes, perform the important function of acting as sentinels that patrol the flow of mineral ions between the blood plasma and the special fluid bathing the bone. Were it not for this guarding network, a small change in the bone's chem-



CALCITONIN MOLECULE in man consists of a specific sequence of 32 amino acid

istry might lead to a flood of calcium into or out of the blood, either of which could be lethal.

The involvement of osteocytes in bone resorption was clearly disclosed recently in radiographic studies by Leonard F. Délanger of the University of Ottawa. It had been supposed that bone was broken down only by the giant digesting cells called osteoclasts. Examining thin sections of bone by means of radiography (using alpha radiation and X rays), Délanger produced pictures showing the matrix and indicating where it was calcified and where it was not. He found that in bone that had been treated with the parathyroid hormone both the matrix and the calcium were dissolved away around each osteocyte [*see illustration on page 45*]. Apparently calcium is so tightly trapped in the matrix that it is not readily released unless the matrix structure is broken down.

The experiment thus demonstrated that the parathyroid hormone promotes bone resorption by acting on the osteocytes; in some way it incites these cells to engulf and destroy the bone tissue around them, with a consequent release of dissolved calcium into the blood stream. We undertook a different set of experiments to probe the counteracting behavior of calcitonin. Conceivably this hormone might lower the blood's calcium by speeding up the laying down of calcium in new bone, but it seemed much more likely that it acted by inhibiting bone resorption.

In looking into the effects of calcitonin we had the benefit not only of the radiographic techniques for examining what went on in bone tissue but also of a newly developed chemical measure of the rate of bone resorption in living animals. The collagen of bone is a protein rich in a peculiar amino acid: hydroxyproline. Darwin J. Prockop of the University of Pennsylvania School of Medicine had learned that hydroxyproline is not incorporated in a protein in the usual way—with the help of transfer RNA. There is no specific transfer RNA for this amino acid. A precursor molecule, protocollagen, is first synthesized and then specific proline units within it are acted on by an enzyme that adds the hydroxyl group to some of the prolines. In this way hydroxyproline is produced at the necessary positions on the collagen molecule. When the collagen molecule is broken down, hydroxyproline shows up in urine. It serves as a distinctive signal; the amount of hydroxyproline in the urine has in fact been found to reflect the rate of bone resorption in an animal. We found it useful in determining whether or not the resorption rate was influenced by calcitonin.

We removed the thyroid and parathyroid glands from rats and then injected the parathyroid hormone, so that the animals had an ample supply of this hormone but no calcitonin. The rats excreted large amounts of calcium and hydroxyproline in their urine, showing a high rate of bone resorption, and radiographic examination of sections from their bones confirmed that the osteocytes had destroyed bone tissue around them and had greatly swelled in size. When we carried out a parallel experiment with another set of rats, this time supplying them with injections of calcitonin as well as the parathyroid hormone after their thyroid and parathyroid glands were removed, the results were strikingly different. The animals' excretion of calcium and hydroxyproline in the urine *decreased*, and radiographic examination of bone sections showed no sign of bone breakdown around the osteocytes. It is also noteworthy that the osteoclasts, the large cells on the surface of bone that are involved in its remodeling, also increase their resorptive activity in response to parathyroid hormone and decrease it in response to calcitonin.

Here, then, was clear evidence that calcitonin performs its role of controlling the calcium content of the blood by inhibiting bone resorption. A simple further experiment demonstrated how

ALA ALANINE
ASN ASPARAGINE
ASP ASPARTIC ACID
CYS CYSTINE
GLN GLUTAMINE
GLY GLYCINE
HIS HISTIDINE
ILE ISOLEUCINE
LEU LEUCINE
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PHE PHENYLALANINE
PRO PROLINE
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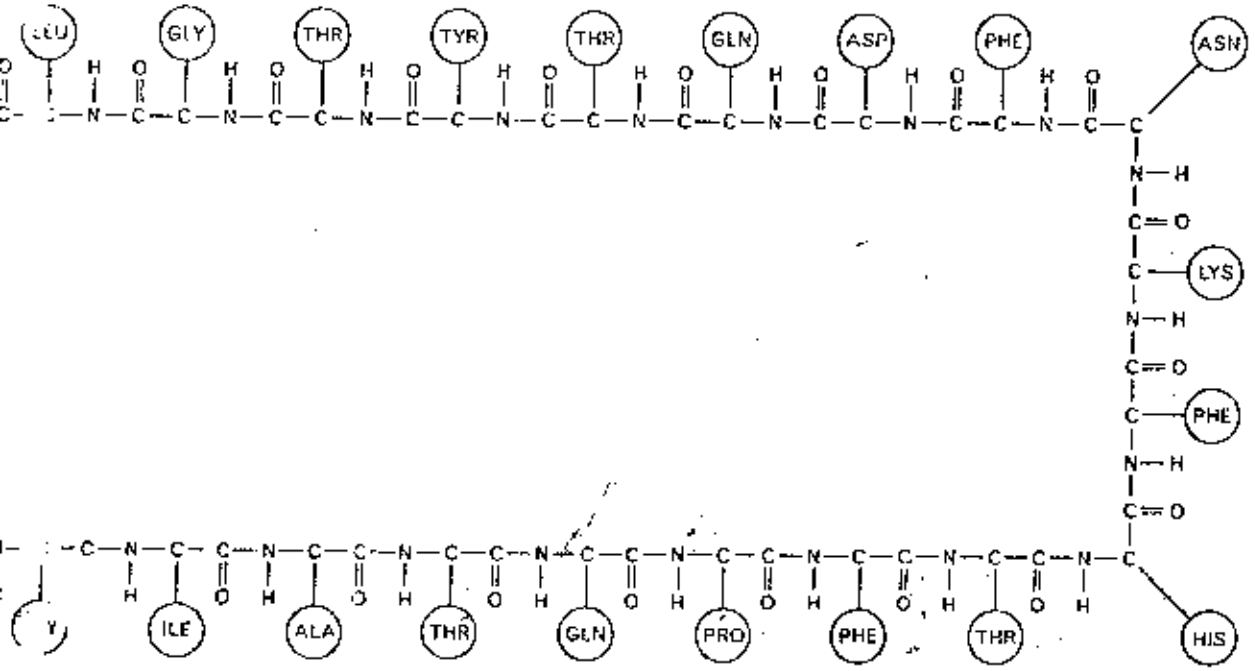
VAL VALINE



subunits. The identity of the individual subunits is indicated by the abbreviations that mark the amino acid side chains. The key at top right gives the full name of each of 17 different amino acid constituents of hormone. The sequence differs in other species.

vital this control is for the animal. In animals that had both glands removed and were then injected with a continuous infusion of calcium and the parathyroid hormone, the calcium concentration in the blood rose rapidly to a high level, urine excretion began to fail after 16 hours, and shortly afterward the animals died. The cause of death, as postmortem examination showed, was a massive accumulation of insoluble calcium phosphate in the kidneys, destroying their function. When, on the other hand, we removed only the parathyroid gland, leaving the thyroid intact, the animals responded to the same infusion of calcium and parathyroid hormone without apparent ill effects. Clearly in this case the thyroid gland saved the day with an output of calcitonin that prevented a marked rise in the blood's calcium level, and we found no damaging deposits of calcium in the kidneys.

In view of calcitonin's now clearly established function as an inhibitor of bone resorption, it is curious to find that some primitive cartilaginous animals, such as the dogfish shark, produce calcitonin (in an ultimobranchial gland) although they have no bony skeleton. We can only speculate about what function the hormone may serve for them. Apparently calcitonin, like some other hormones, is an ancient biological agent that evolution converted from its original function to a new use in the vertebrates.

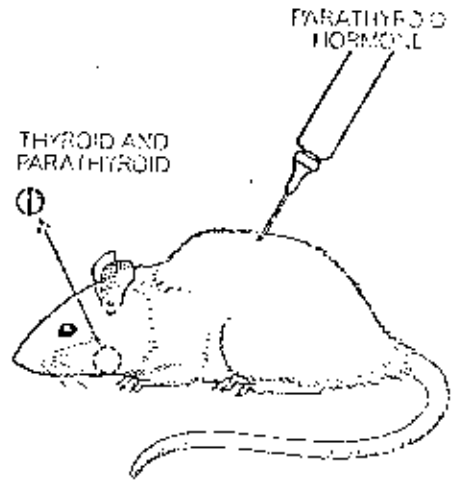
From the physiological study of calcitonin's action we have now gone on to try to learn how the hormone produces its effect in biochemical terms. This is difficult to get at, because bone tissue is structurally so complex and composed of such a diversity of cells that direct exploration by specific chemical experiments is almost out of the question. On the basis, however, of various items of evidence, mostly indirect, we have arrived at a tentative hypothesis about the biochemistry of calcitonin's control of metabolism in bone cells.

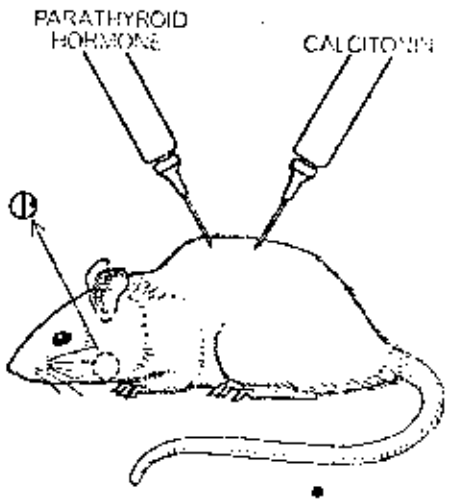
A starting point was provided by recent discoveries concerning the biochemical action of the parathyroid hormone. These discoveries have been made in the years since one of us (Rasmussen) wrote an article in *Scientific American* on that hormone ["The Parathyroid Hormone," by Howard Rasmussen; SCIENTIFIC AMERICAN, April, 1961]. In the urine of rats given parathyroid hormone L. R. Chase and Aurbach of the National Institutes of Health noted an increased content of the remarkable compound known as cyclic 3'5' adenosine monophosphate (cyclic AMP). This substance, first discovered by Earl W. Sutherland, Jr., then at Washington University, had been found to serve as an intermediate in the action of many hormones—the hormone being called the "first messenger" and cyclic AMP the "second messenger."

Sutherland and his coworkers had established that cyclic AMP is produced from adenosine triphosphate (ATP) by an enzyme, adenyl cyclase, located at the surface of cells. From experiments they developed the hypothesis that many hormones interact with specific adenyl cyclases on the surface of specific cells and bring about an increase in the production of cyclic AMP within the cell. For instance, the hormone epinephrine triggers the synthesis of cyclic AMP in liver cells, the parathyroid hormone does so in kidney cells but not in liver cells, and so on. The effect of the second messenger varies according to the special character of the target cell; for example, in the liver it causes an increase in the synthesis and release of glucose; in the adrenal cortex (where cyclic AMP acts as the second messenger for ACTH) it stimulates the synthesis and release of steroid hormones. It is as if cyclic AMP conveyed the messages of the hormones in a generalized form, simply saying to each cell: "Do your thing."

The discovery by Chase and Aurbach that the amount of cyclic AMP in the urine of rats increases following the administration of parathyroid hormone offered an opening for analysis of calcitonin's action. Assume that the parathyroid hormone fosters production of cyclic AMP in bone cells and that the cyclic AMP brings about the resorption of bone. It could be supposed, then, that calcitonin might block the effect of the parathyroid hormone either by preventing the synthesis of cyclic AMP or by inactivating that compound in the cell. (It was known that cyclic AMP could be inactivated by phosphodiesterase, an enzyme in cells that converts it by hydrolysis to 5' AMP.) Looking into the question experimentally, we first infused rats that had been deprived of their thyroid and parathyroid glands with cyclic AMP, using a dibutyryl form of the compound that had been synthesized by Thro Posternak of Case Western Reserve University and had been found to be invulnerable to hydrolysis by phosphodiesterase.

The urine of these animals showed that the injection of the cyclic AMP increased bone resorption just as injections of the parathyroid hormone did. We then found that injections of calcitonin blocked the bone-resorption effect of cyclic AMP, just as it blocked bone resorption induced by parathyroid hormone. The results of these experiments clearly indicated that neither of the hypotheses about calcitonin's action was correct: obviously it did not block bone resorption simply by preventing the parathyroid hormone from producing cyclic AMP, because it was effective against the action of that compound itself in the absence of the parathyroid hormone, and our use of the unhydrolyzable form of the cyclic AMP showed on the other hand that calcitonin did not inactivate cyclic AMP by causing its hydrolysis. Chase and Aurbach produced support-





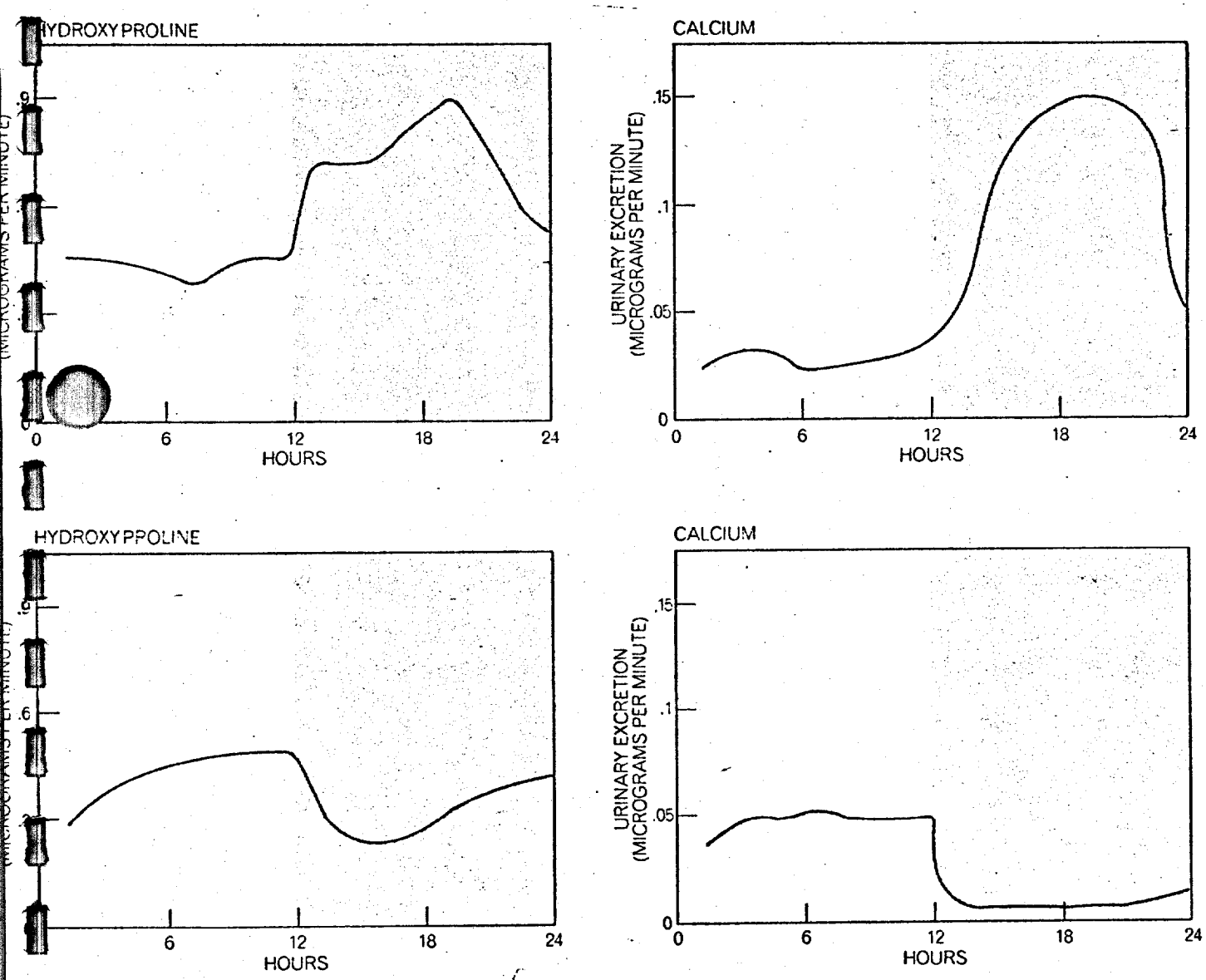
RAT EXPERIMENTS demonstrate clearly that calcitonin controls calcium concentration by inhibiting the resorption of bone induced by the parathyroid hormone. In first experiment (*top*) both the parathyroid and the thyroid glands are removed from the rat (*left*). First graph shows the level of hy-

ing evidence for these conclusions: they demonstrated that the parathyroid hormone brings about an increase in the concentration of cyclic AMP in bone cells and that this increase is not affected by a simultaneous presence of calcitonin.

Hopefully the answer to the problem of how bone resorption was inhibited had to be sought not in some action on the cyclic AMP system itself but in metabolic events in the cell that presumably were influenced by cyclic AMP. Andre B. Borle of the University of Pittsburgh School of Medicine produced a clue that carried the search forward. Growing cells in a tissue culture, he found that when he added the parathyroid hormone to the culture medium, the cells increased their uptake of calcium. Did this effect also take place in cells growing normally in a living organism? Following up Borle's clue, one of us (Rasmussen) and a co-worker, Naokazu Nagata, established that the hormone did indeed affect calcium uptake in natural conditions, and we learned some details of the associated events.

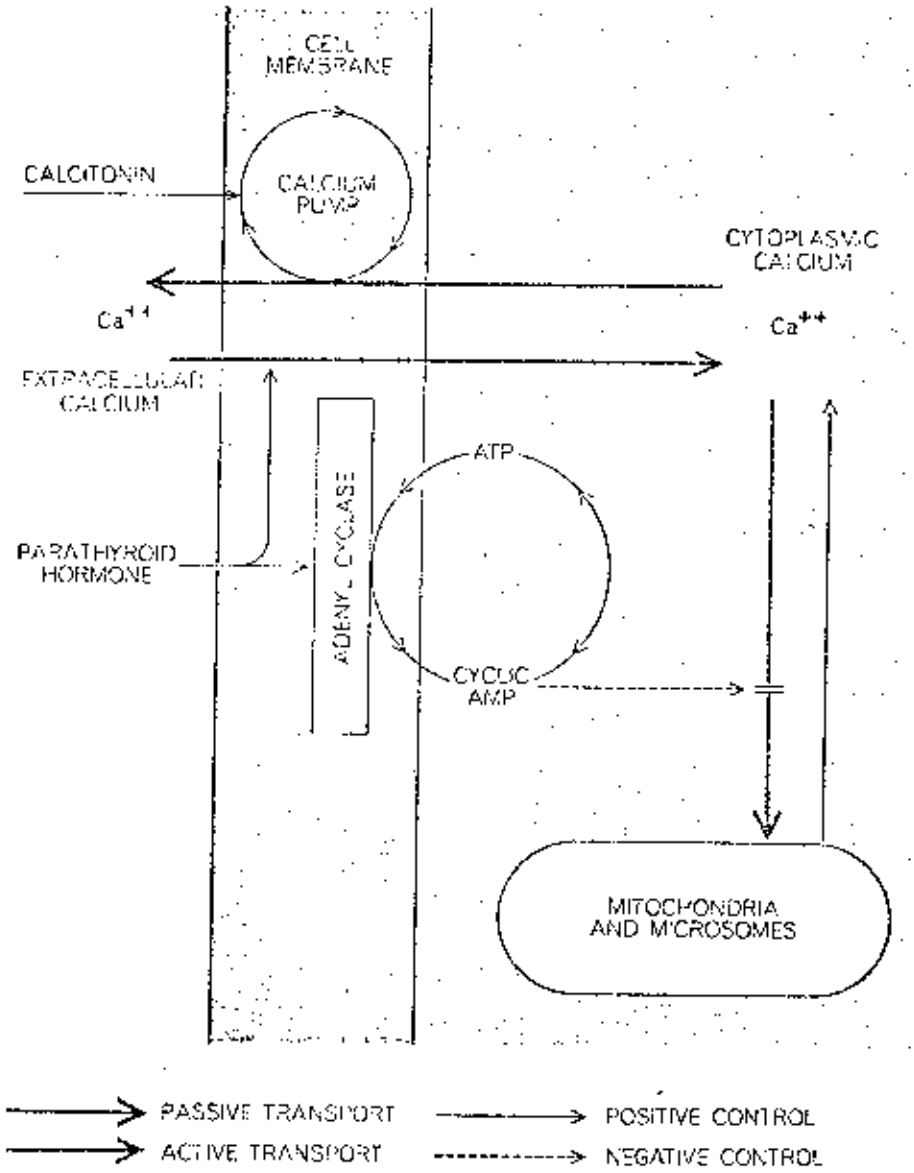
Our first experiments were on whole animals. After removing the thyroid and parathyroid glands from rats, we injected some of the animals with the parathyroid hormone and others with calcium chloride. We then rapidly removed the kidneys, froze them to stop the many metabolic reactions going on in the cells, inactivated all the enzymes with cold perchloric acid and extracted the collection of metabolites, and then by a complex of techniques determined the concentrations of the various important metabolic intermediates.

We found that the infusion of calcium salt produced much the same metabolic picture as that resulting from the parathyroid hormone infusion. In order to obtain a more detailed picture under conditions enabling us to control some of the variables, we followed up with test-tube studies of kidney tissue, broken down with two enzymes, collagenase and hyaluronidase, that split apart the many thousands of small kidney tubules. The experiments on small segments of



Hydroxyproline, an amino acid produced by the breakdown of bone collagen, in the rat's urine. The second graph shows the calcium level in the urine. The white area in each graph represents control period. The colored area represents the period during which the rat was constantly infused with the parathyroid hormone. During the control period in the first experiment, hydroxyproline levels and calcium levels are low. During the infusion period, however, both calcium and hydroxyproline levels are high, indicating considerable release of bone material. In the second experiment (*bottom*) glandless rat receives both parathyroid hormone and calcitonin. The first graph shows that the hydroxyproline level falls during infusions, indicating that calcitonin has blocked resorption of bone tissue. The second graph shows that calcium excretion also falls, confirming that the resorption of bone has been inhibited.

isolated tubules confirmed that the pattern of metabolism brought about by adding to the cells' calcium supply was almost identical with that produced by supplying the parathyroid hormone. This indicated that both treatments somehow operated on the same enzymes in the cell. Both, for example, speeded up the rate at which the cells convert lactic or malic acid to glucose. The rate depends on the cells' uptake of calcium, acting as a signal, and it appears that the amount of calcium entering the cells can be enhanced either by increasing the supply of calcium outside the cell or by providing the parathyroid hormone. Thus in these cells calcium is an important second messenger relaying the hormonal, or first, message into changes in cellular activity.



ACTION OF HORMONES ON CELLS controls body's calcium levels. When parathyroid hormone (*left*) reaches a cell wall (*dark colored area at left*), it stimulates the uptake of calcium by the cell from the extracellular space. At the same time the hormone activates adenyl cyclase, an enzyme in the membrane. Adenyl cyclase acts on ATP (adenosine triphosphate) to produce cyclic AMP (3'5' adenosine monophosphate). As calcium enters the cytoplasm (*light colored area at right*), cyclic AMP (*center*) interferes with active movement of calcium out of the cytoplasm and into the mitochondria and other organelles (*right*). Since this active transport is interrupted, the calcium remains in the cytoplasm. Meanwhile calcium continues to flow passively from the organelles back into the cytoplasm. As a result calcium accumulates there. This accumulation is the key signal to the cell to begin resorbing the surrounding matrix of bone tissue. Calcitonin (*left*), however, counteracts this chain of events by activating a "calcium pump" represented by circular arrows at top left. This process pumps calcium out of the cell's cytoplasm, across the membrane and into the extracellular space, in effect "canceling" the message. Key at bottom shows that colored arrows indicate active transport of calcium and heavy black arrows passive transport. Small black arrows represent positive control; broken arrow shows negative control.

How does the parathyroid hormone produce that effect on cell calcium? Here we may find an answer to the still mysterious question of cyclic AMP's function. Alan M. Tenenhouse of McGill University and one of us (Rasmussen) have suggested as a working hypothesis that the parathyroid hormone has two simultaneous effects on responsive cells: it increases the uptake of calcium and it stimulates the production of cyclic AMP. The increased intracellular concentration of cyclic AMP has two important effects related to calcium. It activates certain enzymes that now become calcium-sensitive, and it alters the intracellular distribution of calcium among various cell organelles.

In the light of this hypothesis and all the experimental findings, we can now see a possible explanation of the counteracting effect of calcitonin. That hormone may block the uptake of calcium by bone cells, a passive process and the one increased by parathyroid hormone, or alternatively it may somehow bring into play energy for active pumping of calcium out of the cells, thereby altering the changes induced by parathyroid hormone in calcium transport and bone resorption. The latter alternative seems more likely, and recently J. Radde of the University of Toronto reported early results in experiments on red blood cells that indicate calcitonin does stimulate the calcium pump in those cells.

The Ciba group of investigators announced several months ago that they had achieved total synthesis of the human form of calcitonin. This has opened the way for large-scale trials of the hormone for the treatment of various bone diseases. Some important uses in diseases characterized by derangement of calcium and bone metabolism had already been established. Unfortunately calcitonin does not appear promising as a cure for osteoporosis, by far the most common of the metabolic bone diseases. (It is particularly common in women over the age of 55 and in severe cases frequently leads to the fracture of bones because they cannot withstand ordinary mechanical stresses.) The discovery of calcitonin has, however, given a great impetus to the study of the fundamental processes of bone metabolism and turnover of the bone substances, and the rapid growth of knowledge in this field may soon bring forth a rational therapy for the disease. If so, the bringing to light of this remarkable new hormone will have served a most important catalytic function in medicine.

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THE CELLULAR BASIS OF METABOLIC BONE DISEASE

HOWARD RASMUSSEN, M.D., PH.D., AND PHILIPPE BORDIER, M.D.

TO a generation of physicians brought up with an Albrightian view of metabolic bone disease,[1] bone turnover or remodeling was a consequence of the activities of osteoblasts (formation) and osteoclasts (resorption) on the bone surface. These two cell types were thought to cover the metabolically active bone surfaces and were considered the sites of action of such hormones as the parathyroid hormone. Of especial import, these cells were thought to be rather randomly distributed over the bone surface, particularly the endosteal bone surfaces of trabecular and haversian bone, and to represent independent sites of skeletal metabolism.

It has generally been considered that two independent lines of cellular differentiation occur in skeletal tissue, both starting from pluripotent mesenchymal cells, one leading to osteoblasts, bone-forming cells, and the other to osteoclasts, bone-resorbing cells.[2] The osteoblasts ultimately become osteocytes within the newly formed bone. The fate of the osteocytes during the subsequent resorption of bone was debated, but the preponderant opinion held that they fused to become osteoclasts. The fate of the osteoclast was less clear. A widely accepted view was that the osteoclasts represented an end stage in bone cell differentiation — namely, that they died like other multinucleated giant cells after performing their function.

The crucial feature of this dualistic view of bone remodeling was that bone resorption was independent of bone formation. Each activity was controlled by different specialized cells. A number of observations, however, remained unexplained by this dualistic view. The simultaneous measurement of the rates of bone formation and bone resorption showed that these two activities often changed in the same direction — i.e., under conditions in which the rates of resorption were highest the rates of formation were also highest, as exemplified by Paget's disease. Furthermore, in experimental hyperparathyroidism in young animals and in the secondary hyperparathyroidism of renal osteodystrophy in man, a net increase in bone mass was often observed even though the parathyroid hormone was thought to enhance bone resorption and inhibit bone formation. In addition, at the histologic level it became clear that new bone is only laid down at sites of previous resorption; in Paget's disease, which is characterized by large numbers of osteoclasts, no death of osteoclasts was seen, and in studies employing tritiated thymidine to trace the course of DNA synthesis in the various bone cells, incorporation occurred first into osteoclasts, then into "preosteoclasts" and preosteoblasts, and finally into osteoblasts.[3] These findings called for an explanation.

THE BONE REMODELING UNIT, THE BONE METABOLIC UNIT AND A SEQUENTIAL MODEL OF CELLULAR EVENTS UPON THE ENDOSTEAL BONE SURFACES

While attempting to correlate the known physiologic and biochemical evidence with the results of morphologic examinations of bone from human beings suffering from a variety of metabolic bone diseases, we were led to re-examine the classic view described above.[2] On re-examination, additional shortcomings of the old theory were found. Consequently, a new point of view was developed. In its development, three additional facts were of major importance.

The first concerned the effect of calcitonin on osteoclast structure and number. After calcitonin was administered to young rabbits, whose endosteal bone surfaces display normally a large number of multinucleated osteoclasts, the number of these cells decreased markedly within 15 minutes. The osteoclasts were replaced by a considerable number of mononucleated cells, which appeared to be preosteoblasts and, within hours, young osteoblasts.[3] Osteoclasts did not die but underwent, instead, a fission into mononucleated cells.

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A rare intermediate form was even discerned — namely, an osteoclast undergoing the fission process.

Secondly, studies of the mitotic events in skeletal remodeling indicated that the only bone cells that undergo mitotic division were the mesenchymal cells and their immediate progeny. Preosteoblasts do not undergo mitosis.

The third fact was recognized by Frost[4]: the normal sequence of events in endosteal bone remodeling is activation of a new group of cells, followed by resorption of bone by the daughters of these cells, and later by sequential formation of new bone at the site of the previous bone resorption. Thus, the sequence of events on the bone endosteal surface was always a progression of activation (A) ——→ resorption (R) ——→ formation (F). Formation never occurred without prior resorption.

These three facts, in particular, led to the following view of the events occurring at the endosteal bone surface: a sequential progression of cells takes place from surface mesenchymal cells ——→ daughter cells (preosteoclasts) - - - → osteoclasts ——→ postosteoclasts (or preosteoblasts) - - - → osteoblasts ——→ osteocytes (Fig. 1). This constitutes the cellular counterpart of Frost's A ——→ R ——→ F. Mitotic division is a property of only the mesenchymal cells and their immediate daughter cells, and in this sense, the activation of a new unit of bone cells engaged in surface remodeling is a differentiation. The fusion of the daughter cells to form osteoclasts and the fission of the single osteoclasts to form numbers of preosteoblasts and then osteoblasts represents sequential modulations of cell function but not differentiation.

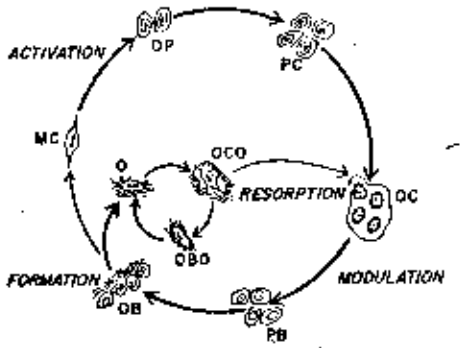

Figure 1. Schematic Representation of the Sequence of Cellular Events in a Bone Remodeling Unit on the Endosteal Bone Surface.

The initial step (left) is the activation of mesenchymal cells (MC) to become osteoprogenitor cells (OP), which by further division become preosteoclasts (PC), which then fuse to become osteoclasts (OC). These eventually undergo modulation to become preosteoblasts (PB), which go on to become osteoblasts (OB), which after completing their synthetic function become osteocytes (O). Once the sequence of events has transpired, the osteocytes in a bone metabolic unit function in maintaining mineral homeostasis. In carrying out this function, they recapitulate, in a sense, a similar cell cycle of resorption (osteoclastic osteocyte — OCO) and formation (osteoblastic osteocyte — OBO). In severe hyperparathyroidism, the osteoclastic phase of the osteocytic cell cycle may be exaggerated to the point where several adjacent osteocytes remove all the bone between them, and then fuse to become osteoclasts.

There is an alternative model that appears less likely but cannot be completely ruled out by present evidence. In this alternative model the daughter cells arising from the mitotic divisions of the osteoprogenitor cells could give rise independently to osteoclasts or osteoblasts — i.e., there are, as previously proposed, two independent pathways for bone cell differentiation, but one or more metabolic products arising from the activity of osteoclasts would regulate the progression of preosteoblasts to osteoblasts. In an operational sense, the physiologic consequences of this alternative model would be identical to the first model. The crucial feature of both would be that the cell pools on the remodeling surfaces are inter-related and do not operate as independent cell populations of osteoclasts and osteoblasts.

Frost has described such a unit of bone surface remodeling as an "active bone metabolic unit." By this term he has implied that activity occurs during the period in which either surface resorption or formation take place. Once new bone formation has ceased, the unit becomes inactive. This point of view implies that once remodeling activity has ceased, the unit of newly formed bone is no longer metabolically active, but this was a premature conclusion. It is now clear that the osteocytes within and upon the surface of such a unit are metabolically active and have an important physiologic function. We therefore propose as a better term, "bone remodeling unit." This unit is equivalent to Frost's active bone metabolic unit. Its lifetime is concurrent with that of a newly activated group of mesenchymal cells in the progression through their osteoclastic and osteoblastic phases. Once bone formation, including matrix synthesis and its mineralization, has ceased, the remodeling activity of this particular unit ceases.

One of the most important aspects of a bone remodeling unit is the delineation of the time course of events within it. Activation of a new group of cells takes place normally over a period of hours or days; the resorptive or osteoclastic phase lasts one to a few weeks, and the final formative phase continues for three or more months. Once it has gone through this sequence, each such unit becomes a bone metabolic unit that can be recognized morphologically either in cortical bone as a haversian system or in trabecular bone as the distinct volume located between the cement line and the bone surface. The intercellular communication within each bone metabolic unit emphasizes the unitary basis of its organization. The osteocytes within each metabolic unit have extensive intercellular connections with adjacent osteocytes and with osteocytes on the bone surface, forming an extensive lacunar-canalicular system within the unit. The osteocytes in one metabolic unit, however, do not communicate with those in the adjacent ones.[2]

Before considering the implications of this new model for the analysis of metabolic bone diseases, one

must define the separate functions of bone remodeling units and bone metabolic units: remodeling units are the functional units of skeletal remodeling, and metabolic units are those most immediately involved in mineral homeostasis. The two units represent sequential functional states of the same group of bone cells.

A recently recognized feature of osteocytic activities in these bone metabolic units is that these cells recapitulate the sequence of cellular events characteristic of a remodeling unit (Fig. 1). Osteocytes undergo cyclic transitions from bone-resorbing to bone-forming cells under the influence of hormonal and ionic factors. This minicycle of osteocytic states normally alters only the small volume of lacunar and canalicular bone immediately adjacent to the osteocyte and its extensions. This specialized lacunar bone compartment is composed of poorly organized, metabolically labile bone tissue. It serves as the immediate pool of bone mineral for the maintenance of mineral homeostasis within the organism. Normally, only a small percentage of osteocytes within a given metabolic unit can be characterized histologically as being engaged in osteocytic osteolysis. In either primary or secondary hyperparathyroidism this percentage increases, and is, in fact, the most sensitive osseous indicator of increased circulating parathyroid hormone. If excessive parathyroid hormone secretion is maintained for long periods, not only will there be a shift to osteolytic phases, but some osteocytes may eventually resorb sufficient adjacent lamellar bone to fuse with their neighbors, and thus rejoin the pool of active osteoclasts on the bone surface. This osteocytic cell cycle and its control are important components of bone physiology. Its role, however, will not be detailed because the present article pertains to the relation between bone remodeling units and metabolic bone diseases.

A consideration of the model of cellular events shown in Figure 1 leads to the conclusion that the ultimate consequence of changes in surface remodeling (and therefore in skeletal structure and mass) depends on the number and activities of two cell pools — osteoclasts and osteoblasts — but that, in contrast to previously held views, the relative sizes of these two cell pools are dependent rather than independent variables.

In any discussion of their relation it is simplest to begin by considering three possible consequences that may ensue during the lifetime of a bone remodeling unit (Fig. 2). In the most ideal case (ideal from the point of view of skeletal homeostasis) the amount of new bone synthesized during the formation phase replaces exactly the bone removed during the resorption phase. Alternatively, the new bone formed may be either less than or greater than that removed, leading correspondingly to a positive or negative balance of bone within the unit. It is obvious that gross skeletal balance will represent the sum of the individual balances of the numerous bone remodeling units that are active at a given time.

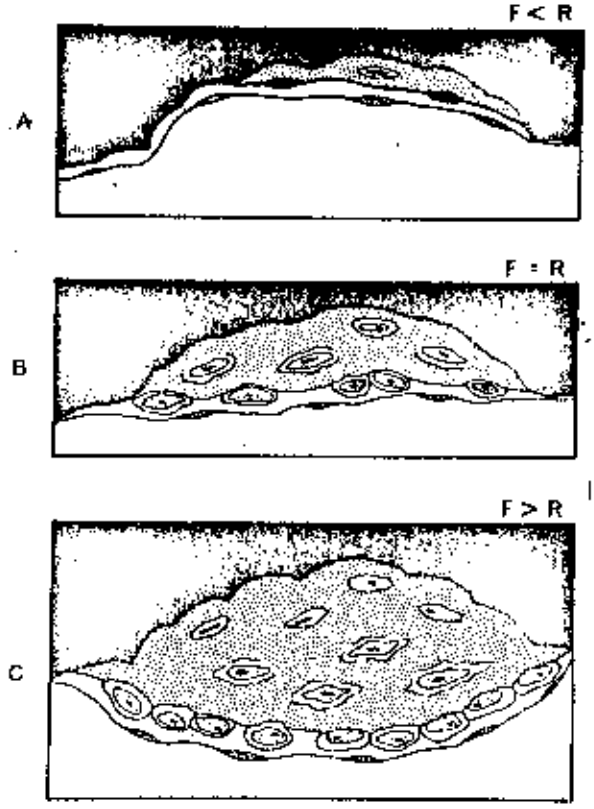

Figure 2. The Three Possible Results of Remodeling Events in a Single Bone Remodeling Unit.

The formation (F) phase may not replace the bone during the resorption (R) phase (*A*); the two may be balanced (*B*), or the formation phase may add more bone than that removed during the resorption phase (*C*).

Returning to a consideration of the model depicted in Figure 1, it is clear that the extent of bone resorption will depend on the number and activity of the osteoclasts, and the extent of formation on the number and activity of the osteoblasts. The size of the osteoclast pool will be determined both by the rate of activation of new mesenchymal cells and by the rate of modulation of osteoclasts to osteoblasts. By contrast, the size of the osteoblast pool will be determined by the rate of modulation of osteoclasts to osteoblasts and by the time during which these latter cells are actively synthesizing new bone matrix.

Hormonal and Ionic Control of the Bone Remodeling Units

To induce a change in the balance of remodeling events in the skeletal system as a whole requires either a change in the balance of events in the pre-existing bone remodeling units or a change in the rate of activation of new units (*or* both). Thus, a duality of control can be envisioned, one that alters rapidly the activity of pre-existing units, and the other that changes the average number of such units over a longer period. It can be anticipated that many of the agents that control events on the bone surface will alter both cell activity and cell number (Table 1). For example, raising the concentration of circulating parathyroid hormone might in-

crease net bone resorption as follows: by increasing the activity of pre-existing osteoclasts; by increasing the activation of new osteoprogenitor cells; by decreasing the activity of pre-existing osteoblasts; and by reducing the rate of modulation of osteoclasts to osteoblasts. In fact, there is evidence that parathyroid hormone exerts all four such actions.[2]

Table 1. Effects of Hormones, Ions and Physical Activity on the Differentiation, Modulation and Activity of Bone Cells.*

Agent	Activation of Mesenchymal Cells to Osteoclasts	Modulation of Osteoclasts to Osteoblasts	Activity	
			Osteoclasts	Osteoblasts
PTH	++		+	--
Ca^{++}	--	++	-	?
T_4	++	+	+	+
Cortisol	0 or -	-	?	--
Physical activity	+	++	?	?
$HPO_4^{=}$	?	+	?	+

*PTH indicates parathyroid hormone, Ca calcium, T_4 thyroxine, & + a stimulatory, - an inhibitory, 0 no effect, & ? unknown action of the particular agent

One must distinguish between transient and steady-state changes in the balance of remodeling activities. Since the average lifetime of a remodeling unit is two to three months, if the administration of a therapeutic agent (e.g., calcitonin) caused an immediate increase in the modulation of osteoclasts to osteoblasts while simultaneously blocking the activation of new progenitor cells, biopsies taken a month later would show a shift in bone surface activities in favor of bone formation. After three months, however, no new bone remodeling units would have been formed, and very low rates of both resorption and formation would be found. Skeletal balance would be re-established, but the total rate of skeletal remodeling would be greatly reduced. This means that periods of six months or more are necessary for valid assessments of the efficacy of drugs employed to treat diseases of bone remodeling.

In addition to parathyroid hormone and calcitonin, the factors of major importance in the control of the size of the pools of active bone cells and of their functions are thyroid hormone, cortisol, phosphate ion, growth hormone and mechanical stress. The distinctive effects of these factors are summarized in Table 1. Instead of discussing each in detail, we will consider the effects of some in relation to one or more metabolic bone diseases, without including all the humoral agents or all metabolic bone diseases. We will concentrate on a sufficient number to illustrate how the newer model of cellular events accounts for the changes seen in the various disease states. The effects of vitamin D deficiency and the pathogenesis of the various forms of osteomalacia will not be considered although they are important subjects; being extremely complicated, their discussion would require more than our available space.

Metabolic Bone Diseases Classified in Terms of the Sequential Model of Bone Surface-Cell Activity

Up to this point events at the endosteal bone surface have been emphasized because the rates of bone remodeling are considerably greater at this surface (which includes both haversian and trabecular bone surfaces) than at the periosteal surface. A distinctive feature of the periosteal surface is that although bone turnover is very slow in the adult, it results in a slight but progressive net formation of bone throughout life. After the age of 25 to 35, in contrast, there is a progressive net resorption of bone on the endosteal surface. A question yet to be fully answered is whether the sequence of cellular events that is postulated to occur on the endosteal surface (Fig. 1) also takes place on the periosteal surface. It is possible that the net formation of periosteal bone is achieved either because some local factors normally enhance the rate of modulation of osteoclasts to osteoblasts, or, alternatively, because the periosteal surface activation of osteoprogenitor cells leads directly to preosteoblasts without an obligatory osteoclastic phase. Despite evidence in support of both points of view the ensuing discussion of the cellular basis of metabolic bone diseases in man is based almost entirely on observations of events taking place on the endosteal bone surface.

Paget's Disease

A logical starting point in our discussion of bone diseases is to consider Paget's disease of bone even though it is not generally considered a metabolic bone disease. In his original description Paget gave it the name of osteitis deformans because this disease leads to skeletal deformity. He considered it to be an inflammatory process, but there is, as yet, no support for inflammation as a cause. A more probable etiology originates from a consideration of two of its distinctive features: there is generally a direct correlation between rates of bone resorption and bone formation (i.e., patients with the highest rates of bone resorption also exhibit the highest rates of formation); and there is a marked heterogeneity of cell size, shape and staining characteristics of the active bone cells. Examination of the osteoclasts in bone from normal human beings, or from patients having either thyrotoxicosis or severe primary hyperparathyroidism with high rates of bone remodeling reveals that these cells are of rather uniform size and contain from two to 10 nuclei per cell (average, four). In contrast, the size of osteoclasts in bone sections from patients with Paget's disease varies enormously and the osteoclasts often contain 100 or more nuclei. Likewise, the osteoblasts in biopsies from patients with primary hyperparathyroidism are uniform in size, shape and staining qualities, giving the impression that these osteoblasts are "all soldiers at dress parade," in contrast to the osteoblasts seen in biopsy specimens of bone in Paget's disease.

These two features, taken in conjunction with the model of cellular events shown in Figure 1, lead to the conclusion that Paget's disease is a neoplasia affecting the bone remodeling units (Fig. 3). The primary event is the activation of a greater than normal number of new osteoprogenitor cells, which exhibit considerable heterogeneity. These then go on to form osteoclasts and osteoblasts, which continue to exhibit the heterogeneity of the original osteoprogenitor cells. Bone resorption and formation increase concomitantly owing to a marked increase in remodeling units that exhibit sequentially resorption and formation phases. They frequently exhibit a net imbalance of activities in favor of formation, primarily because the newly laid bone is less highly organized, and therefore occupies a larger volume than a similar amount of normal bone.

In support of this point of view is the fact that when calcitonin is given to patients with Paget's disease, the most immediate change is a decrease in bone resorption because calcitonin acts not only to inhibit osteoprogenitor cell activation but also to stimulate the modulation of osteoclasts to osteoblasts. There is then an appreciable fall in bone resorption associated with a transient increase in bone formation. This lasts for a few weeks to a month or more and is followed by a progressive decline in the rate of bone formation as a consequence of the preceding inhibition of osteoprogenitor cell activation. Thus, it is logical to consider Paget's disease a benign hormone-sensitive tumor of bone.

Medullary Carcinoma of the Thyroid

Investigation of the cause of the dramatic hypocalcemia produced by calcitonin in young animals led to the conclusion that it acted by inhibiting bone resorption (Table 1). Attempts to demonstrate that calcitonin also enhanced bone formation were not convincing. However, the observation that patients with medullary carcinoma of the thyroid gland have high concentrations of calcitonin circulating in their plasma led to the examination of bone specimens from these patients. The extent of bone surfaces at which either active bone formation or resorption is occurring was found to be drastically reduced.[2] Chronic excessive circulating calcitonin leads to a profound reduction in the rate of activation of new bone remodeling units—i.e., a reduction in activation of osteoprogenitor cells. As a consequence, both resorption and formation surfaces are equally reduced, skeletal balance is maintained, but the overall rate of skeletal remodeling is greatly reduced (Fig. 3). A prolonged excess of calcitonin does not lead to an increase in bone formation, but because of the dual effects of the hormone, its acute administration may lead to a transient positive skeletal balance.

Primary Hyperparathyroidism

Most studies of the effects of excessive parathyroid hormone have been of a short duration. From them, four well defined effects of the hormone have been noted[2]: an increase in the activation of new osteoprogenitor cells; an inhibition of the modulation of osteoclasts to osteoblasts; an increase in the activity of pre-

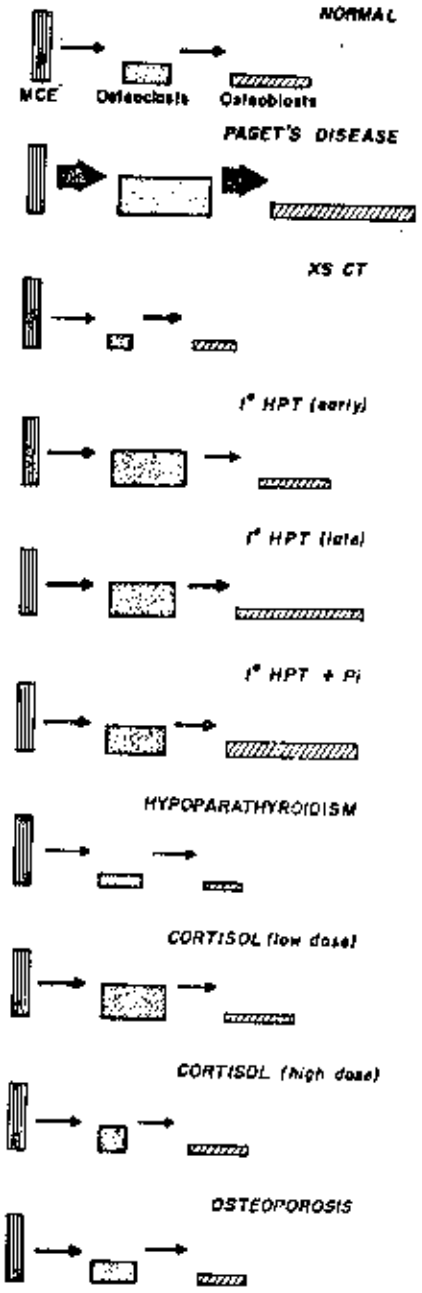

Figure 3. Changes in Cell Activity and in the Size of Osteoclast and Osteoblast Pools in Various Metabolic Bone Diseases or Hormonal States in Man.

Height of blocks represents the extent of cell activity, and width the size of the respective cell pool. Thickness of arrows represents the rate of flow of cells from one pool to the next. In the normal young adult, the rate of flow of cells from cell in the mesenchymal cell envelope (MCE) to osteoclasts and the subsequent flow of osteoclasts to osteoblasts are equal, so that the total activities of the two pools are equal and skeletal homeostasis is maintained. The consequences of the various hormone and pathologic states are discussed in the text. 1°HPT indicates primary hyperparathyroidism, XS CT, excess circulating calcitonin, and Pi, inorganic phosphate.

existing osteoclasts; and an inhibition of the activity of pre-existing osteoblasts (Table 1). The combined effect of all these actions is to cause an increase in osteoclast pool size, a decrease in osteoblast pool size, an increase in bone resorption and a decrease in bone formation. These changes lead to a net negative skeletal balance, but this acute resorption phase is relatively shortlived. Long-term administration of moderate doses of parathyroid hormone or moderately severe primary hyperparathyroidism in man leads to a secondary rise in the size of the osteoblast pool, which is usually sufficient to re-establish relative skeletal balance (Fig. 3), with a marked increase in overall rate of skeletal remodeling — i.e., there are many more bone remodeling units at any given time, but their formative and resorptive activities are in balance, or nearly so. However, the secondary rise in size of the osteoblast pool often does not re-establish exact skeletal balance in patients with this disease. Some increase in rate of loss in skeletal mass is commonly observed. The administration of inorganic phosphate to these patients will often restore skeletal balance completely (Table 1 and Fig. 3). Phosphate administration leads to a rise in plasma phosphate, a fall in plasma calcium and a positive calcium and skeletal balance with little change in urinary hydroxyproline excretion (an index of the rate of bone resorption). Within the skeleton, its most dramatic effects are to increase the activity of pre-existing osteoblasts and increase the modulation of osteoclasts to osteoblasts without changing appreciably the effect of parathyroid hormone on osteoprogenitor cell activation.

Most studies of the effects of phosphate have been short term, and the positive skeletal balance induced by phosphate administration may be a transient rather than a sustained change in skeletal balance. This does not appear likely, however, because patients with renal osteodystrophy (those without evident vitamin D deficiency) have a secondary hyperparathyroidism associated with renal retention of phosphate and a net increase in the mass of cancellous bone — i.e., a sustained positive skeletal balance with a high rate of skeletal remodeling.

Hypoparathyroidism

If parathyroid hormone stimulated bone resorption and inhibited bone formation, as classically thought, in hypoparathyroidism, bone resorption should decrease, and bone formation increase. In this condition, however, both bone formation and bone resorption decrease (Fig. 3). The overall rate of skeletal remodeling is greatly reduced, and the bones appear similar to those seen in patients with medullary carcinoma of the thyroid. Yet in chronic untreated hypoparathyroidism, a small net positive skeletal balance is often found owing to a net positive balance of bone formation at the endosteal surface. Thus, in the few bone remodeling units that do become activated the rate of modulation of osteoclasts to osteoblasts is increased, possibly because of the high extracellular phosphate concentration, and net formation is observed. Events at the endosteal bone surface in the hypoparathyroid subject are similar to events on the periosteal bone surface in normal subjects.

Cortisol-Induced Osteoporosis

One of the best documented causes of osteoporosis in man is excessive circulating adrenal glucocorticoid, either in Cushing's disease or secondary to the therapeutic administration of these hormones. In laboratory animals initial attempts to produce osteoporosis by the administration of large doses of cortisol failed. This apparent species difference has been resolved by the careful study of Jee et al.,[5] who found dose-related effects of cortisol on the sequence of events induced by stimulation of new bone remodeling units in the periodontal ligament of the mature rat. Small doses of steroids (0.5 to 5.0 mg per kilogram per day) caused an increase in the size of the osteoclast pool and a decrease in the osteoblast pool (Fig. 3). Other studies have shown that these steroids also inhibit the synthesis of bone collagen by pre-existing osteoblasts (Table 1). The result of these changes is a net balance of skeletal activity in favor of resorption. In the acute phase of action of low doses of adrenal steroids on bone remodeling units the changes are similar to those seen after parathyroid hormone administration, in keeping with the fact that cortisol administration leads to increased parathyroid hormone secretion. In parathyroidectomized animals, cortisol leads to a slight decrease in the size of the osteoblast pool, but no longer stimulates the activation of osteoprogenitor cells to osteoclasts. Thus, a major factor in the pathogenesis of the osteoporosis produced by low doses of cortisol is a secondary hyperparathyroidism. This condition leads to the activation of new osteoprogenitor cells, and acting in concert with the steroid hormone, inhibits the modulation of osteoclasts to osteoblasts, as well as the activity of the few osteoblasts that form. The only difference between this state and the state of moderate hyperparathyroidism is that the adrenal hormones prevent the secondary rise in osteoblast pool size and bone formation rate, which is the usual course of events in hyperparathyroidism. Thus, in the presence of low doses of cortisol there is an uncoupling between resorption and subsequent formation with a rapidly progressing osteoporosis (Fig. 3).

Larger doses of cortisol (25 to 50 mg per kilogram per day) inhibit osteoprogenitor cell activation, as well as the modulation of osteoclasts to osteoblasts (Table 1). Hence, the degree of inhibition of bone formation is as great, but the size of the osteoclast pool and therefore the rate of bone resorption is considerably less (Fig. 3) than during treatment with lower doses of this steroid.

Postmenopausal Osteoporosis

One of the most striking aspects of the aging skeleton is a progressive loss of bone mass.[6] If one examines bone biopsies from the normal human ilium in persons of

different ages there is a slow but progressive loss of both cortical and cancellous bone throughout life. Also, if one examines long bones by quantitative radiographic methods there is a progressive loss of cortical bone mass with age even though the external transverse diameter of the bone increases. This is due to slight net positive skeletal balance on the periosteal surface, but a more marked negative skeletal balance on the endosteal bone surface.

Although at the age of 15 to 19 years the mean skeletal mass of women is greater than that of men, and although there is a progressive loss of bone throughout life in both sexes, the incidence of osteoporosis is considerably lower in elderly males than in age-matched females. There are two reasons for this apparent paradox. The first is that from the age of 20 to 50 or 55 the average rate of loss of skeletal mass in females is slightly greater than in males. Even so, the total skeletal mass is nearly equal in 50-year-old males and females. However, during the 10 to 15 years between 50 to 55 and 65 there is a dramatic and abrupt loss of skeletal mass in females. At the end of this period the average skeletal mass of females is definitely lower than that in males, and from then on the rate of loss of skeletal mass is actually less in females than in males.

It is clear that an underlying factor in the development of postmenopausal osteoporosis has to be this abrupt loss of bone during the fifth and sixth decades of life. Postmenopausal osteoporosis represents only one end of a spectrum in terms of skeletal mass, the end in which skeletal loss has been sufficient to predispose the patient to crush or other types of fractures. The etiologic factors underlying the abrupt loss of skeletal mass in women in later life are not known, but two general theories attempt to account for the negative skeletal balance that underlies the development of postmenopausal osteoporosis. The first, espoused initially by Albright,[1] has proposed a failure of osteoblast function. The second, espoused by a number of investigators, has proposed an increased rate of bone resorption.

A major piece of evidence used to support the theory of increased resorption was that obtained by quantitative microradiography, which estimates the extent of the bone surface occupied by resorption sites and the extent occupied by formation sites by noting the characteristic surface profile that these two types of surfaces project. The most dramatic change observed was a marked increase in total resorption surface. However, a major assumption in this type of analysis is that the microradiographic appearance of a resorption surface indicates an active resorption surface. If this were so, examination of similar bone sections by quantitative histologic methods should show an increased number of osteoclasts on the endosteal bone surface. This has not been the case; osteoclast numbers are either normal or slightly reduced but not elevated in osteoporotic bone. The total extent of resorption surface, however, is found to be increased, just as indicated by microradiography. The important distinction is that by histologic analysis it is possible to distinguish between inactive or arrested resorption surface and active resorption surface. In the bone of most patients with postmenopausal osteoporosis, the extent of active resorption surface is nearly normal, but the extent of inactive resorption surface is substantially increased, and the extent of active bone formation surface decreased. A logical explanation of these changes can be made by reference to Figures 1, 2 and 3. In osteoporosis the activation of new remodeling units continues at a nearly normal or slightly reduced rate. The osteoprogenitor cells form osteoclasts, which, however, contain, on the average, fewer nuclei than normal. In all likelihood these osteoclasts carry out their normal resorptive phase, but after their fission to postosteoclasts or preosteoblasts, either their modulation to osteoblasts is greatly delayed or an important percentage never become active osteoblasts. As a consequence, inactive or arrested resorption surfaces increase in extent, and active formation surfaces decline.

This view is, in reality, an updated and possibly somewhat more sophisticated version of the original theory proposed by Albright.[1] Osteoporosis in postmenopausal women results from the failure of osteoblastic activity. It represents an uncoupling of the normal progression from resorption (osteoclast) to formation (osteoblasts) in bone remodeling units.

The importance of validating this view lies in its therapeutic implications. If this model is correct and if calcitonin acts as proposed, it is obvious that calcitonin cannot be an effective therapeutic agent for the treatment of postmenopausal osteoporosis. Effective therapy must be aimed at increasing the rate of activation of new bone remodeling units and simultaneously increasing the modulation of osteoclasts to osteoblasts. It must somehow re-establish the coupling that normally exists between resorption and subsequent formation.

Conclusions

A model of the cellular events underlying skeletal remodeling at endosteal bone surfaces has been developed that emphasizes the sequential nature of these events: activation ⟶ preosteoclasts ⟶ osteoclasts ⟶ postosteoclasts or preosteoblasts ⟶ osteoblasts. The crucial feature of this model is that the activities of osteoclasts and of osteoblasts are directly and intimately related. The present model accounts for a number of previously unexplained facts and provides a satisfactory basis for describing the nature of the cellular derangements underlying the development of a number of metabolic bone diseases in man. As such, it represents a more logical basis on which to devise effective therapeutic measures for some of these diseases.

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The Effect on the Rat of Prolonged Administration of Large Doses of Electrically Activated Vaporized Ergosterol

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A previous review of the clinical and experimental literature on the use of massive doses of vitamin D in arthritis and other conditions,[1] indicated that electrically activated ergosterol was less toxic than other vitamin D preparations.

Recently, Klassen and Curtis[2] carefully studied the calcium and phosphorus metabolism before, during and after the administration of large doses of electrically activated ergosterol (Ertron) in four patients. Two of these had atrophic spondylitis, and two had degenerative arthritis of the spine. Their ages ranged from 28 to 71. For four three-day periods, beginning with a dose of 200,000 units per day, the dose was increased by 200,000 units during each of the two succeeding three-day periods, so that during the last period each patient received 1,000,000 units per days. None of the patients showed signs of toxicity; there were no gastro-intestinal disturbances; there was no loss of weight, and no marked change in the clinical picture. The blood pressure remained normal. There was no change in the amount of urine excreted daily. There was no appreciable change in the average level of serum calcium during the entire period of treatment. No demonstrable changes occurred in the activity of

[illegible] (continued).

the parythyroids or the thyroids. There was an increase in the appetite of all four patients during and also after the treatment. This experiment tested the effect of massive doses of Ertron on the physiological processes of the body and was not concerned with the therapeutic effects.

Snyder, Squires and Forster have recently reported that, during a period of six years in which they used electrically activated ergosterol (Whittier process) in a group of 250 cases, they had never seen any evidence of serious toxicity as a result of the administration of from 200,000 to 400,000 units per day.

A year previously the same group[1] stated: "The safety of this form of therapy has, we believe, been established. The various criteria for determining toxicity were studied. During the 12-month period of treatment, none of the following occurred: No increased calcification of blood vessels was observed, either roentgenologically or by ophthalmological examination of retinal vessels. Renal function was not impaired as shown by repeated chemical and microscopic urinalyses and by the N. P. N. remaining normal. Gall-bladder function was not impaired. Evidence is presented to suggest some improvement of impaired gall-bladder function occasionally taking place."

Wolf,[2] using electrically activated ergosterol (Whittier process) in the single massive dose treatment of rickets, gave 600,000 units to five children from five to 18 months old. "No ill effects were observed." In 1943[3] he summarizes: "In this series, 75 infants were given 600,000 U. S. P. units of an electrically activated preparation of ergosterol (Ertron), divided in two oral doses in precooked cereal and milk at about the third to the fifth month of life . . . (These doses) were well tolerated, and no toxic manifestations were observed, such as loss of weight, vomiting, or anorexia. Rather, these infants thrived exceptionally well. No diminution in the incidence of respiratory infections was noted. There is no danger in administering single massive doses of vitamin D to young infants, even premature infants. The danger lies in repeating these large doses over a period of time so that several million units of vitamin D are given. Depending on the age and weight of the infant, such large doses may be toxic if repeated daily . . . The administration of a single massive dose of vitamin D for the prophylaxis of rickets is not only of proved effectiveness, but also is safe and time saving." In a more recent paper[7] he reports further clinical and histological evidence of the safety of Ertron. The paper includes an excellent bibliography and review of the litertaure on toxicity, safety of the various vitamin D components and preparations.

Other and earlier evidence of the relative safety of high dosage Ertron therapy may be found in the bibliography of a previous study.[1]

These evidences were so provocative that we undertook to determine the effects of long continued daily administration of large doses of Ertron to the rat.

Our chief interest was in the histological examination of organs and tissues of animals sacrificed at the end of various periods of intensive treatment. Secondarily, x-ray studies were made of half the rats in each group.

Material and Methods

FORTY-SEVEN male white rats weighing 85 to 107 grs. were divided into four treated groups of 10 animals each; seven animals served as controls. All were equally provided with water and Purina dog chow plus lettuce once or twice a week.

The four treated groups were given, by pipette, a daily dose of Ertron as indicated in Table I. The drug was dissolved in corn oil in varied concentration, so that the daily amount of oil administered was the same for each group.

At the end of 100 days of medication two rats from each of groups 1, 2, 3 and 4 and one from group C (control) were killed.

At the end of 127 days three rats from each treated group and three controls were killed.

At the end of 190 days of medication the remaining

TABLE I.

Animal number	I. U. per Kg. daily	Days of treatment
10, 11		100
12, 13, 14	4,000	127
15, 16, 17, 18, 19		190
20, 21		100
22, 23, 24	8,000	127
25, 26, 27, 28, 29		190
30, 31		100
32, 33, 34	12,000	127
35, 36, 37, 38, 39		190
40, 41		100
42, 43, 44	20,000	127
45, 46, 47, 48, 49		190
C1	0	100
C2, C3, C4	0	127
C5, C6, C7	0	190

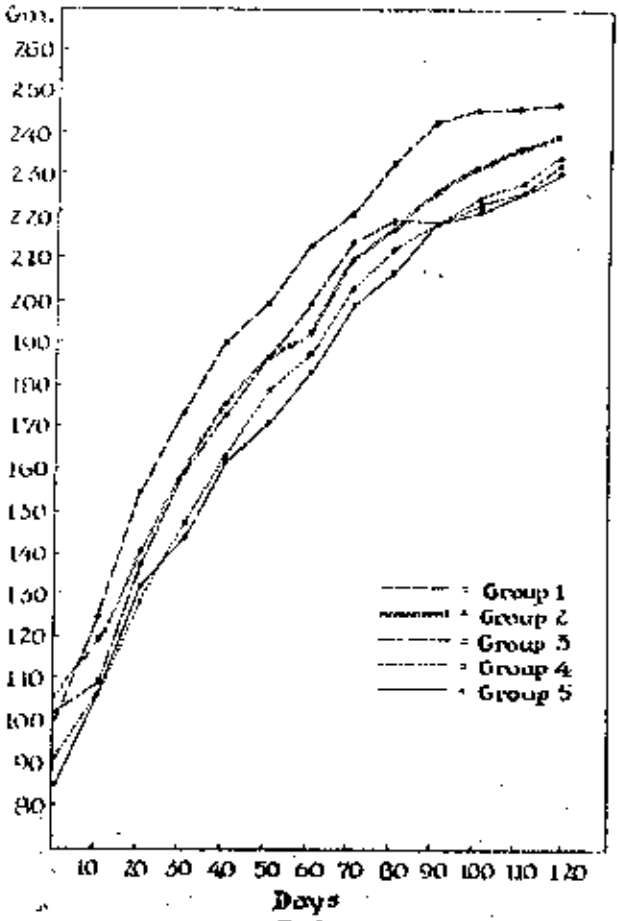
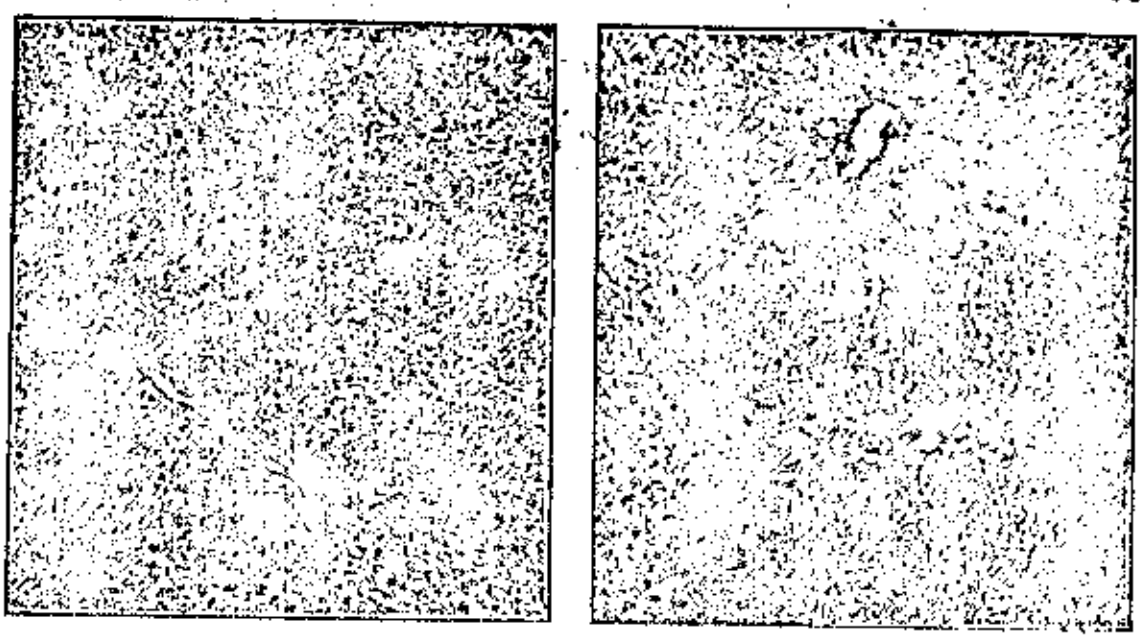


Fig. 1.
Average weight increase of treated groups (1, 2, 3, 4) and controls (5) for first 120 days. See Table I.



A Fig. 2. B

(A) Liver of rat treated with 20,000 I. U. of electrically activated ergosterol per kg. daily for 127 days. The cytoplasm of the cells about the central veins shows clear vacuoles. Hematoxylin and eosin stain. X 100.
(B) Liver of untreated control rat. The cytoplasm of the cells about the central veins shows vacuolization similar to that seen in the treated animal. Hematoxlyn and eosin stain. X 100.

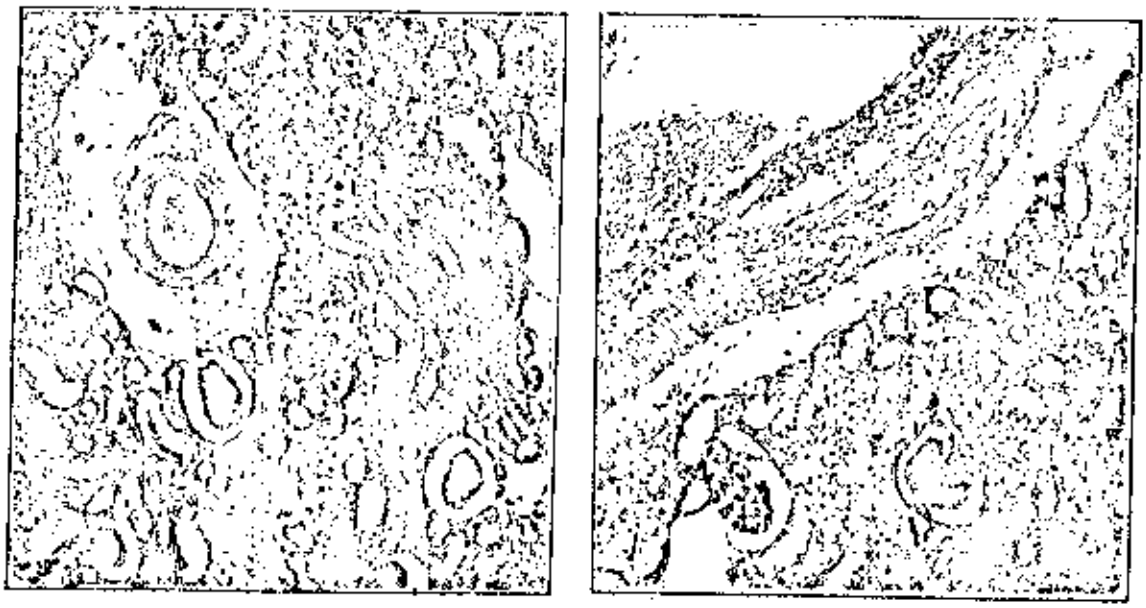
five animals of each treated group and the three remaining controls were killed and promptly x-rayed. All animals were killed rapidly by chloroform in a closed jar.

Histologic studies were made on formalin fixed tissues removed from rats treated for 100 and 127 days and from the control animals in these groups. Paraffin sections, stained with hematoxylin and eosin, were prepared from the lungs, liver, spleen, pancreas, stomach, adrenal glands, kidneys, aorta and brain. Bone sections from the lower end of the radius and ulna, and including the carpal bones of the fore foot were prepared from animals treated for 100 days, and sections from the lower end of the femur were prepared from the animals treated for 127 days. Bone tissues were decalcified in weak HNO_3 solution; embedded in paraffin and stained with hematoxylin and eosin.

Results

GENERAL: The average growth curves for groups 1, 2, 3, 4 and C (control) showed no significant divergences (Fig. 1). No indication of diarrhea, anorexia or lassitude was noticed. All animals survived, apparently in normal health, until sacrificed.

HISTOLOGICAL: As compared to respective control tissues, no changes were noted in the heart, lungs, liver (Fig. 2), spleen, pancreas, stomach, adrenal glands, kidneys (Fig. 3), aorta and brain. The medium-sized and small blood vessels in the organs studied showed no changes. No areas of metastatic



A Fig. 3. B

(A) Kidney of rat treated with 20,000 I. U. electrically activated ergosterol per kg. daily for 127 days. No changes are noted in the glomeruli, tubules, or small or medium sized arteries. Hematoxylin and eosin stain X 100
(B) Kidney of untreated control rat. Hematoxlyn and eosin stain. X 100.

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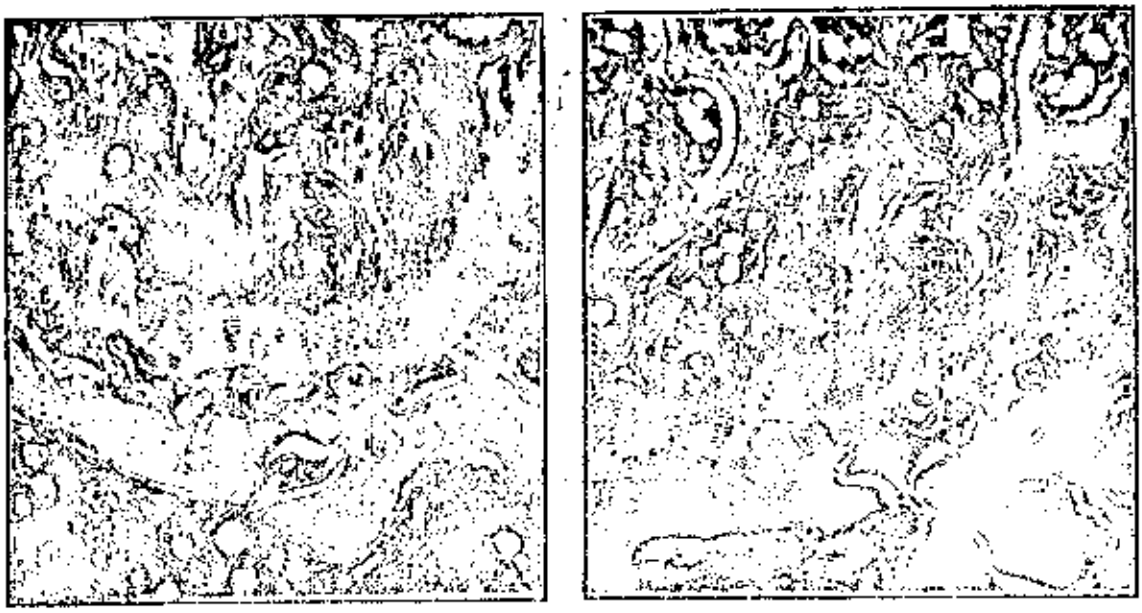

A Fig. 4. B

(A) Lower end of femur of rat treated with 20,000 I. U. of electrically activated ergosterol per kg. daily for 127 days. Regular rows of cartilage cells which become regularly transformed into bone tissue are present at the epiphyseal line. No changes are seen in the trabeculae or marrow. Hematoxylin and eosin stain. X 80.
(B) Lower end of femur of untreated control rat. Hematoxylin and eosin stain. X 80.

calcification were found. Comparative studies of bone tissues showed the epiphyseal lines of both experimental and control animals to be composed of regularly arranged rows of cartilage cells which became sharply transformed into bone tissue. There was no abnormal proliferation of endostical or periosteal bone, nor was any evidence of decalcification noted. The marrow cavities of both experimental and control animals showed blood forming cells mixed with fat tissue (Fig. 4).

RADIOLOGICAL: Roentgenograms of the rats which had 190 days of dosage showed no signs of alteration in bones, joints, cartilages or other structures as compared with controls. (See Figs. 5, 6, 7.)

Discussion

THE experimental periods were considered adequate, as they represent from 1/11 (100 days) to 1/6 (190 days) of the average life of the rat. We wish to emphasize that the material was administered to the

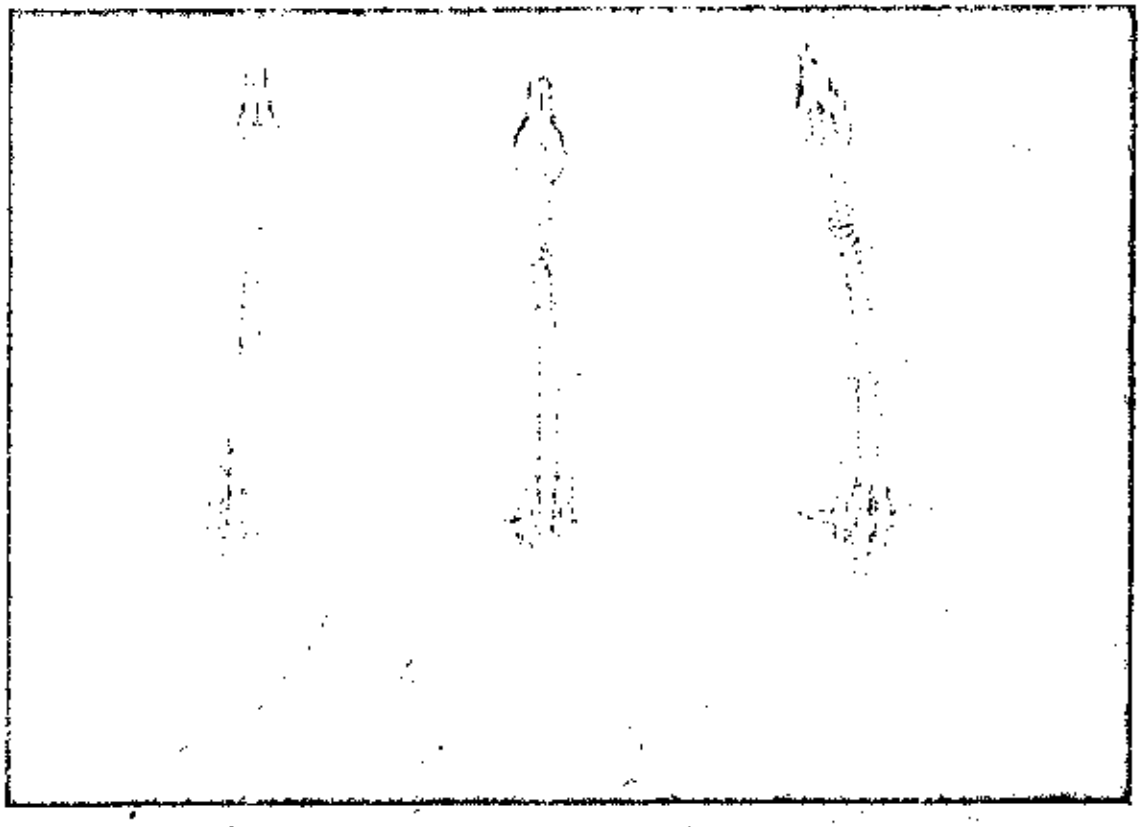

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Fig. 5.
Comparative roentgenograms of treated and untreated rats at the 190th day. See Table I.

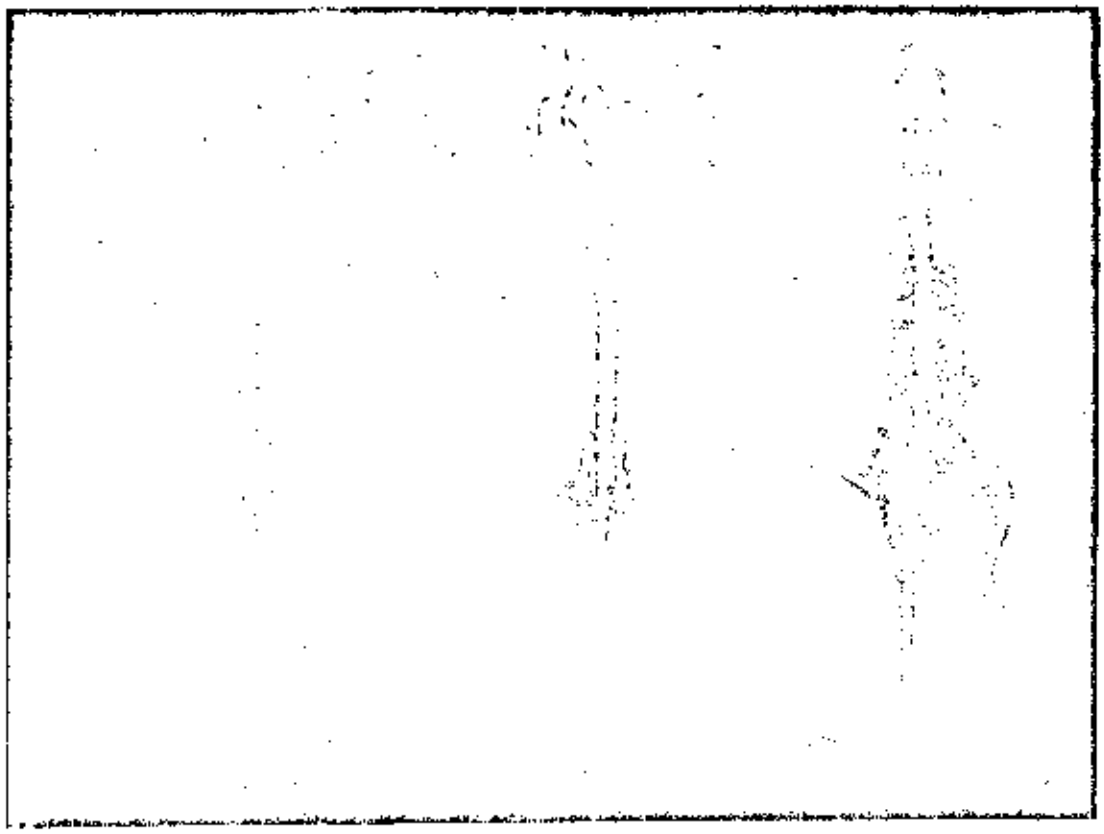

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Fig. 6.

Comparative roentgenograms of treated and untreated rats at the 190th day. See Table I.

animals during their most active period of growth.

Since there are about 10 forms of vitamin D known it should also be emphasized that the above observations are based only on the one preparation used, and are in no sense comparative.

Summary

IN THE RAT, daily doses of electrically activated ergosterol up to 20,000 I. U. per kg. for 127 days produced no histological changes as compared with control animals or those receiving smaller doses.

1. 4,000 I.U. per Kg. 2. 12,000 I.U. per Kg. C. Control 3. 8,000 I.U. per Kg 4. 20,000 I.U. per Kg

Fig. 7.

Comparative roentgenograms of femurs of treated and untreated rats at the 127th day.

2. Similarly, rats receiving up to 20,000 I. U. per kg. daily for 190 days showed no discernible radiological alterations in the body structures.

3. None of the animals showed any signs of toxicity at any time. All survived, apparently in normal health, until the time of sacrifice.

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Comparative Therapeutic Value and Toxicity of Various Types of Vitamin D

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CLINICAL reports of the toxic effects of massive doses of vitamin D, on the one hand, and of the benefit of such therapy to arthritic patients, on the other, have been so conflicting that some interpretation should be offered the medical profession with regard to this form of medication.

Physicians should be reminded that preparations of high potency are the result of activation of vitamin D precursors. There are many of these, of which ergosterol and 7-dehydrocholesterol have so far been found most fruitful. All known precursors have a common chemical relationship, being derivatives of cyclopentenophenanthrene. Yet relatively minor changes in structural conformation and substitutions result in widely different pharmacological activity. The basic structure of the precursor group is found also in the male and female sex hormones,[1] the active principle of adrenal cortex, and a number of cardiac aglucones.

A fact of importance, and apparently not so well known, is that "the progressive irradiation of a single vitamin D precursor can result in the formation of a series of compounds, each member of which has a different degree and type of physiological activity."[1]

As Windaus and associates pointed out some years ago, the various irradiation products of ergosterol may have an action other than antirachitic. While the series, as a whole, has a marked influence on calcium metabolism, its members are not equally antirachitic, and some are many times more toxic than others.

Early impressions of the use of irradiated ergosterol, especially from the German clinics, were that there was little margin between therapeutic and toxic dosage. We can now say with some degree of assurance that this proximity was the result of a non-uniform activation of the precursor, yielding an uncontrolled mixture of calciferol and toxisterol type compounds. By the latter, bone may be decalcified, resulting in hypercalcemia, and causing the unwanted calcifications so stressed in the earlier reports.

The various workers for the most part have been using two different types of activated ergosterol compounds—one, made by ultraviolet irradiation of ergosterol, which was not designed to obtain a product for massive dosage and which should not be used in the treatment of arthritis; the other made by electric current activation of heat-vaporized ergosterol (Whittier process). By the latter procedure, only one-sixth of the yield by the former is obtained.[2] Favorable and relatively safe action has been uniformly gotten with the heat-vaporized electric current activated product. Yet it is interesting to note how many clinicians using these various preparations are unaware that there is a difference in the activating process through which the high potency compounds are obtained.

In this paper we are concerned with two important questions: (a) beneficial effect in arthritic patients; (b) incidence of toxicity, from the use of large doses of vitamin D.

I. The Question of Benefit

There are a great many reports of clinical trial of vitamin D in arthritis which are unfavorable. Among those who have used ordinary ultraviolet irradiated ergosterol, Vrtiak and Lang[3] treated twenty patients with daily doses of viosterol (ultraviolet irradiated ergosterol) of from 150,000 to 250,000 U.S.P. units of vitamin D. Two patients showed marked improvement, six moderate improvement, four slight improvement and eight no improvement. They concluded that these results are similar to those obtained with a number of other methods of treatment or with palliative measures.

Wyatt, Hicks and Thompson[4] used vitamin D prepared as irradiated ergosterol in sesame oil, supplied for experimental purposes by the National Institute of Nutrition, Los Angeles, and viosterol and vitamin D in propylene glycol. The percentage of clinical improvement noted was definite and clear-cut in 20 per cent. In eight patients, or 20 per cent of the total series, treatment was abandoned entirely because of unfavorable reactions, while the remaining 60 per cent showed no specific benefits that could be attributed to vitamin D therapy.

Abrams and Bauer's[5] widely quoted report confirms these findings that the administration of massive doses of vitamin D in rheumatoid arthritis is of little or no value in altering the course of the disease. These authors administered vitamin D in the form of drisdol for a short time to the first five patients in the series. These and the remaining patients subsequently received a more concentrated preparation, crystalline vitamin D in propylene glycol or in sesame oil, with results that were almost entirely negative. They were unable to exceed a dose of 200,000 U.S.P. units daily of the crystalline vitamin D in propylene glycol administered in milk without encountering toxic symptoms.

It may be also concluded from Sternberg's report[6] that an effect of massive doses in chronic arthritis was non-specific, and any improvement noted would fall in line with that following the use of many other non-specific remedies.

In 1939 the Council on Pharmacy and Chemistry of the American Medical Association[7] concluded that clinical evidence does not warrant the claim that massive doses of vitamin D are of benefit in chronic arthritis, in allergic disorders, or in psoriasis. (The Council stated,

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however, that it believed further studies should be conducted, but because of possible toxic effects of large doses of vitamin D, such studies should be made only in clinics where close supervision is possible.)

On the other hand an entirely different picture has been presented in instances where the heat-vaporized electric current-activated preparation (Whittier process) has been used.

Livingston,[8] using Whittier process high concentrate vitamin D, reported that progressive improvement had been noted in from one week to two months of treatment, with decreased pain, greater mobility, reduction of deformity and improved muscular tone associated with increased muscular strength and a general improvement of health. The depressed mental attitude of most of these patients became hopeful and their appetites returned. Twenty-two cases of severe arthritis of various types were treated only with vitamin D or with vitamin D plus fever therapy. Nineteen of these patients showed clinical improvement. One patient discontinued the treatment, and two showed no improvement.

Using the same preparation, Farley[9] reported from a group of 27 private patients that not one of this group had failed to respond in some degree to the high vitamin D therapy. In this group not only were the symptoms of pain and reduced movement of joints relieved, but the general nutritive state was greatly improved. This was evident by the gain in weight, appearance, and, a feeling of euphoria.

Of another series of 87 patients,[10] all who continued treatment for two months or longer, in accordance with the prescribed management, showed considerable improvement. In most cases the improvement was so marked that it enabled these people to resume their business activities and occupations. Most of the discharged patients continued to appear physically normal. There was evidently little or no tendency toward a return of the arthritic process. All patients responded well to the management established. Unfavorable results did not appear in a single instance.

In a testudy[11] of 15 patients who had received or were receiving this same product for arthritis, Farley concluded that the results obtained were at variance with those of Abrams and Bauer, but were in complete accord with those of Livingston and Steck. In no instance did the blood calcium rise to dangerously high levels, and in only one patient was there an elevation of the serum calcium level above 11.27 mg. per cent. In most patients the blood calcium level was less than 10 mg. per cent, even after the administration of large doses of vitamin D for many months or years. Farley found, with others, that the benefit obtained with this vitamin D preparation in the treatment of arthritis cannot be obtained with ordinary ultraviolet irradiated ergosterol or certain other forms of vitamin D.

Always[12] there occurred a definite rise in the number of erythrocytes up to the normal level, while the hemoglobin level followed, in general, the course of the erythrocytes. In most of these cases, there occurred improved functional activity, increased strength, and a general sense of well-being.

In 1940 Snyder and Squires[2] made a preliminary report on a rigidly supervised two-year study of a group of twenty-three patients with severe chronic arthritis who were treated with activated ergosterol prepared by the Whittier method. Their findings indicated that this drug has been of benefit in most of these patients in varying degrees. In a relatively high percentage of cases, the degree of clinical improvement has been marked and sustained.

These authors found the results of this preliminary study sufficiently favorable to justify further intensive study of the particular form of high-dosage vitamin D agent used, as well as the broader problem of vitamin D, in general, in the treatment of arthritis.

In 1941, the same authors[13] reported all of their cases had suffered from arthritis for from two to five years before receiving ertron, which is ergosterol activated by electrical discharge in a low pressure chamber. Each had proved to be resistant to most of the accepted forms of therapy prior to the administration of this drug. Most of the patients during this follow-up period were able to return to their normal gainful activities either without any medication or on a small maintenance dose of ertron.

Snyder and Squires[14] have concluded, as a result of their careful study of this group, that a marked improvement occurred in a sufficiently large number of patients to establish definitely the value of this form of treatment in all forms of chronic arthritis. Following the early improved sense of well-being, there was diminution of soft tissue swelling, lessening or abolition of pain, increased range of motion, restoration of normal function, an increase in weight in the debilitated underweight individuals and a marked increase in the patients' endurance.

Reed, Struck, and Steck[15] treated a group of forty-five patients suffering with atrophic arthritis; seven patients with hypertrophic arthritis and three with a mixed type of infection were treated with 150,000–200,000 ertron units of vitamin D daily with specific measurable improvement in 74.5 per cent of cases, accompanied by reduced swelling and pain, increased mobility (active and passive), improved muscular tonus, and weight gain.

The antiarthritic activity seems to have been found only in the high concentrate preparation made by the Whittier process and is not due to antirachitic effect, per se.

II. The Question of Toxicity-Safety

Many references to severe toxic reactions have appeared in medical literature. These are found to have occurred when large doses of ultraviolet irradiated ergosterol were used. Thus Steck et al[16] reported that early experience with impure preparations of vitamin D, particularly abroad, had led to a great deal of misunderstanding and fear of overdosage on the part of those who have had little knowledge of the fundamental mechanisms involved—a point adequately discussed by Bills. They feel that most of the earlier work should be disregarded in view of the effects produced by the highly purified preparations now available.

According to Bills,[17] the toxic properties peculiar to one particular brand of irradiated ergosterol solution

were the subject of numerous reports in medical literature during 1928 and 1930. The German manufacturer of the offending product had obtained two patents, one British and the other American, the specifications of which are most revealing. Under these specifications, the irradiation of ergosterol was continued just until the absorption spectrum of what is now recognized as toxisterol attained its maximum.

From Pfannenstiehl[18] we learn that after a long period of days during which otherwise normally fed young rabbits were given orally the same small dose of only two drops of vigantol (1.0 per cent solution of irradiated ergosterol in olive oil) a number of animals (nine in all) died with the characteristic signs which he calls hypervitaminosis.

From an editorial in the *Journal of the American Medical Association*,[19] it may be concluded that beyond little doubt massive doses of irradiated ergosterol—quantities exceeding by a thousand times or more the minimal antirachitic requirement—may result in considerable impairment of nutrition, loss of weight, pronounced hypercalcemia, and abnormal calcium deposits in certain tissues and organs.

Vrtiak and Lang[3] treated twenty cases of atrophic arthritis with 150,000–200,000 daily units of viosterol. Nausea developed in all their patients; frequency of urination and nocturia in several. Albright, Butler and Bloomberg,[20] raising their daily dosage of a propylene glycol solution of crystalline vitamin D to approximately 300,000 units, found the serum calcium rose to 12.7. The urine was loaded with calcium and calcium casts as in cases of hyperparathyroidism. Similar figures and findings, including anorexia, nausea, vomiting, epigastric pain or distress, diarrhea, headache, drowsiness, polyuria, polydipsia, and nocturia are reported by Abrams and Bauer.[6] They did not feel justified in using more than 200,000 units.

Meanwhile Tumulty and Howard[20] in a recent review of the subject emphasized the danger of toxicity from ultraviolet irradiated ergosterol. The preparation they used was found to contain 50 per cent calciferol and 50 per cent of a mixture of lumisterol, tachysterol and toxisterol. They described the occurrence of a marked hypercalcemia and damage to the renal tubules in patients receiving this product.

There are contrasting expressions from users of the Whittier processed preparation. Thus Livingston[8] has used high dosage vitamin D therapy in cases with heart lesions, vasomotor instability, and other complications without untoward effects, and concludes from his observations made in this series that there is no contraindication to vitamin D or fever therapy so far as is known at this time. Farley[9,10,11] reported favorable results, seeing only minor symptoms at times when the daily dosage was 400,000. No severe toxic reactions were encountered and the serum calcium level was not elevated above the normal.

This form of vitamin in clinical as well as experimental work appears less toxic than other forms. According to Reed, Struck, and Steck, human toxic effects have been observed so rarely that there is little data on which to base any discussion. In only three patients out of 150 that have received it have symptoms of toxicity occurred.

Snyder and Squires,[2,13,14] who encountered no serious toxic manifestations, report that the occasional signs of intolerance that have followed the use of ertron have all been temporary. They have made a careful study of over 200 arthritic patients during the past six years. Rigid ophthalmological examination of the retinal vessels, as well as X-ray studies of the larger vessels have shown no demonstrable increase in the degree of calcification. This suggests that the preparation not only does not cause arteriosclerosis, but may be instrumental in arresting the process. Blood calcium levels were not appreciably altered, even during twelve months' treatment.

These and other indications of low toxicity are further corroborated by findings in other than arthritic conditions.

Reports of Use of Massive Doses of Vitamin D (Whittier Process) in Other Than Arthritic Conditions

Vollmer[21] thus has treated cases of rickets and tetany with single massive doses without evidence of toxicity or damage, while, on the other hand, Hess and Lewis[22] found abnormally high percentages of blood calcium, sometimes associated with untoward symptoms.

Toxic effects produced by massive doses of irradiated ergosterol are also reported by Poncher et al.[23]

Further statistics by Wolf corroborate Vollmer, who encountered no toxic or ill effects. In Wolf's comprehensive study of this subject, active rickets in infants between the ages of 5 and 18 months was treated with a single massive dose (600,000 units) of vitamin D in the form of ertron. Such treatment effected a rapid therapeutic improvement in the patient clinically, serologically, and roentgenographically with no observable ill effects.

Since this work was published, Wolf (in press) administered single massive doses (600,000 units) of ertron to about one hundred additional babies for the prevention and treatment of rickets. He observed the same therapeutic effectiveness and complete absence of any toxic manifestations.

Meanwhile Krafka[25] has treated twenty-five patients with psoriasis over a period of two years without untoward symptoms or complaint of discomfort.

Discussion and Summary

A study of the administration of large doses of vitamin D leads to the belief that contradictory findings indicate that various workers were using different types of preparations. It is strikingly evident, however, that massive doses of irradiated ergosterol bring about the development of toxic effects without clinical improvement of arthritic patients, while use of the electric-discharge activated heat-vaporized ergosterol (Whittier process) has consistently been followed by clinical improvement with frequent rehabilitation, and with negligible or no toxic manifestations even over prolonged periods of intensive treatment.

Such findings may be accounted for pharmacologically by the following points:

1. The difference in degree and type of physiological activity of a series of compounds resulting from progressive irradiation of a single vitamin D precursor.[1]

2. The presence of a large amount of toxisterol type compounds which has been demonstrated in some of the irradiated preparations.[17,20]

3. The fact that electric current activation is applied to heat *vaporized* ergosterol in the Whittier process. The yield of end products is much smaller (one-sixth) than that made by other methods. Activation of a homogeneous vapor may yield a concentrated usable vitamin D, without the complicating presence of diluent or toxic undesirable by-products.

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The Hypomagnesemia of Vitamin D Administration.* (29891)

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Little is known about the effects of vitamin D_2 on magnesium metabolism. Recently, in a short-term balance study in rats given large doses of vit D_2, Hanna(1) demonstrated hypomagnesemia accompanied by increased intestinal absorption and increased urinary excretion of magnesium. The occurrence of this hypomagnesemia has since been confirmed by others(2). During the period of vitamin administration, Hanna's experimental animals were in net positive magnesium balance, whereas their controls which received physiologic amounts of vit D_2 were in a slight negative balance. These data suggest the possibility that the hypomagnesemia induced by administration of vit D_2 followed as a consequence of a redisposition of this cation from the extracellular phase to bone or some cellular depot. The present experiments were undertaken to evaluate this problem in more detail. The experimental design was constructed so as to be most certain of the nature of the balance of magnesium.

Methods. Forty-eight 200 g male Sprague-Dawley rats were randomly placed into 2 groups. Both groups were pair-fed a basal diet previously described(3) that was deficient in magnesium, sodium, potassium, chloride and phosphate but contained calcium carbonate. By direct analysis the diet was found to contain 0.16 mEq of magnesium and 16.4 mEq of calcium per 100 g. All rats had free access to distilled water, and all were given by gavage once each day 5 ml of a solution containing 2 mMoles each of sodium and potassium, 2.5 mMoles of chloride and 0.75 mMoles of phosphate.

The animals were housed in metabolic cages which were placed over funnels containing mineral oil. Urine and feces were collected over intervals of 5 days just prior to and during the administration of vit D_2.

After 10 days on the special diets, just prior to initiation of the administration of vit D_2, 9 animals randomly chosen from the 2 identical groups were killed and the contents of their intestines, from stomach to rectum, immediately removed and analyzed for magnesium. The remaining animals labeled initially as the experimental group were then given 5 consecutive daily subcutaneous injections of 100,000 units of vit D_2 in sesame oil in the form of activated ergosterol (Whittier Labs—Ertron®). The animals initially labeled as controls received injections of sesame oil alone. At the end of the 15th day of the experiments, 24 hours after the fifth and last series of injections, all animals were anesthetized with hexobarbital, Na 5-(1-cyclohexen-1-yl)-1, 5-dimethylbarbiturate (Winthrop Labs—Evipal®) and exsanguinated from their abdominal aortas. Thigh and back muscles were removed and analyzed separately from the remainder of the carcass for their ion concentrations. Again, intestinal contents were examined for magnesium.

Serum and muscle were analyzed for sodium and potassium with an internal standard flame photometer, for chloride by the Cutlove chloridometer, for serum total CO_2 content by a manometric method, for urea nitrogen and phosphate with the autoanalyzer(4) and for serum total proteins by a temperature-compensated hand refractometer (American Optical—T.S. meter)(5). Serum calcium and magnesium, as well as urinary, fecal, muscle and carcass magnesium, were determined by flame photometry in a Zeiss spectrophotometer with double monochromator(6).

Results. When compared with their controls, the animals which received the large doses of vit D_2 demonstrated significant

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TABLE I.

	Serum								Muscle				Carcass
	Ca	Mg	Na	K	CO_2	Cl	Total prot	Urea N	Mg	Na	K	Cl	Mg
	(mEq/l)						(g%)	(mg%)	mEq/100 g FFDS†				mEq
Control(14)*	5.17	1.71	141	4.7	22.2	105	5.8	19.4	6.23	8.3	41.9	5.9	11.7
Vit D (15)	6.45	1.37	141	4.2	21.2	101	5.7	21.7	6.08	8.7	40.5	5.6	11.2
P	<.01	<.01		<.01		<.01		<.05					

* No. of animals in each experiment.
† Fat-free dry solids.

hypercalcemia and hypomagnesemia (Table I). The differences between the urinary magnesium excretions of the 2 groups, during the time of vitamin administration, were not significant (Table II). Neither were there significant differences between fecal magnesium excretions (Table II) or between the final intestinal magnesium contents (Table III).

The muscle magnesium concentrations per 100 g fat-free dry solids and total carcass magnesiums of the 2 groups were not significantly different (Table I).

The mean serum potassium and chloride levels of the vit D_2-treated group were slightly but significantly lower, and the mean blood urea nitrogen levels were somewhat higher than those of the controls. There were no significant differences in the concentrations of sodium, bicarbonate or total proteins.

The muscle potassium expressed as mEq per 100 g fat-free dry solids of both groups were similar (Table I). These values are lower than those ordinarily reported for rats and are consistent with the low values of muscle K previously observed in magnesium depletion(3).

TABLE II. Cumulative Magnesium Excretions During Administration of Vitamin D.

	Urine	Feces
	(μEq total)	
Control(14)	90	353
Vit D(15)	98	348

TABLE III. Gastrointestinal Luminal Magnesium Content μEq Total.

Prior to admin of Vit D		After admin of Vit D	
Control	150	Control	197
		Vit D	118

Discussion. This experiment was done to study the hypomagnesemia consequent to administration to rats of large doses of vit D_2. Specifically scrutinized was the relative importance of acute changes in external magnesium balance as opposed to possible changes of the distribution of the cation within the body. Prior work had established the occurrence of this hypomagnesemia(1,2). The balance study of Hanna(1) done on rats fed normal amounts of magnesium and injected with large amounts of vit D_2 demonstrated hypomagnesemia, a net positive magnesium balance and hypermagnesuria. These findings in themselves suggest that the hypomagnesemia was primarily the result of a redistribution of the cation within the body.

In the experiment presented here, magnesium-depleted rats receiving an essentially magnesium-free diet were used to accentuate any possible differences in urinary or fecal magnesium excretion.

Definite hypomagnesemia was observed in the animals given the vit D_2 injections. Neither before nor during the period of administration of the vitamin was there any significant difference between the urinary or fecal magnesium excretions, or magnesium content of the intestines of the two groups. Clearly this would preclude any *necessary* action of vit D_2 on gastrointestinal absorption or secretion or urinary excretion of magnesium in producing the hypomagnesemia.

The lack of difference in total protein content of serum suggests that the hypomagnesemia is not readily ascribable to hemodilution. The possibility remains that there may have been some diminution in protein binding of magnesium induced by vit D_2 *per se*, or by the hypercalcemia consequent to its administration. Another possible explanation for the

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vit D_2-induced hypomagnesemia would appear to be a redistribution of this cation from the extracellular phase to some tissue, presumably bone, independent of any specific influence on protein-binding of magnesium. The lack of difference between the muscle and carcass levels of magnesium in the two groups is compatible with this suggestion.

The slight but significantly depressed level of serum potassium of the vit D_2-treated group is interesting in view of the known adverse effect of this vitamin on renal conservation of potassium(1), but, unfortunately, urine potassium excretion was not determined.

Summary. The hypomagnesemia consequent to administration of large doses of vit D_2 to rats was studied. Two groups of animals were pair-fed a magnesium-deficient diet, and one was then given injections of vit D_2. Hypomagnesemia resulted in the vit D_2-treated group. During the period of vitamin administration there was no significant difference between the urinary or fecal magnesium excretion of the 2 groups. Nor were the differences significant between the levels of magnesium in the total carcass and muscle. The level of serum proteins also was not different in the 2 groups. It is suggested that the hypomagnesemia is due to a redistribution of magnesium from the extracellular phase to some other depot, which may well be bone. One mechanism might involve a reduced binding of magnesium by serum proteins due to some specific effect of vit D (or perhaps to the resultant hypercalcemia). An alternative hypothesis is that vit D_2 has some other specific influence that promotes a deposition of magnesium in bone or some other depot.

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VITAMIN D_2 (type 500) (F.C.C.) 0241

DEFINITION: A dry, water dispersible preparation of pure, crystalline vitamin D_2, also known as activated ergosterol or calciferol, having a potency of 500,000 U.S.P. units per gram.

MATERIAL: Pure crystalline calciferol, or activated ergosterol, is a physiologically active compound having the formula $C_{28}H_{44}O$, usually prepared by irradiation of sterols.

WORKMANSHIP: The vitamin D_2 shall be uniformly mixed with a suitable carrier in such quantities that the potency of the mixture shall be equal to 500,000 U.S.P. units per gram. The product shall be free-flowing, water dispersible, and free from off odors, impurities, or foreign matter of any kind. All materials and processes employed in the preparation of this product shall be in accordance with good commercial practices, and under strict sanitary conditions.

CONTAINERS: Sound, adequately sealed containers, capable of protecting the contents against impairment of quality (especially with respect to sanitation, moisture content and vitamin activity) under various conditions of handling, transportation and storage. Containers must be free of dirt, foreign matter, or any adulterating substances.

NET WEIGHT: As specified on purchase order.

LABELING: Each container shall be clearly and properly labeled, indicating the name of the manufacturer, net weight and the ingredient name.

SHIPPING: To be shipped in first class transportation equipment which has been thoroughly cleaned to prevent any possible contamination of product or its containers.

REQUIREMENTS: Deliveries shall comply in every respect with the Federal Food, Drug and Cosmetic Act, state laws, and with the requirements given in this specification. The product shall also meet all the requirements of the Food Chemicals Codex.

SAMPLES: Upon buyer's request before purchase, seller shall furnish representative samples for inspection. Such samples should be drawn from actual lots which the buyer may purchase.

TESTING: Acceptance will be subject to sampling of the material actually delivered, and to testing of these samples according to the laboratory instructions in this specification.

METHODS: Methods of analysis will be in accordance with the methods of the Association of Official Agricultural Chemists, or any other standard method deemed advisable by the buyer.

J Pediatr. 83, (1973)

2/ 25-HYDROXYVITAMIN D_3 ($25\text{-}OHD_3$): PLASMA LEVELS IN MOTHERS AND THEIR PREMATURE INFANTS WITH NEONATAL HYPOCALCEMIA (HC). J.P. Jacobs, M.Poria Ray, S.Rothberg, and L.Lilienfeld. Montefiore Hosp. and Med. Ctr., Northside City Hosp., Albert Einstein Coll. of Med., N.Y. 10467; and Nassau County Med. Ctr., N.Y.

To define further the possible role of vitamin D metabolism in the pathogenesis of HC of prematurity, the major circulating metabolite of vitamin D_3, $25\text{-}OHD_3$, has been measured by competitive radioassay in mothers and their affected premature infants. From December to February 1972-3, 15 unselected, consecutive premature infants with HC (calcium values in serum of 7.5mg/100ml or less within 48 hours of life) entered this study. Surprisingly, in 11 of 15 mother-premature pairs (group I), plasma levels of $25\text{-}OHD_3$ (mean ± S.E.M., ng/ml) were strikingly low in both mother and infant: 11 ± .6 and 7.0 ± .7, respectively. Plasma levels of $25\text{-}OHD_3$ in control mothers and their full-term infants were 28 ± 2 and 23 ± 2, respectively. However, in 4 of 15 premature infants with HC, no correlation was observed between the low levels of serum calcium and normal levels of $25\text{-}OHD_3$, though the concentration of phosphate in serum was significantly lower in these premature infants, compared to group I infants. Common to all groups, if the infant's plasma level of $25\text{-}OHD_3$ was low or normal, the mother's level was correspondingly depressed or normal but was still 4-7ng/ml above her infant's value. Nutritional vitamin D deficiency in group I mothers was suggested by the association of low plasma levels of $25\text{-}OHD_3$ with hypocalcemia, hypoalbuminemia, and considerably lower intake of vitamin D. The presented data suggest the postulate of a direct role of vitamin D deficiency as one of several pathogenetic factors in HC of prematurity, and that nutritional vitamin D deficiency may be relatively common in these mothers whose pregnancies are more likely to end prematurely. Future studies will presumably determine if more effective prevention of vitamin D deficiency in mother and baby may be provided by $25\text{-}OHD_3$ or other vitamin D analogs. (Supported in part by a grant from the John A. Hartford Foundation, Inc.)

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Suppression by Vitamins D_2 and D_3 of Hamster Cheek Pouch Carcinoma Induced with 9,10-Dimethyl-1,2-benzanthracene

With a Discussion of the Role of Intracellular Calcium in the Development of Tumors[1]

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Abstract. Local application of vitamins D_2 and D_3 caused inhibition of tumor formation in the hamster cheek pouch, induced by topical treatment with DMBA. These results and data from the literature are used in an attempt to explain the mechanism of carcinogenesis by DMBA, and the inhibition of this process by vitamin D.

In tumor cells the amount of alkaline phosphatase and the amount of calcium bound to the cell membrane are both decreased. It is probable that the protein molecule alkaline phosphatase can also act as calcium binding protein. The decreased amount of calcium bound to the membrane of tumor cells leads to increased permeability, with an increased and less controlled flow of metabolites to and from the cytoplasm, resulting in a decreased control of the mitotic process.

Vitamins D cause induction of the synthesis of alkaline phosphatase/calcium binding protein, and this may explain the marked inhibition of DMBA carcinogenesis in animals treated simultaneously with DMBA and vitamin D. This effect of vitamin D is probably not specific, and there are indications that other substances which cause induction of alkaline phosphatase also inhibit tumor formation.

Key Words
Chemical carcinogenesis
Calcium metabolism
Cell membranes
Calcium binding protein
Alkaline phosphatase

Introduction

Carcinoma of the small intestine is rare in man [19, 20, 23, 31] as well as in animals [11, 38]. Cats, dogs and cattle appear to be virtually immune to this lesion [35].

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DE LUCA [14] and CORRADINO and WASSERMAN [7] showed that calcium is actively resorbed in the small intestine by means of a calcium binding protein (CBP), and that group D vitamins are able to induce the synthesis of this protein. In tissue cultures of chick embryo intestine the resorption of ^{45}Ca by the epithelial cells increased after addition of vitamin D_3, correlated with an increase in the amount of CBP in the cells. NORMAN *et al.* [22] and HAUSSLER *et al.* [17] showed that administration of vitamin D_3 to chickens resulted in increased resorption of calcium from the intestine, and in a simultaneous increase in the amount and activity of alkaline phosphatase (AP) in the intestinal cells. According to these authors this enzyme may be identical with CBP, and HAUSSLER *et al.* [17] suggested, more specifically, that CBP is identical with Ca^{++}-ATPase. PILEGGI *et al.* [24] demonstrated that in rachitic animals the activity of AP is decreased in epithelial intestinal cells. It is known that the amount of calcium in the walls of different types of cancer cells is markedly lower than in normal cells from the same tissues [8].

It seems feasible to suggest that a relation may exist between the low frequency of small intestinal carcinoma, the active resorption of calcium in this organ, and the low concentration of calcium in tumor cell walls. It, therefore, seemed of interest to determine whether carcinogenesis might be influenced by vitamins D_2 and D_3, which are known to induce the synthesis of CBP. As model we chose carcinogenesis in the hamster cheek pouch induced with 9,10-dimethyl-1,2-benzanthracene (DMBA) [27, 33].

Material and Methods

Animals. A total of 64 male Syrian golden hamsters of a local strain was used. They were 8–10 weeks old at the start of the experiment, and weighing 70–80 g. The animals were kept 4 or 5 in a cage and received Purina laboratory chow and tap water *ad libitum*.

Materials. DMBA, vitamin D_2 and vitamin D_3 were dissolved in liquid paraffin and kept at room temperature. The following solutions were prepared: (1) 0.5% DMBA; (2) 0.8% vitamin D_2; (3) 0.8% vitamin D_3; (4) 0.5% DMBA combined with 0.8% vitamin D_2, and (5) 0.5% DMBA combined with 0.5% vitamin D_3. Previous studies showed that DMBA has an optimal carcinogenic effect in a concentration of 0.5% [27, 33]. On the basis of the assumption that competition occurs between DMBA and vitamin D for active sites on the DNA, the solutions of the vitamins were prepared in the same molarity as that of DMBA. The concentrations of the vitamins were not increased further in view of possible toxic effects.

Spectrometric studies. In order to exclude the possibility of an *in vitro* reaction between DMBA and vitamin D, absorption spectra were made of solutions of DMBA,

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vitamin D_2 and vitamin D_3 alone, and of DMBA with each of the vitamins. The findings showed that the arithmetic sum of the spectra of the solutions of the separate substances was equal to that of the combined solutions. In addition, no change was found in the amplitude of the absorption lines of the different substances when they were combined in a solution, also not when this examination was repeated with 2-week old solutions. These measurements were made with a Cary 14 spectrophotometer of the Applied Physics Corp. in the range of 2,000–26,000 A.

Method of application. The different solutions were applied three times per week to the right cheek pouches of the hamsters with a soft paint brush. A preliminary study had shown that at each painting approximately 0.2 ml of the solution was deposited in the pouch. This treatment was continued for 8 or 10 weeks.

Examination of the animals. At the end of the treatment periods the animals were sacrificed. The cheek pouches were examined macroscopically and the tumors were counted. After fixation in Bouin's fluid, multiple sections were taken for histological studies.

Results

The tumors which were found during the macroscopic examination of the cheek pouches all proved to be squamous cell carcinomas with varying degrees of infiltrative growth. In the animals treated with vitamins D_2 or D_3 only, no tumors or other pathological changes were found. The findings in the remaining groups are summarized in table I. An interesting additional finding was the weight loss occurring in animals which received vitamins D only; animals which received DMBA only or in combination with vitamin D showed an increase in body weight (table II).

Discussion

The results demonstrate that chemical carcinogenesis in the hamster cheek pouch is inhibited by simultaneous topical administration of vitamin D. We shall attempt to discuss the mechanism of DMBA-carcinogenesis and its inhibition by vitamin D on the basis of these results and of data from the literature.

General Introduction

Vitamin D is not only active in the small intestine, but also in bone [37], kidneys [15] and skin [16]. On the basis of another report [28] and of the fact that in the present study vitamin D had an effect in the hamster cheek pouch, we assume that its action is also present in other tissues. In addition we assume, basing ourselves on the above quoted paper [17], that the protein which is synthesized as the result of the action of vitamin D can act either as the enzyme AP or as CBP. This assumption

Table I. Incidence of cheek pouch carcinoma in hamsters treated topically with DMBA or with combinations of DMBA and vitamins D

Treatment	Duration, weeks	Number of animals at start/survivors	Number of animals with tumor	Total number of tumors in group	Average number of tumors per treated cheek pouch
DMBA 0.5%	8	20/13	13	75	5.8
DMBA 0.5% + vitamin D_2 0.8%	8	12/10	2	2	0.2
DMBA 0.5% + vitamin D_3 0.8%	10	10/8	1	2	0.3

Table II. Changes in body weight of hamsters treated with DMBA, vitamin D_2 or combinations of both

Treatment	Weight at start of study in g (range/average)	Weight at conclusion of study in g (range/average)
0.5% DMBA	70–80/76	85–110/94
0.8% vitamin D_2	70–80/76	45–75/61
0.8% vitamin D_3	70–80/76	45–70/54
0.5% DMBA + 0.8% vitamin D_2	70–80/76	85–105/93
0.5% DMBA + 0.8% vitamin D_3	70–80/75	80–100/89

is also supported by the finding that the AP in osteoblasts is of importance for the process of ossification, which consists amongst others of precipitation of calcium salts [3].

Whether the carcinogen is a virus, radiation or a chemical substance, it supposedly induces a change in the nucleus which prevents vitamin D from reaching its active site on the DNA. Vitamin D is then unable to induce the synthesis of a sufficient amount of CBP. This will result in a decreased ability of the cell to bind calcium to its membrane. The importance of calcium for the integrity and the maintenance of normal permeability of cell membranes is well known [30, 39]. The decreased calcium binding potential will result in increased cellular permeability, and thus in an increased flow of metabolites to and from the cell. This facilitates increased and less controlled mitotic activity. The increased permeability does not exist for those ions which are transported actively, e.g., K^+, Na^+ and Ca^{++}; for the transport of these ions energy is consumed in processes in which ATPase, amongst others, acts as an enzyme. As stated earlier, there is a decrease in this phosphatase in malignant cells, and, therefore, the flow of these actively transported substances will not increase, but on the contrary decrease in malignant cells [7].

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In the following part we shall attempt to explain the results of the present study, dealing separately with those processes which take place in the nuclear environment, and other processes which take place in the vicinity of the cell membrane.

Processes in the Nuclear Environment

There is a marked structural resemblance between vitamins D, steroids and various carcinogenic agents (fig. 1). Molecules of vitamin D approach the active site on a nucleic acid molecule. This active site is probably identical with that of other steroids, since these substances are also inducers of AP [10], and because of the resemblance in structure.

According to the model proposed by JACOB and MONOD [18], the inducer molecule – in the present case vitamin D – attaches to the RNA molecule which acts as a specific repressor responsible for the inhibition of the formation of a certain protein – in the present case CBP –, and the inducer neutralizes the repressive activity. This facilitates the initiation of the process of transcription of the messenger RNA on the DNA. The messenger RNA which is formed passes into the ribosomes, and here the second phase of the process of transcription takes place and the specific protein is synthesized.

We assume that the DMBA molecule is also able to fit stereometrically to the active site on the repressor, and that this results in blocking of the active site but not in inhibition of the repressive activity. It is also possible that DMBA acts directly on the DNA, inhibiting the first stages of the process of transcription, due to a strong affinity between DMBA and certain sites of the DNA.

There appears to be competition between vitamin D and DMBA for one or more active sites on the nucleic acid. This is supported by an incidental finding in the present study (table II). Animals which received vitamin D only did not grow, and most of these animals even decreased in body weight. This effect of vitamin D has also been noted by others [28, 29, 41]. On the other hand, in animals receiving the same amount of vitamin D as the previous groups, but with an equivalent amount of DMBA, the body weight increased as in animals treated with DMBA only. It appears that excess vitamin D resulted in the induction of an abnormally large quantity of CBP, which bound an excessive amount of calcium to the cell membranes in different tissues. This resulted in decreased permeability of these membranes with a subsequent decrease in the flow of metabolites to and from the cells, leading to underdevelopment of the

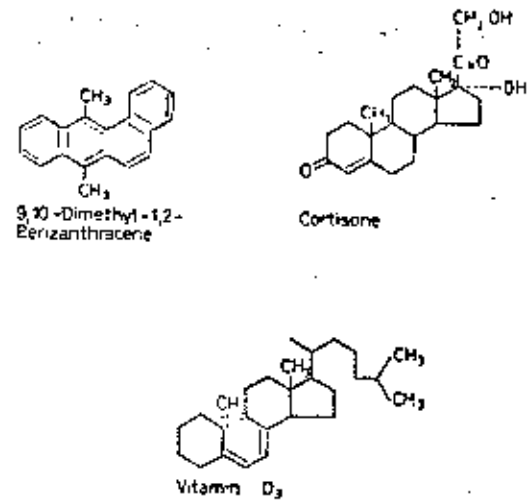

Fig. 1. Structure of DMBA, cortisone and vitamin D_3, demonstrating resemblance between these compounds.

tissues and subnormal growth of the animals. DMBA – which apparently competes with vitamin D – prevented the induction of abnormally large amounts of CBP by vitamin D, and thus the normal permeability of the cell membranes was preserved. The development of the tissues of these hamsters was not inhibited, and their growth was normal.

Processes in the Vicinity of the Cell Membrane

COMAN [6] showed that tumor cells separate more easily than normal cells, and this was confirmed by others [1]. The electrical charge of tumor cell membranes is more negative than that of normal cells [1, 2, 36]. Decreased levels of calcium are found in tumor cells [9, 40], and there is a regular decrease in the calcium level in the course of carcinogenesis. Under the influence of EDTA amphibian embryos are dispersed, probably due to the removal of calcium by chelation [2]. Cell membranes of different types of tumor cells contain less AP than membranes or normal cells [4].

The cell membrane consists of three layers, i.e. two protein layers which enclose a layer composed of lipoid material (fig. 2). The lipoid layer has an average thickness of 50 A. The cell membrane has pores which form a communication between its two sides. The outer and inner protein layers may be regarded as plates of conducting metals, and the lipoid center as an isolating layer. The membrane is thus similar to an electrical con-

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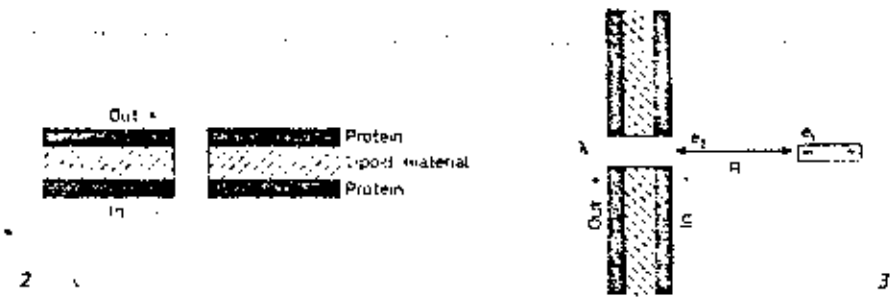

Fig. 2. Schematic representation of the cell membrane, consisting of two protein layers and a lipoid core. Note the presence of a pore.

Fig. 3. A rejecting force exists between the sector λ of the cell membrane with the charge e_2 and a protein molecule with the charge e_1. The force is equal to the product of the charges, divided by the square of the distance R which separates them.

densator [12]. Normally there is a difference in potential between the inner and outer side of the membrane in the range of −70 mV, the inside being negative in relation to the outside. This difference in potential can also be predicted on the basis of theoretical considerations [13]. The above is of course not true for muscle and nerve cells, where different potentials exist at rest and during activity.

The molecule of AP or CBP is synthesized by the ribosomes, and from this moment onwards it is exposed to the sum total of mechanical, thermal and electrical forces acting in the cell. The resultant vector of these forces will eventually determine where the molecule will arrive.

1. The Electrical Force

The AP protein molecule has a negative carboxyl and a positive amide pole. As shown in figure 3, a rejecting electrical force exists between the AP molecule and the sector λ of the cell membrane to which the molecule approaches. Theoretically there is in this case an electrical dipolar moment. However, since the exact polar charges are not known, it is only possible to make the crude quantitative statement – true from the physical viewpoint – that the force which exists in this case results in repulsion, and that the magnitude of this force is equal to the product of the electrical charges, divided by the square of the distance between the charges, according to the formula of Coulomb

$$F_e \sim \frac{e_1 \cdot e_2}{R^2},$$

where e_1 is the polar charge, e_2 is the charge of the part λ of the membrane under discussion, and R is the distance between these two charges.

In addition there exists in the cell the electromagnetical force which results from the movement of charged particles, i.e. ions and polar molecules, in the cytoplasm. This force is mentioned here for the sake of completeness, but for the purpose of the present discussion it may be included in the general electrical force F_e.

2. The Mechanical Forces

a) Viscosity. This force always opposes movements of the molecule, and its magnitude is given by the formula of STOKES [34]

$$F_{vis} = 6\pi\eta r v,$$

where η is the viscosity coefficient, which depends on the nature of the medium in which the molecule moves, and on the temperature; r is the radius of the molecule in the plane perpendicular to the direction of its movement, and v is the speed of the movement. In the present model η is a composite factor, since it must also include the resistance offered to the movement by the endoplasmatic reticulum and other intracellular structures.

b) Gravity. This may be ignored for practical purposes, since there is no evidence of a possible difference in the influence of gravitation on different parts of the cell or its membrane.

c) Thermal force. This gives the molecule its Brown movement. The thermal force is an exclusive function of the absolute temperature, and results in diffusion of a substance from a point where it is concentrated, and in its distribution throughout the medium. The molecule receives both a translatory and a rotatory movement. The latter can result in a condition where the negative pole of the molecule is pointed towards the cell membrane despite the fact that this is in opposition to the electrical force. As a result of the translatory movement the molecule may also move towards the membrane in opposition to the electrical force.

When the thermal force gives the molecule a translatory velocity V, the force acting in this direction is $F_T = \frac{d}{dt}(mV)$, where F_T is the thermal force, m is the mass of the molecule, and V is its velocity; $\frac{d}{dt}$ is the differential of this expression.

The complex of forces acting on a single molecule consists, therefore, of the electrical force F_e, the mechanical force F_{vis}, and the thermal force F_T.

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In order for these forces to enable the molecule to reach the cell membrane, one of the following three conditions must exist:

(A) The thermal and electrical forces are directed towards the membrane, and their sum is greater than the mechanical force of the viscosity, which acts in the opposite direction:

$$\vec{F}_e + \vec{F}_T > \vec{F}_m .$$

(B) The thermal force, which acts in the direction of the cell membrane, is greater than the total of the electrical and mechanical forces, which both act in the opposite direction:

$$\vec{F}_T > \vec{F}_e + \vec{F}_m .$$

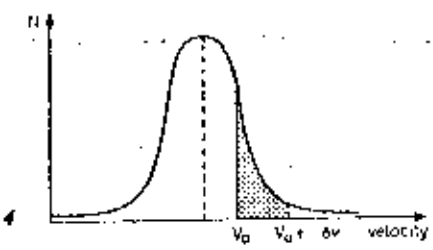
(C) The electrical force, which acts in the direction of the cell membrane, is greater than the total of the thermal and mechanical forces, which both act in the opposite direction:

$$\vec{F}_e > \vec{F}_T + \vec{F}_m .$$

Until now we considered the case of a single molecule. It is obvious that in the case of a number N of molecules which are exposed to a certain temperature, not all will have the same thermal velocity. A different Gaussian curve actually exists for each temperature, representing the distribution of the velocities. Only part of the molecules in the medium will have the minimum velocity V_o required to fulfill the conditions of one or more of the above equations. We shall assume that at a certain moment there are in the cytoplasm N molecules of AP. The number of molecules with a velocity $\geq V_o$ may be estimated by means of statistical mechanics. According to Maxwell's law of the distribution of velocities, among a population of N molecules with a molecular weight m, in a medium with the absolute temperature T, the number n of molecules with a velocity equal to or greater than V_o is expressed by the equation

$$n = N \frac{(2m^3)^{1/2}}{\pi k^3 T^3} \cdot V_o^2 \cdot e^{\frac{-mV_o^2}{2kT}} \delta v ,$$

where k is Bolzmann's coefficient and δv is the interval of the velocities greater than V_o in a given situation. In figure 4 the integral within the limits $x = V_o$ and $x = V_o + \delta v$ describes the proportion of molecules which have a velocity $\geq V_o$. It follows that the number n of molecules with a velocity $V \geq V_o$, which can thus fulfill one of the above equations A, B or C, is correlated with the number N of molecules present in the cell. We may thus assume that in the cell the following processes can



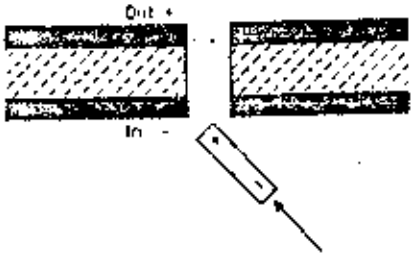


Fig. 4. Gaussian curve describing the distribution of the velocities as the result of the Brown movement, among a number N of molecules exposed to a certain temperature. Between the limits V_0 and $V_0 + \delta v$ there is a number n of molecules with a velocity $\geq V_0$.

Fig. 5. Formation of a bond between the positive pole of a polar molecule and a negative group at the inner aspect of the membrane.

take place: (1) the polar molecule approaches with its positive pole to a negative group at the inner aspect of the cell membrane, and a bond is formed (fig. 5); (2) the polar molecule approaches a pore with its negative pole, and penetrates in such a way that the negative pole is bound to a positive group on the outer surface of the membrane, and the positive pole is bound to a negative group on the inner aspect (fig. 6); (3) as a result of its high kinetic energy the molecule collides elastically with the membrane and returns to the intracellular pool (fig. 7), and (4) a molecule with high kinetic energy passes through a pore and leaves the cell (fig. 8).

When we accept these possibilities, we can with their aid attempt to explain the differences between membranes of normal cells and tumor cells which were mentioned above.

We shall first consider a normal cell. Figure 5 represents a condition where the protein molecule is bound only with its positive pole to the cell membrane, and its remaining surface is exposed to the cytoplasm. It can now act as the enzyme AP, since there is no stereometric interference with the approach of suitable substrates. This is in agreement with the results of different staining methods for AP, which demonstrate activity of this enzyme especially in the vicinity of the cell membrane [4, 21]. In this condition the molecule is unable to bind calcium present in the intercellular space.

When the condition depicted in figure 6 is present, the whole protein molecule is now localized within the pore, where it hinders the passage of nonlipid-soluble metabolites such as amino acids, electrolytes, carbo-

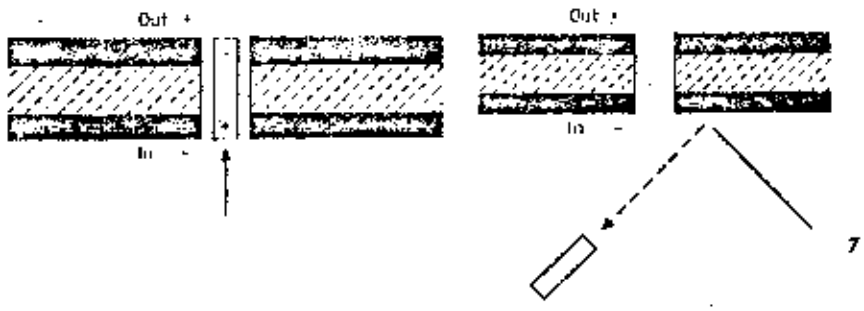
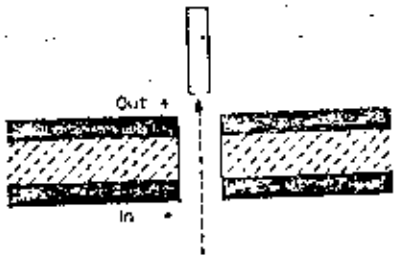


Fig. 6. Penetration of a polar molecule into a pore, with its positive pole at the negative side of the membrane, and vice versa.

Fig. 7. Elastic collision of a molecule possessing a high kinetic energy with the cell membrane, and return to the intracellular pool.

hydrates and water-soluble vitamins, which are transported via the pores [5]. In this position the molecule is lying contrary to the general arrangement of the electrical charge of the cell membrane, i.e. with its positive pole adjacent to the negative side of the membrane and vice versa. It now neutralizes part of the electrical charge, and the result is a decrease in the membrane potential. The negative pole of the molecule is directed towards the intercellular space, so that electrical bonds can be formed with different cations present in this space, e.g. calcium. Bonds may thus be formed between the protein molecule (now acting as CBP) and one valence of a Ca^{++} ion, while the second valence of this ion is bound to the negative pole of a CBP molecule in a pore of the membrane of a neighboring cell, as shown schematically in figure 9. If many such bonds are present, they can overcome the electrical forces of repulsion between the cells, and it is feasible that they can hold the cells together, at least temporarily, until protoplasmatic bonds are formed. This was also proposed by AMBROSE [2], who showed that fetal tissues disintegrate into separate cells under the influence of chelating agents which remove Ca^{++} ions. Since the molecule is wholly localized within a pore, this interferes stereometrically with the approach of substrate to its active sites, and thus it will not act as AP but as CBP. HAUSSLER *et al.* [17] did indeed succeed, by means of butanol extraction and destruction of the lamellar structure of the cell membrane, to liberate the protein molecule from its position in the membrane; at the same time the activity of AP in the medium, which was previously low, increased. This strongly supports the assump-



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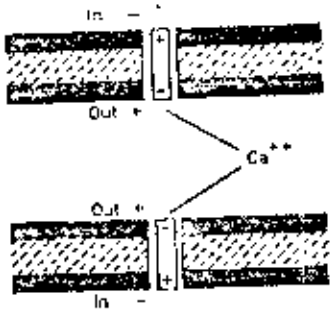

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Fig. 8. A molecule with a high kinetic energy passes through the pore into the extracellular fluid.

Fig. 9. Formation of bonds between extracellular calcium and negative poles of polar molecules localized in the pores of the membranes of adjacent cells.

tion that AP may be present in the cell membrane, without showing enzyme activity, and it may explain why the presence of a certain enzymatic protein does not mean that there must necessarily be a corresponding level of specific activity of this enzyme. Figure 8 explains the physiological presence of the enzyme in the extracellular fluids.

We shall now attempt to explain the processes which take place in the tumor cell, using the above model, and basing ourselves on the assumption that the tumor cell is unable to synthesize sufficient amounts of AP or CBP, an assumption supported by a number of reports [4, 21].

In the tumor cell the entity N – the total number of molecules – from Maxwell's law is smaller than normal. As a result, n – the number of molecules with a velocity $\geq V_n$ – also decreases. This leads directly to a decrease in the number of molecules which are caught in the pores of the cell membrane, with the following consequences:

(a) The permeability of the cell membrane increases, and the transport of metabolites will be less controlled. As is known [32], certain enzyme inducers lead to increased activity of specific permeases, and this facilitates the transport of corresponding specific substances. This presumably takes place through a number of pores which are not occupied and occluded or narrowed by molecules of AP/CBP, and which are, therefore, available for metabolite transport. Under the presently discussed conditions an increased number of pores is available for this purpose, leading to an overall increase in permeability, involving all transport mechanisms which take place via the pores.

(b) Due to the decreased amount of CBP in the pores, there will be less binding of calcium to the cell membrane, resulting in decreased intercellular adhesion and increased tissue friability. It is feasible that this may be related to the potential of tumors to metastasize.

(c) Less polar molecules are oriented on the membrane contrary to the distribution of electrical charges, and thus less neutralization of the membrane potential will take place. The latter will thus be more negative in a tumor cell than in a normal cell. This in itself may explain in part the existence of greater separating forces between tumor cells than between normal cells [2].

(d) The phenomenon of increased activity of AP despite a decreased number of molecules of this enzyme may be related to the fact the inner aspect of the tumor cell membrane is more negative than that of a normal cell. The probability that the enzyme molecule will approach the membrane with its negative pole is now smaller, and relatively more molecules will be found either in the intracellular pool or bound to the inner aspect of the membrane with their positive pole, as depicted in figure 5. In none of these two conditions will there be stereometric interference with the approach to the active sites which determine the activity of the protein as an enzyme molecule, and thus it will now act as AP. The possibility of such a discrepancy between the amount and the activity of an enzyme has been proven for AP by HAUSSLER *et al.* [17].

The concept that carcinogenesis is related to a defect in AP is also supported by the finding that corticosteroids, known as inducers of AP [10], depress experimental tumor formation [25, 26]. The membrane-stabilizing effect of corticosteroids may also be related to the induction of the protein molecule which can act as the enzyme AP. If it is true that this protein may under different circumstances act as CBP, the increased binding of calcium to the cell membrane resulting from the induction of this protein is probably responsible for the decreased membrane permeability [30].

This work was carried out in partial fulfilment of the requirements for the M.D. degree of the Hebrew University (D. RUBIN).

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METABOLIC ACTIVITIES OF VITAMIN D IN ANIMALS

I. DECREASE OF SULFATE METABOLISM IN VITAMIN D-DEFICIENT CHICKS

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Concerning the enzymatic role of water-soluble vitamins many investigations have been published, but few papers are available on the mechanism of the action influencing lipid-soluble vitamins. Our recent interest in the biochemistry of lipid-soluble vitamins is an outgrowth of a curiosity about the correlation between vitamin D and sulfate metabolism.

It was early reported by the present authors on the bioassay of vitamin D with rats fed on sterol-free synthetic diets and it was confirmed that no influence of vitamin B_{12} on the degree of healing in vitamin D-deficient rats was observed (1).

Thereafter many papers have been published on metabolic function of vitamin D, *e.g.*, relation to calcium and phosphorus metabolism, relation to citrate metabolism and other metabolic relationships. Sano (2) summarized his researches on the rickets in Japan, *e.g.*, thiamine and pyruvate levels in rachitic children, riboflavin levels in blood and organs of experimental rachitic rabbits, phosphorus metabolism in young normal and rachitic rats, serum citrate levels in rachitic children and healing effect of cortisone on rachitic children.

Keane *et al.* (3) reported that vitamin D markedly increased the absorption and retention of Ca^{45} given orally to chicks but no effect of the intramuscularly injected Ca^{45} was observed. It was concluded that the effect of vitamin D on calcium metabolism was primarily due to the absorption from the intestinal tract. Gershoff and Hegsted (4) fed diets containing various Ca/P ratios to chils and found that the ratios had no significant effect on the intestinal absorption of Ca^{45} if an adequate amount of vitamin D was present. Otherwise, the calcium absorption was inversely proportional to Ca/P ratios. Cramer and Steenbock (5) found that the rise in calcium intake in the presence of vitamin D changed the negative calcium balance induced in rats by a low phosphorus diet to a positive one.

The relationship between vitamin D and citrate metabolism at the enzymatic level were investigated by DeLuca *et al.* (6). Addition of the vitamin to rachitogenic or nonrachitogenic diets reduced the oxidation of citrate by kidney homogenates or mitochondria, but no effect was found on the oxidation of other Krebs cycle intermediates. These findings were presumed by the authors to account for

the increase in the citrate levels in certain tissues. DeLuca *et al.* (7) showed further that vitamin D reduced the oxidation of citrate but not that of oxaloacetate. Armstrong and Singer (8) studied *in vitro* the exchange of citrate in the bone using C^{14}-citrate, and a competition between the phosphate and the citrate on the mineral crystal surfaces of the bone was demonstrated.

However, no papers have appeared concerning the studies on the relationships between bone osseomucoid and vitamin D metabolisms. The interest of the authors has led to research this topics.

Lipmann (9) reported the mechanism of sulfate activation in chondroitin sulfate synthesis and of enzymatic transfer of "active sulfate" to steroids, galactose and hexosamine derivatives. Further, it is a well-known fact that there is a metabolic interrelation between estrogenic substances and active sulfate, as the hormones appear in female urine as the esters of glucuronic and sulfuric acids (10).

Araki *et al.* (11) found with a histochemical method employing the fluorescence that vitamin D in the mucous epithelium of the intestinal tract within two to three hours after an oral dose. The vitamin appeared in the liver within four hours after administration. It was also present in kidneys, lungs, adrenal cortices and adipose tissues. However, all the manifestations of vitamin D deficiency are due to defective bone formation, but it is not possible to explain adequately the metabolic role of the vitamin. It is a well-known fact that irradiation of yeast ergosterol yields an extraordinarily potent antirachitic agent in mammals, whereas the compound is much less effective in chicks. On the other hand, irradiatted 7-dehydrocholesterol, vitamin D_3, is equally active in curing the rickets of both chicks and rats (10).

Guided by these studies, the present authors have recently attempted the biochemical studies on the metabolic activities of vitamin D in chicks. The first experiments were performed for further confirmation of the difference in biopotency between vitamin D_2 and D_3 to chicks. A marked antirachitic activity was always seen in the chicks receiving vitamin D_3, but the effect was low in the birds supplemented with D_2 (12). The antirachitic potency was determined by estimating the total ash, phosphorus and calcium in the bone with conventional methods. All the results indicated an excellent biological potency of vitamin D_3. The second experiments were made to elucidate the correlation between vitamin D and citrate metabolism in chicks. The pyruvic acid levels in blood and kidneys were determined by Friedemann's method (13) and the citrate levels in both tissues by Ettinger's method (14). The data showed high citrate and pyruvate levels in the chicks receiving vitamin D_3 (15). The first experiments were carried out to study the relationships between vitamin D potency and bone sulfate level. The experiments were repeated for chemical analysis of sulfate, and the data were further confirmed by injecting S^{35} sulfate. Thus the decrease in bone sulfate level in vitamin D-deficient chicks was recognized by the radio-autograms of tibia.

Further researches are in progress for the elucidation of the action mechanism of vitamin D in sulfate metabolism in chicks (16, 17).

EXPERIMENTALS AND RESULTS

1. *Production of Vitamin D Deficiency in Chicks*

Day-old White Leghorn cockrels (about 30 g) were used. Chicks were housed in the electrically heated brooder with a wire floor, and kept away from sunshine and other source of actinic light that might influence calcification. The animals received the experimental diet and water *ad libitum*. The birds were divided at random into three groups, one of which was fed a vitamin D-deficient diet, a modified method of the Association of Official Agricultural Chemists (AOAC) (Table I). The other groups receiving orally 500 i.u. of vitamin D_2 or D_3 per kg of the basal ration served as positive controls. During the feeding experiments, life-growth curves were recorded and the body weight was estimated at the end of four weeks as shown in Table II.

TABLE I

Composition of Vitamin D-Deficient Diet

Ingredient	Group receiving D_3	Group receiving D_2	D-deficient group
	Basal diet		
	per cent	*per cent*	*per cent*
Ground yellow corn	57.5	57.5	57.5
Wheat middling	25.0	25.0	25.0
Casein	12.0	12.0	12.0
Tricalcium phosphate	3.0	3.0	3.0
NaCl (0.02% KI added)	0.5	0.5	0.5
Brewer's yeast	2.0	2.0	2.0
	Additives		
$MnSO_4 \cdot 4H_2O$	0.2 g/kg basal diet	0.2 g/kg basal diet	0.2 g/kg basal diet
Vitamin A	6000 i.u./kg basal diet	6000 i.u./kg basal diet	6000 i.u./kg basal diet
Vitamin D	500 i.u. D_3/kg	500 i.u. D_2/kg	None

TABLE II

Body Weight Gain of Vitamin D-Deficient Chicks

No. of experiment	Group receiving D_3		Group receiving D_2		D-deficient group	
	$\bar{x}$		$\bar{x}$		$\bar{x}$	
1[b]	117.7	± 31.0[a]	95.1	± 23.1	78.0	± 25.1
2[c]	120.4	± 45.4	147.0	± 50.1	135.6	± 34.5

[a] Standard deviation.

[b] Statistically significant ($P < 0.01$) between D_3 and D_2; statistically significant ($P < 0.01$) between D_3 and D-deficient.

[c] Statistically significant ($P < 0.05$) between D_3 and D_2; statistically significant ($P < 0.01$) between D_2 and D-deficient.

2. *Tests for Vitamin D Deficiency*

After feeding for 1–6 weeks, the birds were killed, and the bones were collected by the ordinary method. Total ash was estimated by the AOAC method. Calcium content of the left tibia of the 2-week deficient animal was colorimetrically determined by permanganate titration, and phosphate of the 6-week deficient

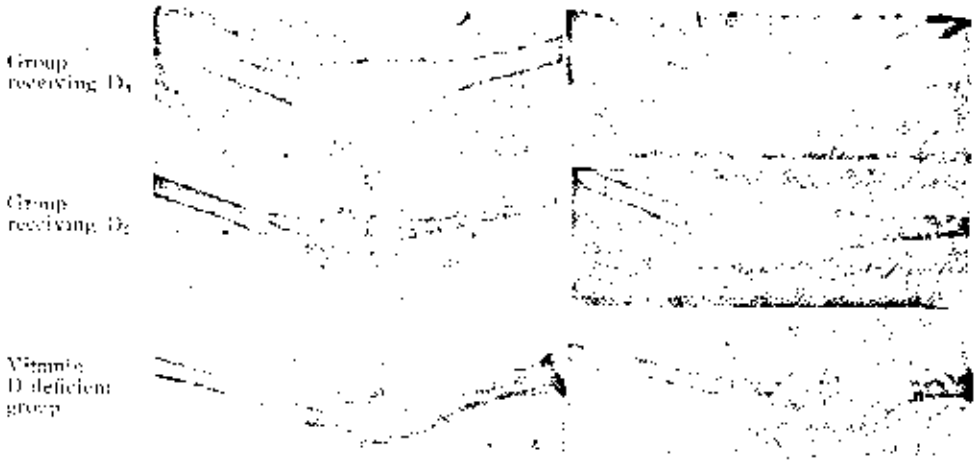

FIG. 2 *Influence of Vitamin D_2 on the Antirachitic Potency of Chicks*

TABLE III

Total Content of Ashes, Calcium and Phosphorus in the Left Dry Tibia

	No. of experiment	Group receiving D_3	Group receiving D_2	D-deficient group
		per cent	*per cent*	*per cent*
Ash	1[b]	51.6 ± 0.8[a]	44.1 ± 6.1	32.9 ± 5.2
	2[c]	51.8 ± 3.9	43.3 ± 2.7	38.5 ± 3.8
Calcium	1[d]	21.9 ± 1.5	13.6 ± 1.9	10.4 ± 1.0
Phosphorus	1[d]	14.4 ± 3.1	7.2 ± 1.6	3.1 ± 1.0

[a] Standard deviation.

[b] Statistically significant ($P < 0.05$) between D_3 and D_2; statistically significant ($P < 0.05$) between D_3 and D-deficient.

[c] Statistically significant ($P < 0.01$) between D_3 and D_2; statistically significant ($P < 0.05$) between D_2 and D-deficient.

[d] Statistically significant ($P < 0.01$) between D_3 and D_2; statistically significant ($P < 0.01$) between D_2 and D-deficient.

animal by Takahashi's method (18) (Fig. 1), with the results given in Table III.

3. *Test for Pyruvate Levels in Blood, Liver and Kidneys*

The blood was collected in the morning after about 12 hours at the end of 4-week feeding, and the pyruvate level in the total blood was estimated by the Friedmann's method (12) (Fig. 2). Pyruvate levels in the total blood was always found to be higher in the group receiving vitamin D_3, but scarcely detectable in the kidneys and liver (Table IV).

4. *Tests for Citrate Levels in the Blood and Kidneys*

Further, determination of citrate was performed by Ettinger's method (14). The highest level was seen in the chicks receiving vitamin D_3 and the lowest in the vitamin D-deficient group.

5. *Chemical Analysis of Sulfate in the Bone*

Total sulfate in the left tibia was estimated by Egami's method (19). The

TABLE IV

Pyruvate and Citrate Levels in the Blood and Kidneys of Vitamin D-Deficient Chicks

Acid	Organ	Group receiving D_3	Group receiving D_2	D-deficient group
		γ/ml	γ/ml	γ/ml
Pyruvic	Blood[b]	48.7 ± 2.6[a]	42.2 ± 7.8	33.3 ± 9.5
	Kidney	Trace	Trace	Trace
	Liver	"	"	"
Citric	Blood[c]	75.5 ± 21.0[a]	50.0 ± 6.5	37.3 ± 6.7
	Kidney[d]	34.6 ± 15.3	36.9 ± 7.3	28.6 ± 7.4

[a] Standard deviation.
[b] Statistically nonsignificant between D_3 and D_2; statistically significant ($P<0.01$) between D_2 and D-deficient.
[c] Statistically significant ($P<0.05$) between D_3 and D_2; statistically significant ($P<0.01$) between D_3 and D-deficient.
[d] Statistically significant ($P<0.05$) between D_3 and D_2; statistically insignificant between D_2 and D-deficient; statistically significant ($P<0.05$) between D_3 and D-deficient.

TABLE V

Sulfate Content in the Left Tibia of Vitamin D-Deficient Chicks

	Group receiving D_3	Group receiving D_2	D-deficient group
	mg/g	mg/g	mg/g
SO_4^{2-}	220.2 ± 156.8	130.4 ± 14.0	92.3 ± 31.6

Statistically significant ($P<0.01$) between D_3 and D_2; statistically significant ($P<0.01$) between D_3 and D-deficient

highest level was found in the chicks receiving vitamin D_3, while the lowest in the vitamin D-deficient ones (Table V).

6. Tests for Bone Sulfate Levels Using S^{35}-Sulfate

At first, vitamin D-deficient chicks were previously prepared, and at the end of the feeding experiment (after 5-week feeding) S^{35}-sulfate was injected into the crop. After 24 hours, the birds were rapidly killed, and the radioactivities were carefully estimated by radioautography and serveymeter. (Fig. 2, Table VI)

TABLE VI

S^{35}-Sulfate of the Left Tibia in Vitamin D-Deficient Chicks
$H_2S^{35}O_4$ 0.01 N HCl solution, 0.02 mci was added

	No. of photograph	Body weight	Radioactivity A	Radioactivity B	Radioactivity C
		g	Counts per minutes		
D-deficient group	1	169	15	9	10
	2	158	12	7	10
Group receiving vitamin D_3	3	196	35	27	31
	4	174	22	13	20

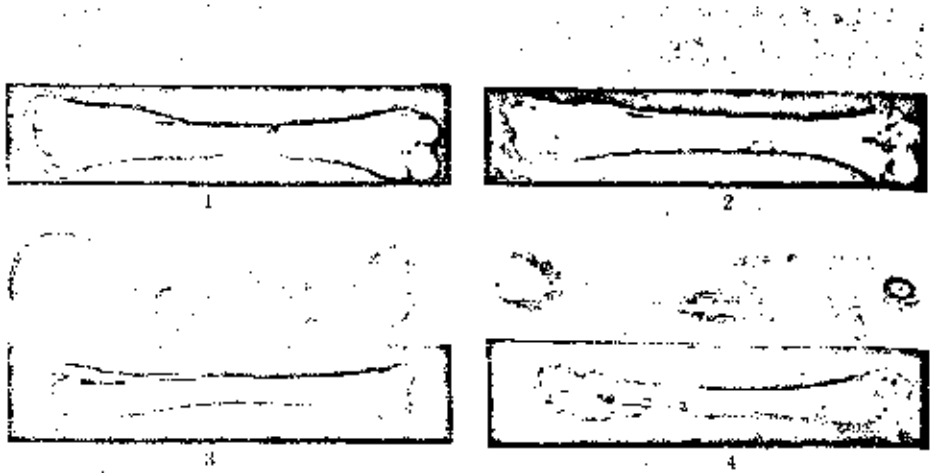

FIG. 2. *Radioautograms of S^{35}-Sulfate which Appeared in the Tibia of the Chicks after Receiving Vitamin D_3*
1, 2, vitamin D-deficient group; 3, 4, group receiving vitamin D_3

SUMMARY

1. The difference of antirachitic potency between vitamin D_2 and D_3 was repeatedly studied using vitamin D-deficient chicks. Marked effect was confirmed in the chicks receiving vitamin D_3, but it was low in the birds supplemented with vitamin D_2 as judged by the chemical analyses of total ash, calcium and phosphorus.

2. The correlation between vitamin D and citrate metabolism in chicks was investigated, and the citrate and pyruvate levels in the blood and kidneys were determined and high citrate and pyruvate levels in the chicks receiving vitamin D_3 was confirmed.

3. The relationship between vitamin D potency and sulfate metabolism was further studied, and the high tibia sulfate level in the chicks receiving vitamin D_3 was demonstrated using S^{35}-sulfate. This finding suggests that there is an intimate correlation between vitamin D and sulfate metabolism in chicks.

ACKNOWLEDGEMENT

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Influence of vitamin D on calcium absorption in the chick[1]

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SALLIS, J. D., AND E. S. HOLDSWORTH. *Influence of vitamin D on calcium absorption in the chick.* Am. J. Physiol. 203(3): 497-505, 1962. The site of absorption of Ca^{45} was studied in rachitic chicks and rachitic chicks given vitamin D_3. Vitamin D_3 markedly increases absorption from the small intestine and, in vivo, similar amounts of calcium are absorbed along the entire small intestine. With everted gut sacs, the distal third of the small intestine transported much more calcium than did the duodenal and middle sections. Thus, interpretations of in vitro results may not always depict the normal in vivo process. Vitamin D_2 had little activity in the chick, but AT-10 series 2 and AT-10 series 3 were almost as active as vitamin D_3 in calcium transport. These results suggest an "active carrier" may be formed by addition of hydroxyl or hydroxyl groups to the opened ring B of vitamin D_3, giving a carrier capable of reversible oxidation-reduction or keto-enol tautomerism. Using metabolic inhibitors, active calcium transport in vitro relied on glycolysis for its energy supply. The transport was independent of the sodium pump.

IN A PREVIOUS PAPER (1) we described a technique for measuring the in vivo absorption of calcium from the small intestine of the chick. When given in small amounts (100 IU) to rachitic chicks a few hours before the experiment, vitamin D_3 increased the amount of calcium absorbed two- to threefold over that absorbed by untreated rachitic chicks. However, we were unable to show this effect in vitro with isolated intestine. Since Schachter and Rosen (2) and Harrison and Harrison (3) have demonstrated the effect of vitamin D on the in vitro absorption of calcium in the rachitic rat, we have reinvestigated the phenomena in chicks. We also studied the effect of vitamin D_2, dihydrotachysterol, and various inhibitors on the process of absorption. These results and some of those in the accompanying paper were communicated to the Fifth International Congress on Nutrition (4).

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MATERIALS AND METHODS

White Leghorn cockerels were obtained locally on the day of hatching and housed in electrically heated tier brooders in an airconditioned room from which sunlight was excluded. Only rachitic chicks were raised (in one exception, normal chicks were raised to calculate the ash content of tibia), by feeding the following rachitogenic ration for a period of approximately 4-5 weeks.

Diet	% Composition
Ground wheat	26
Ground maize	30
Soya meal	15.5
Casein (N.B.C. vitamin free)	9
Dried yeast	3
Dried lucerne	4
Salt	1
Wood charcoal	0.5
Manganese sulphate ($4\ H_2O$)	20 mg/100 g diet
Calcium phosphate (B.D.H. precipitated)	2
Vitamin A	2,000-3,000 IU/450 g diet
Vitamin E	13 IU/450 g diet
Arachis oil	1

The vitamins dissolved in the arachis oil were triturated with the calcium phosphate before adding to the remainder of the diet.

The chicks were allowed food and water at all times. Rachitic symptoms became evident after 18 days and the degree of rickets was assessed from the ash content of the dried fat-free bone. Typical figures for rachitic chicks were 26-30% ash of the fat-free bone, whereas chicks raised to the same age on a diet supplemented with adequate vitamin D_3 from birth gave an ash content of from 40 to 44%. For experimental purposes, the chicks were selected as groups of approximately equal weight from the 26th to 35th day of hatching.

A positive control group was included in all our experiments and, unless otherwise stated, this group received 100 IU vitamin D_3 in arachis oil 18 hr before the test, since we have previously shown this would lead to calcium uptake comparable to that of normal chicks (1). Similarly, rachitic controls received the vehicle

alone in the manner described for each particular experiment.

Vitamin D and Related Steroids

Crystalline vitamin D_2, D_3, and dihydrotachysterol$_2$ (AT-10$_2$) were obtained from L. Light & Co. Ltd., Colnbrook, England. Pure crystalline samples of dihydrotachysterol series 2 (AT-10$_2$) and dihydrotachysterol series 3 (AT-10$_3$) were kindly provided by Dr. Westerhof of Philips-Roxane, Amsterdam. For oral dosing, steroids were dissolved in arachis oil and administered by stomach tube. When given as an intracardial injection, the steroid was dissolved in ethanol propylene glycol mixture, the final concentration of ethanol being less than 0.5%.

Calcium 45 Chloride

The $Ca^{45}Cl_2$ was obtained from the Radiochemical Centre, Amersham, England, and was diluted for use with unlabeled calcium chloride to give a specific activity of approximately 2 μc/mg calcium. Unless otherwise stated, the calcium was administered as the chloride at pH 6.0 and each chick received 4 mg calcium and 8.0 μc/loop.

Sodium 22 Chloride

$Na^{22}Cl$ was obtained from the Radiochemical Centre, Amersham, England, and was diluted for use with unlabeled NaCl (0.15 M) to contain 2 μc/ml.

Phosphorus 32

The carrier-free orthophosphate, from the Radiochemical Centre, Amersham, England, was diluted with sodium phosphate to give 10 μc/mg PO_4.

Radioactive Counting Procedures

Blood. For the counting of Ca^{45} or P^{32} in the blood, the material was collected from a wing vein into a heparinized syringe. P^{32} was estimated on whole blood, whereas plasma was prepared for Ca^{45} counting. An 0.1-ml sample was spread on a 1-cm² aluminum planchet, allowed to dry, and finally counted, using a G.E.C. EHM$_2$ end-window counter.

Bone. Ashed tibia were dissolved in 2 N HCl and the calcium precipitated as oxalate in the conventional manner. Calcium^{45} oxalate was counted at infinite thickness on 1-cm² polyethylene planchets, using a G.E.C. EHM$_2$ end-window counter.

Ca^{45} in tissue fluid. Serosal fluid (0.1 ml) was added directly to 5.0 ml of scintillator phosphor, as developed by Bray (5), and the $Ca^{45}Cl_2$ estimated by scintillation counting in an LKCO N664A counter. In the case of mucosal tissue, the loops were cut into small pieces, extracted overnight with 5 N perchloric acid and finally heated at 80 C for 2 hr. The precipitated protein was removed by centrifugation and $Ca^{45}Cl_2$ estimated by scintillation counting. The necessary quenching corrections were made for the final evaluation.

Na^{22}. Whole blood from the wing vein (0.4 ml) was pipetted directly into a deep-well polyethylene scintillation tube and Na^{22} was counted in an LKCO N597 NaI crystal scintillation counter.

Chemical Estimation of CaCl

Organic material from intestinal loops was destroyed by the method of wet oxidation (6). After diluting to a known volume, calcium was estimated essentially as described by Herrmann (7). After precipitation of an aliquot of the digest as calcium oxalate, the ethylene diaminotetraacetic acid (EDTA)-calcein titration method was used. The final assay system was comprised of: 1.4 ml, .025 M EDTA; 1.0 ml calcein indicator, 0.3 ml 2 N KOH containing 1% KCN, 0.5-ml sample; and one drop octyl alcohol. This was titrated with standard calcium chloride solution (1 mg/ml). Recoveries of 98% were estimated from this method.

Lactic Acid Determination

Lactic acid was estimated by the method of Barker and Summerson (8). After incubation of the everted intestinal sacs, the mucosal and serosal fluids were pooled and kept at 0 C. The intestinal tissue was homog-

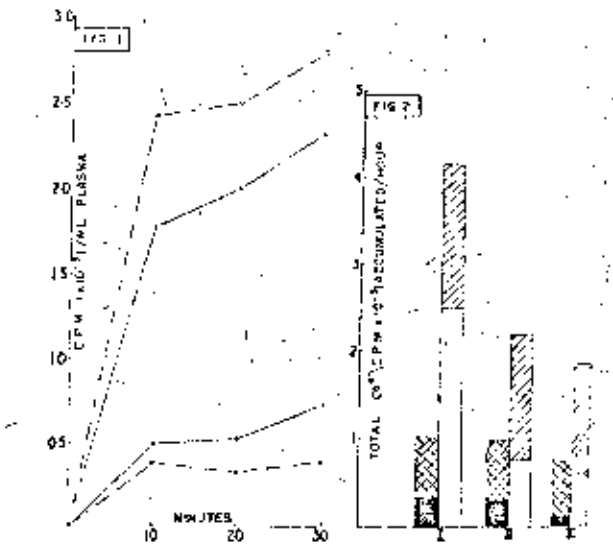

FIG. 1. Plasma Ca^{45} levels during absorption from duodenal and distal loops. Ca^{45} was measured in plasma after preparing in vitro loops of small intestine from rachitic and vitamin D_2-treated chicks. Each point on graph is the mean of 4 birds in a typical experiment (1 of 3). ○------○ = vitamin D_2-treated distal loops. △ — △ = vitamin D_2-treated duodenal loops. ◐ — ◐ = rachitic duodenal loops. ×------× = rachitic distal loops.

FIG. 2. Site of Ca absorption in vitro. Accumulation of Ca^{45} into mucosal tissue and serosal fluid of everted small intestine. Each histogram is the mean of 8 sacs. *Segments I, II,* and *III* are distal, middle, and proximal sacs, respectively. Blocked histograms are rachitic serosal fluid. Cross-hatched histograms are rachitic mucosal tissue. Blank histograms are vitamin D_2-treated serosal fluid. Diagonal-hatched histograms are vitamin D_2-treated mucosal tissue.

ized in ice-cold distilled water, using a Potter-Elvehjem Teflon homogenizer before adding to the pooled fluids and making to a constant volume. Perchloric acid filtrates were prepared and neutralized before copper-calcium treatment. Lactic acid was eliminated on an aliquot of the supernatant.

Surgical Procedure

The preparation of in vivo loops of small intestine was essentially that described in an earlier paper (1). The distal loop subsequently used in most of this work was a section of small intestine measured upwards from the entrance of the cecum to, in most cases, a few centimeters past the yolk sac. The effective length used in the experiments was 10–15 CID.

In Vivo Perfusion Experiments

The perfusion apparatus consisted of a small peristaltic pump connected to the gut through glass cannulas. The pump and connections were enclosed within a thermostatically controlled chamber. Nembutal was used as an anesthetic in conjunction with a fitch ether. After the introduction of the cannulas, the portion of small intestine, with blood supply and tissue still intact, was washed with physiological saline at 37° C, drained, and immediately replaced within the abdominal cavity, the incision being kept covered with pads of moist cotton wool. Circulating fluid consisted of 0.15 M NaCl to which had been added glucose in a concentration of 0.02 M. Perfusion was carried out at 37° C and, at the completion of the test period, the apparatus and gut were thoroughly drained to enable the volume to be measured. The absorption of saline was measured by following the disappearance of Na^{22} from the perfusion fluid and by counting samples of blood taken at varying time intervals from a wing vein.

In Vitro Everted Intestinal Sac Technique

Sequents of chick small intestine were prepared essentially as described by Harrison and Harrison (3). Standard incubation medium consisted of Krebs-Henseleit bicarbonate buffer with glucose added in a concentration of 0.02 M (4).

The everted sacs were filled with 1 ml of the buffer fluid containing 90 μc Ca^{45}/ml, i.e., 2.25×10^{-9} M. These were then placed in 5 ml of mucosal fluid containing 8½ μc Ca^{45}/ml, i.e., 2.125×10^{-9} M. Thus, the serosal surface was bathed with a slightly higher concentration of unlabeled Ca^{++}. The preparations were incubated for 2 hr at 38° C in an atmosphere of 95% O_2, 5% CO_2 in a metabolic shaker bath. At the end of the incubation the sacs were removed, rinsed externally with cold 0.9% saline and dried with absorbent tissue. The serosal fluid was drained into a weighed container and rinsed through with exactly 1 ml saline. The pooled fluids were weighed to estimate the volume of serosal fluid.

TABLE 1. *Effect of vitamin D_3 on Ca transport from different portions of small intestine*

Segment	Rachitic		Vit. D_3-Treated	
	% Ca uptake	Bone Ca^{45}, counts/min × 10⁻³/g ashed tibia	% Ca uptake	Bone Ca^{45}, counts/min × 10⁻³/g ashed tibia
Duodenal	16±3.5 (20)	10.65±3.08 (8)	48±3.9 (20)	27.78±4.12 (8)
Distal	8±2.1 (12)	5.12±2.39 (8)	56±6.5 (12)	45.06±3.15 (8)

Uptake and deposition of Ca was measured after 2 hr from in vivo loops containing 4 mg Ca^{45}. Treated birds were given 100 IU vitamin D_3 16 hr prior to Ca test. Values are means ± SE; figures in parentheses represent number of birds.

RESULTS

Site of Absorption

Loops of small intestine were prepared in vivo to locate the site of maximum absorption of calcium. Figure 1 presents the results obtained by following plasma Ca^{45} levels at varying time intervals after placing 4.0 mg Ca^{45} into either the duodenal or the distal loops of small intestine of rachitic and vitamin D_3-treated chicks. A marked increase in circulating calcium occurred from either loop under the influence of vitamin D_3. However, a greater ratio (D:rachitic) was obtained with distal loops (7:1) as compared with duodenal loops (3:1). Further evidence that this plasma Ca^{45} level was a reliable indication of the absorption rate is shown by the measurement of calcium disappearance from the loop and its subsequent deposition or exchange in bone, as revealed in Table 1. Vitamin D_3 increased the uptake from duodenal loops threefold and this calcium was deposited in bone. An even greater (sevenfold) increase in uptake occurred from distal loops. The amount of calcium absorbed by distal loops after 2 hr in vivo was slightly greater than the amount absorbed from duodenal loops.

Similar experiments, but using isolated everted loops, added support to the in vivo observations. Accumulation of Ca^{45} into both mucosal tissue and serosal fluid was measured after 1 hr of incubation. The greatest amount of transport occurred with distal segments of small intestine from vitamin D_3-treated chicks (fig. 2). Using the results expressed as Ca^{45} accumulated in the serosal fluid, the D:rachitic ratio was 7:6 with distal loops. If the method of Harrison and Harrison (3) was used for assessing total transport, i.e., the sum of the serosal fluid plus the mucosal tissue Ca^{45}, then the ratios were 4:1 distal, 2.4:1 middle, and 2.4:1 for duodenal loops. It is interesting to note that similar amounts of calcium were accumulated by mucosal tissue in all parts of the small intestine of vitamin D_3-treated chicks. The absolute amount of calcium transported by the distal segment was 35 μg Ca/hr/g for rachitic gut, and 150 μg Ca/hr/g with vitamin D_3-treated chicks.

On occasions, variations from the above ratios have been observed and are due mainly to the age of the batch of chicks selected for the experiment, since the severity of rickets produced depends on the age of the chick. Similarly, the type of pretreatment with vitamin D_3 influences the ratios. Of the many hundreds of chicks examined, however, the minimum ratio observed, using distal loops after 16 hr pretreatment with 100 IU vitamin D_3, was 3.1 in vivo and 2.6 in vitro.

Factors Influencing Ca Transport In Vivo

pH effect. We have confirmed that the pH of duodenal loop contents is 6.9, increasing to 7.2 in the distal loop, as reported in an earlier paper (1). Since pH has an important influence on the solubility of some calcium salts, we studied the effect of a more acid medium on calcium transport. Figure 3 shows that no difference could be observed in plasma Ca^{45} levels using in vivo duodenal loops containing medium at pH 4.0 or pH 6.0.

Chelation. Chelation of Ca present in duodenal loops by means of EDTA caused a marked decrease in Ca absorption from both rachitic and treated groups (Fig. 4). The much weaker chelating agent, citrate, had no effect on uptake, as reported earlier (11). Thus, it appears to be ionic Ca that is absorbed.

Amino acids. It has been reported that L-lysine and L-arginine aids the absorption of Ca in rats (10), but not in chicks (11). The addition of 10 mg L-lysine or 10 mg L-arginine (pH 6.0) to in vivo duodenal loops did not increase the plasma Ca^{45} levels when tested on rachitic chicks.

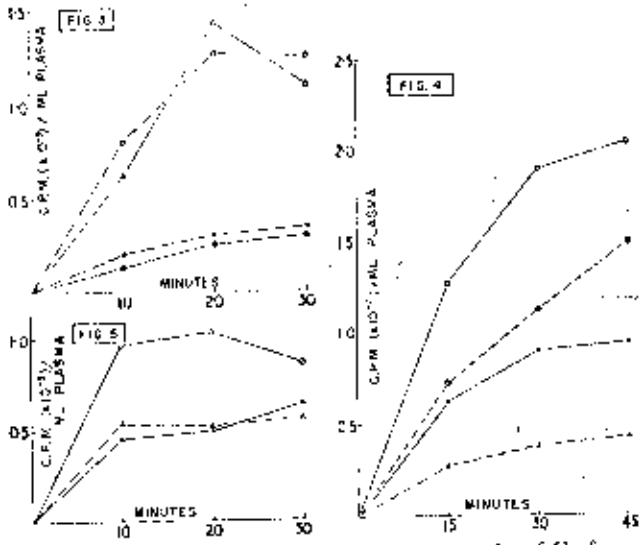

FIG. 3. Influence of pH of the medium on uptake of Ca from duodenal loops. Ca^{45} was measured in plasma after preparing in vivo duodenal loops from rachitic and vitamin D_3-treated chicks. Loops contained 4 mg Ca^{45} in 0.2 M citrate buffer. Each *point* is the mean of 4 birds. ○——○ = vitamin D_3-treated, pH 6.0. △——△ = vitamin D_3-treated, pH 4.0. ×——× = rachitic, pH 6.0. ●——● = rachitic, pH 4.0.

FIG. 4. Effect of EDTA on Ca absorption in vivo. Ca^{45} was measured in plasma after preparing in vivo duodenal loops from rachitic or vitamin D_3-treated chicks. Loops were given 0.1 mmoles Ca^{45} EDTA and control loops 0.1 mmoles $Ca^{45}Cl_2$ in saline. Each *point* is the mean of 5 birds. ●——● = vitamin D_3 treated and EDTA. ○——○ = vitamin D_3-treated controls. △——△ = rachitic and EDTA. ×——× = rachitic controls.

FIG. 5. Effectiveness of short-term administration of vitamin D_3 on Ca absorption in vivo. Ca^{45} was measured in plasma after preparing in vivo distal loops from rachitic or vitamin D_3-treated chicks. Vitamin D_3, 1,000 IU in propylene glycol, was injected into the duodenum. Each *point* is the mean of 4 birds. ○——○ = 4 hr after giving vitamin D_3. ×——× = 2 hr after giving vitamin D_3. △——△ = rachitic control.

TABLE 2. *Influence of vitamin D_3 with time and route of administration on deposition of Ca^{45} into bone*

Route of Administration	No. of Birds	Amount of Vit. D_3	Bone Ca^{45} counts/min/g. of ash. Ashed tibia
	4	0	3.5 ± 1.4
Intracardial	4	10,000 IU	[illegible]
Oral	4	10,000 IU	[illegible]

Vitamin D_3 was administered 2 hr before giving 2 mg $Ca^{45}Cl_2$ by mouth. Tibia was removed 4 hr later.

Influence of Vitamin D_3 With Time of Administration

We have extended the attempts to show immediate or short-term effects of vitamin D administration. A large dose of 10,000 IU vitamin D_3 was given as a colloidal aqueous suspension either *a)* by stomach tube or *b)* intracardially, 1 hr before giving 2 mg $Ca^{45}Cl_2$ by stomach tube. After 2 hr, the left tibia was removed and the Ca^{45} counted. In a further experiment, the vitamin D_3 was given as above, but 2 hr before the Ca^{45}. The tibia was removed at the end of 3 hr. The results of this latter experiment are shown in Table 2 and, in spite of the large unphysiological amounts of vitamin D_3 in circulation, no significant increase in bone counts was detected.

The administration of vitamin D_3 by stomach tube might lead to a delay in the absorption of the vitamin, due mainly to its rate of passage through the intestinal tract. To eliminate this difficulty, 1,000 IU vitamin D_3 in propylene glycol was injected into the duodenum and, after 2 and 4 hr, Ca^{45} was placed in in vivo distal loops. The increases in plasma Ca^{45} levels obtained are shown in Fig. 5 and indicate no effect of vitamin D_3 after 2 hr, but an increase following the 4-hr period. Direct addition of 10,000 IU vitamin D_3, together with $Ca^{45}Cl_2$, to in vivo distal loops or to the mucosal fluid in vitro had no effect on calcium uptake.

Effect of Vitamin D_2, Dihydrotachysterol

The effects on calcium absorption from duodenal loops after oral dosage of vitamin D_2 and of AT-10 (Lights) are compared with those of vitamin D_3 in Fig. 6. The plasma Ca^{45} levels reveal that on an equal weight basis, vitamin D_2 in comparison to vitamin D_3 had only a slight effect on calcium uptake. Only with large unphysiological levels of vitamin D_2 could the plasma level of Ca^{45} be raised to a value similar to the

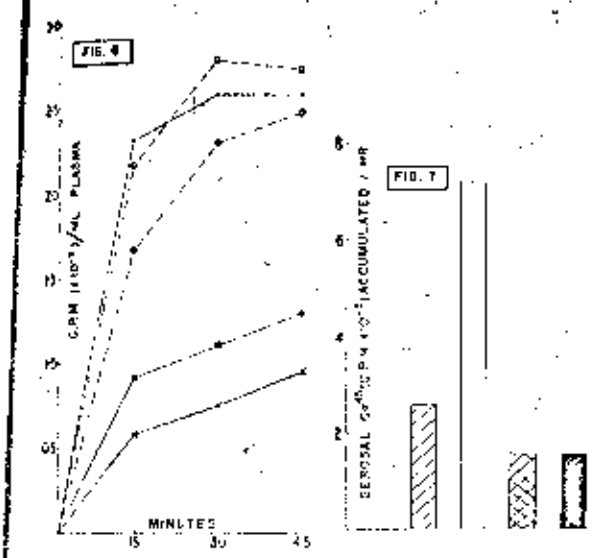

FIG. 6. Effect of dihydrotachysterol and vitamin D_2 on Ca absorption in vivo. Ca^{45} was measured in plasma after preparing in vivo duodenal loops. Number of birds is given in parentheses. ● — ● = vitamin D_2, 2.5 μg (4). ○ ------ ○ = vitamin D_2, 2.5 μg (4). △ ------ △ = AT-10₂, 10 μg (4). ▲ — ▲ = AT-10₂, 2.5 μg (8). × ------ × = rachitic controls (8).

FIG. 7. Unidirectional transport of Ca by in vitro distal loops. Ca^{45} was measured in serosal fluid after 1 hr. Histograms represent the mean of uptake from 6 sacs. Diagonal hatched: rachitic everted distal intestine. Blank: vitamin D_2-treated everted distal intestine. Cross hatched: rachitic noneverted distal intestine. Blocked: vitamin D_2-treated noneverted distal intestine.

effect of 100 IU vitamin D_2. AT-10₂, a steroid used therapeutically for raising serum calcium levels was much more active than vitamin D_2 and, when given at the dose level of 10 μg by mouth 16 hr before the experiment, plasma Ca^{45} levels were comparable to those of chicks which had received 2.5 μg vitamin D_2. If the deposition of Ca^{45} in bone was taken as a measure of activity, then Table 3 shows that AT-10₂ was more active on a weight basis than vitamin D_2.

AT-10₃ (Philips-Ruxane), given by mouth 16 hr prior to the test, had the same activity as AT-10₂ (Philips-Ruxane). Both AT-10₂ and AT-10₃ were ineffective in increasing calcium uptake when given at a level of 2.5 μg intracardially 1 hr before the in vivo test. In vitro studies with everted distal segments of intestine, after treatment of the chicks with 2.5 μg AT-10₃ (Philips-Ruxane) or AT-10₃ (Philips-Ruxane) by stomach tube 16 hr before the experiment, indicated that these steroids were equally as active in increasing calcium accumulation in the serosal fluid. However, the level of calcium transported above the rachitic value was only twofold, compared with the fivefold increase with vitamin D_2-treated chicks.

Influence of Vitamin D_2 on Transport of Other Ions

Sodium transport. When attempts were made to follow Na uptake by placing 1.0 ml isotonic saline (labeled with Na^{22}) into loops of intestine in vivo, rapid absorption took place, so that it was impossible to detect differences in uptake due to vitamin D_2 action. For this reason, in vivo duodenal loops were perfused from a reservoir of 10 ml isotonic saline containing Na^{22}. The data in Table 4 show that the transport of sodium was not affected by vitamin D_2.

Phosphate transport. The medium introduced into the in vivo duodenal loops was modified to include 1 mg $P^{32}O_4$ (10 μc P^{32}) and unlabeled calcium and the absorption of the phosphate ion was followed by counting the total P^{32} in whole blood. The results from rachitic and treated chicks (100 IU vitamin D_2) are compared in Table 5. A small but insignificant increase has been recorded. The residual P^{32} in the loop at the conclusion of the experiment was also counted, but again no significant effect of vitamin D_2 could be detected. Normal values for calcium absorption were recorded during this experiment, being 16% for the rachitic duodenal loop and 48% for the vitamin D_2-treated loop.

Mechanism of Calcium Transport

Evidence for active transport. To show that the accumulation of Ca is a unidirectional process, we studied the transport of Ca in noneverted isolated distal segments. The noneverted gut sacs were subjected to the same conditions as described for everted loops and the results are shown in Fig. 7.

The noneverted rachitic sacs transported less Ca than the everted rachitic sacs. The decreased transport may be a consequence of the lower transport of water observed, which is a net gain of 0.6 g water/hr per everted gut, but a net loss (from the original 2 g) of 0.3 g water/hr for noneverted sac. More interesting was the absence of any influence of vitamin D_2 on the transport of Ca, using noneverted gut. Evidence for the participation of enzyme action in Ca transport can be seen from the fact that, at 0 C, transport of Ca was only 5% that of normal. Further, Ca transport was taking place against a slight concentration gradient.

Effect of metabolic inhibitors. Various inhibitors were used in an attempt to identify the source of energy used in calcium transfer and the results are shown in Table 6. These results have been expressed as per cent inhibition of the "active transport process," by which is inferred the difference between vitamin D_2-treated and rachitic transport. This seems justified because the rachitic transport, which is probably made up of passive

TABLE 3. *Effect of AT-10₂ on Ca^{45} deposition in bone*

Dose of Steroid, μg	Bone Ca [illegible] × 10⁻³ Ashed Tibia
	2.45±0.37
2.5 D_2	6.50±0.90
2.5 AT-10₂	10.99±1.70
10.0 AT-10₂	12.83±3.50

Steroids were given by mouth 16 hr before preparing in vivo duodenal loops. Tibia was removed after 2 hr. Values are means ± SD for 4 birds.

TABLE 4. *Influence of vitamin D_3 on Na transport*

No. and Type of Birds	Na^{22}, counts/min × 10^{-3}/ml Blood		
	15 Min	30 Min	45 Min
12 Rachitic	1.12	1.04	6.14
12 Vitamin D_3-treated	1.30	2.02	6.11

Duodenal loops were perfused in vivo with $Na^{22}Cl_2$. 100 IU vitamin D_3 was given 16 hr prior to perfusion to the treated birds.

diffusion and exchange processes, is not inhibited by 2×10^{-4} M iodoacetate under anaerobic conditions, whereas the vitamin D_3-enhanced transport is greatly inhibited. Although the well-known inhibitory effects on active Ca transport, the greatest effect was obtained with an inhibitor of glycolysis, viz., 2×10^{-3} M iodoacetate under anaerobic conditions. The results obtained with mercuric chloride cannot be interpreted because this substance caused the mucosal fluid to become turbid with sloughed cells.

Starvation and effect of glucose. In some early experiments it appeared that starvation decreased the ability to transport calcium. In vitro experiments with gut sacs from chicks starved for 24 hr and incubated in standard medium show a slightly diminished ability to absorb calcium. Similarly, gut sacs from chicks not starved, but incubated without glucose in the standard medium, showed that the endogenous substrate can support 62% of the normal rate of absorption. These results, which are presented in Table 7, were obtained with vitamin D_3-treated chicks.

Measurement of glycolysis. To determine whether vitamin D_3 had an effect on glycolysis, everted distal gut sacs were incubated in standard medium, but the normal aerobic conditions were replaced with a 95% N_2/5% CO_2 atmosphere to prevent further breakdown of the lactic acid formed. The reaction was stopped after 1 hr and lactic acid determined as outlined under MATERIALS AND METHODS. Table 8 shows that similar amounts of lactic acid were produced by the rachitic and vitamin D_3-treated gut sacs.

DISCUSSION

Several investigators (2, 3, 12–14) have shown from in vitro studies that isolated everted segments of rat small intestine can actively transport Ca, as measured by the final concentration of Ca inside the everted sac to that in the mucosal medium. This process is dependent on the vitamin D status of the animal. Dietary factors may also exert a major influence on the result of such experiments, as recently suggested by Kimberg et al. (13). In earlier experiments by Schachter and Rosen (2), rats were fed a vitamin D-free diet containing a low P, high Ca ratio and these animals developed rickets in 14 days. When Ca transport was measured, the final I/O for control animals was 3.2 ± 4.2 and active transport could only be shown to occur in the proximal one fifth section of small intestine. However, a more recent investigation (13) indicates that when rats were fed a low Ca, high P diet, rickets were not observed within the same time period, and the I/O was 2.0 and active transport could be demonstrated in all parts of the small intestine.

Under these circumstances then, the rat does not seem to be very suitable for work related to vitamin D and calcium metabolism, as rickets can only be produced by an imbalance of the Ca/P ratio of the diet. Further, rickets so produced can be healed by restoring conditions to those found in natural diets even without vitamin D. It would seem then that the variable I/O ratios reported by Schachter and Rosen (2) may be difficult to interpret due to this interplay of dietary effects and vitamin D action. Many of these difficulties are eliminated, however, when chicks are taken for experimental studies of this nature, as was shown by Gershoff and Hegsted (15). The chicks used in our experiments became rachitic after 18 days on a diet containing approximately 0.8% Ca and 0.4% P; this diet was complete with the exception of vitamin D. Calcium absorption can be restored to levels approaching that in normal birds by very small amounts of vitamin D_3 (1) and is in contrast to the large nonphysiological amounts of vitamin D_2 usually used in rat experiments (2).

We have studied the in vivo absorption of Ca from a loop of chick small intestine by measuring the disappearance of Ca from the loop, or by the appearance of Ca^{45} in the serum, or by counting the Ca^{45} in the bone ash at the conclusion of the experiment. With distal loops from rachitic chicks, only 8% of the initial 4 mg calcium was absorbed, but this was increased sevenfold to 56% when 100 IU vitamin D_3 was given 16 hr earlier, whereas with duodenal loops the increase recorded was only threefold (16 to 48%). It is interesting to note, however, that vitamin D_3 distal loops give only 17% better absorption than similarly treated duodenal segments, which confirms our earlier observation. Ca^{45} measurements indicated comparable ratios in plasma and the absorbed Ca was deposited in bone, giving a D_3/rachitic = 3:1 from distal loops.

The transport of Ca obtained with segments of intestine in vitro is qualitatively similar to that in vivo. We prefer to assess the amount of Ca transported as that appearing in the serosal fluid of the everted loops, since this is more comparable to measuring serum Ca^{45} in

TABLE 5. *Influence of vitamin D_3 on phosphate uptake*

No. and Type of Birds	P^{32}, counts/min × 10^{-3}/ml Blood			
	10 Min	20 Min	30 Min	120 Min
6 Rachitic	3.47	2.69	1.95	2.64
6 Vitamin D_3-treated	3.78	4.31	2.40	3.08

1 mg $P^{32}O_4$ was inserted into in vivo duodenal loops of rachitic and vitamin D_3-treated birds. 100 IU/di hr.

vivo. At the usual dosage of 100 IU vitamin D_3 16 hr before the experiment, the ratio D_3/rachitic in distal loops was 3.5. In an experiment designed to measure the maximum amounts of Ca absorbed and transported, when 2,000 IU vitamin D_3 was given over a period of 48 hr, the ratio of D_3/rachitic increased to 7.6. The greatest transport occurred with distal loops. Schachter and Rosen (2) report similar findings on Ca uptake in the hamster. The rather constant amount of Ca bound by the mucosal cells from each of the three equal segments of intestine suggests that Ca may be saturating a combination of tissue Ca-binding material (e.g. protein) and a Ca-carrier system. The increase in binding of calcium after giving vitamin D_3 may be due to an increase in both these mechanisms, but more probably to an increase in the carrier system. A more rapid rate of Ca turnover seems to occur with the distal portion of the intestine, leading to a greater transfer of calcium to the serosal fluid.

Both in vivo and in vitro, the greatest effect of vitamin D_3 has been obtained with distal loops and, for many purposes, the use of the distal loop for studying Ca absorption was the more sensitive technique. If only in vitro studies had been made it would have been easy to conclude that, in the chick, the greatest amount of absorption of Ca takes place in the distal part of the small intestine. However, the in vivo results show that the duodenal portion from vitamin D_3 everted chicks is only slightly less effective than the distal segments and suggests that, under natural conditions, Ca absorption takes place effectively along the entire small intestine. These findings confirm our earlier investigations when we concluded that there was no special portion of the gut concerned with Ca uptake (1). Similarly, with the rat, although it has been established (12) that the proximal fifth of the small intestine is very largely responsible for Ca uptake in vitro, Kimberg et al. (13) have shown that Ca absorption can take place along the entire small intestine. The pattern of in vivo Ca absorption in the rat has yet to be fully established. Nicolaysen (16) has suggested that, under the influence of vitamin D_3, Ca absorption takes place mainly from the upper half of the small intestine. Harrison and Harrison (17) believe, however, that only Ca absorption from the distal portion is affected by vitamin D treatment.

TABLE 6. *Effect of inhibitors on active transport of Ca*

Inhibitor and Conc.[a]	No. of Birds	% Inhibition
N/CO_2 in gas space	7	37
Sodium cyanide, 5×10^{-5}	4	8
Sodium cyanide, 1×10^{-4}	4	39.4
2,4 Dinitrophenol, 2×10^{-4}	8	92
Sodium iodoacetate, 2×10^{-3}	8	61
Sodium fluoride, 2×10^{-1}	7	51
Sodium arsenite, 2×10^{-3}	4	55
Mercuric chloride[b] 2×10^{-3}	4	95
Urethane, 6.8×10^{-3}	11	[illegible]

Active transport was taken to be the difference between Ca transported into serosal fluid by everted distal sacs from rachitic chicks as compared with similar sacs from chicks treated with 100 IU D_3 16 hr previously. Inhibition is expressed as a per cent of the active transport. The inhibitors were added to mucosal fluid and are recorded as final concentrations. [b] Cells sloughed off.

TABLE 7. *Effect of starvation or absence of glucose on Ca transport in vitro*

	No. of Birds	% Absorption
Rachitic (control)	6	28
Rachitic (starved 24 hr)	4	27
Rachitic (no glucose)	4	25
Vit. D_3-treated (starved 24 hr)	4	65
Vit. D_3-treated (no glucose)	4	62

Transport was measured by accumulation of Ca^{45} in serosal fluid from everted distal sacs. Sacs from vitamin D_3-treated chicks, 100 IU-16 hr, incubated in standard medium were assigned an arbitrary value of 100%.

Attempts to show an immediate effect of vitamin D_3 on calcium absorption by having the vitamin present as an aqueous colloidal suspension, either in loops in the live bird or added to in vitro preparations, were unsuccessful. This confirms our earlier work (1). The shortest time for which we could show an effect of vitamin D_3 was 4 hr. The intracardial method of administration ensured large circulating levels of vitamin D_3 in the blood and would thus eliminate any time lag due to the absorption of the vitamin. That there is a time lag before the vitamin can be shown to influence calcium transport would suggest that either there is a change in the mucosal cells themselves, or that vitamin D_3 itself is converted into a more active form, or indeed, that both these transformations are required for complete calcium transport.

We have been unable to demonstrate any influence of vitamin D_3 on sodium transport in vivo and this gives support to a similar type of in vitro study (3). Similarly, there was no significant effect of vitamin D_3 on phosphate ion absorption in vivo, as originally shown by Nicolaysen and Eeg-Larsen (18). Evidence that the transport of Ca under the influence of vitamin D_3 is an active process is based on the following findings. Calcium was transported against a slight concentration gradient in our in vitro experiments. There was little transport of calcium at 0 C, when enzyme activity would be negligible. The passage of Ca from serosal to mucosal fluids (nonverted gut sacs) was small and unaffected by vitamin D_3 and this small amount we regarded as a measure of the amount of passive diffusion and exchange that was taking place. Rachitic everted sacs transport slightly more Ca than nonverted rachitic sacs, and this may be due to the difficulty of ensuring that the chick is completely deficient in vitamin D_3. The unidirectional increase in transport above the rachitic value brought about by vitamin D_3 we ascribe to the active transport of calcium.

Conditions in the small intestine under normal dietary status would appear to be unfavorable to the presence of high concentrations of Ca^{++} in solution, and Ca uptake by diffusion must therefore be a very slow process. A mechanism that rapidly removes the small

TABLE 8. *Accumulation of lactic acid by chick everted intestine*

No. of Chicks	Type of Birds	μg Lactic Acid in Gut Distal Loop
6	Rachitic	16.2±1.7
6	Vitamin D₂-treated	16.3±2.25

In vitro everted sacs from rachitic and vitamin D-treated chicks, 300 U vitamin D, were incubated in standard medium under anaerobic conditions. Values are means ± SD.

amount of Ca^{++} across the membrane would allow further dissociation of the sparingly soluble Ca salts to take place and would lead to a more rapid uptake of Ca. Thus, speaking teleologically, we can see why the vitamin D active transport mechanism is necessary for the transport of this small ion.

There is certain disagreement in the literature concerning the effect of inhibitors on the in vitro transport of Ca by everted sacs. In an early paper Schachter and Rosen (2) claimed that N_2 inhibited the active accumulation of Ca, whereas in a recent paper (19), it was found that, although N_2 inhibited transport in the duodenal segments, N_2 stimulated Ca uptake in the lower segments. The results of Harrison and Harrison (3) show that N_2 CO_2 only partially inhibited Ca uptake. Similarly, there was disagreement on the action of cyanide.

We found only 8% inhibition of active transport by 5×10^{-4} M CN in agreement with Harrison and Harrison (3) and 37% when the atmosphere was N_2 CO_2. Thus, there appears to be some contribution to energy production via oxidative phosphorylation but, since iodoacetate 2×10^{-4} M inhibited transport by 80%, it would appear that in vitro the major energy supply is from glycolysis. Breuer and Whittam (20) and del Monte (21) have also suggested that glycolysis supplies the energy for the transport of glucose, water, sodium, and potassium. That vitamin D_3 does not affect transport by altering the amount of glycolysis is shown by the production of equal amounts of lactic acid when using rachitic or vitamin D_3-treated chick intestine.

Daniel and Robinson (22) state that ouabain interferes with sodium and potassium exchange in rabbit uterine segments. Csaky, Harzog, and Frankel (23) have shown that this drug completely inhibits the active transport of α-methyl glucose and these authors suggest that the inhibitory action of ouabain is primarily on the "sodium pump." If this is correct, our finding of no inhibitory effect by ouabain on calcium transport would indicate that the uptake is independent of the sodium pump.

Vitamin D_2 is known to have only a small percentage of the biological activity of vitamin D_3 when tested with chicks (24) and our results confirm this finding. The structural difference between these two vitamins lies in the side chain (C_{17}) and it is interesting to note that $AT\text{-}10_2$ (since chain like vitamin D_2) and $AT\text{-}10_3$ are equally active in increasing Ca absorption in chicks and are only slightly less active than vitamin D_3 when tested in vivo. However, there are some puzzling features regarding the effect of dihydrotachysterol on Ca metabolism, since our results show that the Ca^{++} absorbed under the influence of this steroid can be deposited in bone (Table 3), and yet the same batch of $AT\text{-}10_2$ showed less than 1 IU/mg when tested for antirachitic potency in chicks (Dr. M. E. Coates, personal communication). The precise stereochemistry of these sterols is still a matter of controversy, but our results suggest that the constitution of the side chain is not of prime importance in calcium transport. However, the change of vitamin D_2 to the active carrier form may be slow in the chick, possibly due to the difference in the side chain, whereas in the rat both vitamin D_2 and vitamin D_3 are equally as active.

The introduction of two atoms of hydrogen into the opened ring B of vitamin D_2 (to give AT_{10}) leads to greatly enhanced activity (for Ca transport) in the chick. It is possible that a change in this part of the molecule is the kind of change required to produce the active form. Raoul (25) has prepared compounds from both vitamin D_2 and D_3 which appear to be diols with OH groups in positions 19 and 7 of the opened ring B. These diols form lipid-soluble Ca complexes that were reported to be antirachitic in the chick. Kodicek and Ashby (26) were unable to detect the presence of ketone 250 in biologically active fractions from rat tissues and further, the existence of the proposed ketone precursor that Raoul prepared has been challenged (27). Nevertheless, the possibility of a change in the vitamin D molecule to an active form of the type proposed by Raoul presents an attractive theory, since there are opportunities of oxidation and reduction or keto-enol transformation that would permit the carrier to release its calcium, e.g., in the manner required in the theory of cation transport put forward by Robertson (28). A proposed site for the formation of such a carrier will be considered in a further paper.

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GERMAN TRANSLATION

EFFECT OF IRRADIATED ERGOSTEROL AND RELATED PRODUCTS ON EPITHELIAL TISSUE

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Source: Zeitschrift fur Krebsforschung 45 (4):335-346, 1937.

In a series of tests carried out by Sander in 1932 at the Pathological Institute of Tubingen University, epithelial proliferations on the rabbit ear were induced under the effect of irradiated ergosterol (Merck). These tests were supplemented by an observation reported by Nordmann and Horger at the same institute, according to which the growth of tissues is accelerated under the action of Vigantol in the tissue culture. Control tests performed at that time with nonirradiated ergosterol gave negative results. In various other studies, the promoting effect of vitamin D on epithelial growth was similarly established (Kemmler, Windaus, Genkin, Dessauer, Seyderhelm), and the use of vitamin-containing ointments to effect a more rapid healing of wounds (Lohr) is based on these experiments. In 1933, a study by Matsudaira was published, which described the action mechanism of vitamins A to D on the basis of animal tests. Matsudaira found that the growth of tumors (adenocarcinomas) can be affected by administration of various amounts of vitamins. These tumors were induced by inserting small stones into the gall bladder of guinea pigs. The typical structure of adenocarcinomas reported by Matsudaira, however, cannot be definitely characterized as such on the basis of the figures included in his study.

The observations reported by Askanazy and Bellow that malignant tumors can be induced by tomato juice were also at first frequently attributed to a vitamin action. Later, saprophytes (Subtilis group) were made responsible for this fact, and the above tests could also not be confirmed by all other authors. Caspari and Plonskier had also obtained true tumors. In a series of tests with 100 animals fed almost exclusively tomato juice, I was unable to note any kind of pathological findings whatsoever. In a series of 100 rats which were given an intraperitoneal injection of juice from tomatoes that had been in part exposed to intense sunlight and in part subjected to artificial irradiation, we never found anything more than a few occasional absorption granulomas. A malignant transformation of such a granuloma was never observed.

Following these negative results, I therefore turned again to tests with pure vitamin D and attempted to find another, perhaps more suitable, test object than the rabbit ear (Sander). On the advice of Prof. Dietrich, I selected the gall bladder of the guinea pig. The above-mentioned study of Matsudaira, which describes the same test object, was unknown to us at that time and had not yet been published.

Our tests were performed with ergosterol (Merck), both in irradiated and nonirradiated form, and in addition AT 10 was used, and also pure linseed oil as a control.

A. Method

We used a Vitamin D solution, which was prepared as follows, according to the directives of Haffner. To obtain a 0.2% stock solution, 0.1g highly

pure (purissimum) ergosterol (Merck) was dissolved in a quartz flask in 50 cc absolute alcohol; the flask was closed and sealed with paraffin after passing CO_2 through the solution. The dissolved ergosterol was irradiated for 2 1/2 hours with a quartz lamp at a distance of 15 cm and under repeated rotation of the flask. The contents of the f k, following irradiation, were transferred into a distillation flask, and the alcohol evaporated under slow heating, whereby the temperature did not exceed 40°C. Yellowish crystals formed, which were dissolved in 5 cc pure ether, and the ether was evaporated in a desiccator. The crystals were collected, weighed, and dissolved in a corresponding amount of sterile linseed oil. The solution obtained in this manner has a limited stability, and was therefore freshly prepared by us each time after 14 days, and, in addition, was stored in a dark cabinet.

B. Animal Tests

The test animals were always adult guinea pigs of both sexes. After checking the weight of the animals, by means of a curved incision under ether narcosis the gall bladder was pushed into the opened epigastric region by exerting pressure on the thorax. After clamping the tip of the gall bladder, the ductus cysticus was tied up (ligated), the bile was tapped, and, after replacing the syringe, the same amount of vitamin solution was injected. After sealing the puncture point and washing the operation site with Tyrode's solution, a suture was applied. The operation was mostly well tolerated by the animals.

A total of 6 test series was carried out.

Series I. Injection of pure linseed oil.

Series II. Injection of nonirradiated 0.2% ergosterol.

Series III. Injection of irradiated 0.2% ergosterol, also dissolved in pure linseed oil.

Series IV. Control animals under similar external conditions.

Series V. Injection of AT 10 (Merck) into guinea pigs.

Series VI. Injection of AT 10 (Merck) into dogs.

In order to check the absolute reliability of the performed ligature of the ductus cysticus and thus be sure that the injected product exerts its effect over a long period of time, the gall bladder of one test animal was filled with Thorotrast (a contrast agent). At three-week intervals, we determined, by means of X-ray photographs, how long the contents of the gall bladder could still be detected. An autopsy of the animals performed after 14 months, showed that small amounts of Thorotrast were still present in the gall bladder, and the preparation could be continuously identified in X-ray photographs. It is far from our mind to compare in anyway this absorption with that of ergosterol, but this absorption constitutes a proof of the fact that the changes in the gall bladder described further in this article should not be considered as being due solely to an operational or even a chemical stimulus. Histologically, the gall bladder filled with Thorotrast did not differ in any way from the picture of a normal gall bladder described below.

The following is a description of the way tests were carried out and of the general results of some tests, the details of which are further discussed below on the basis of some selected examples.

Series I. Injection of Sterile Linseed Oil.

A total of eight animals received this injection, and one animal died from wound infection. Autopsy performed after 12 months showed a full gall bladder, with no thickening of its wall. In the gall bladder, small concrements (calculi) were found, in which the injected oil could be detected.

Microscopically, the structure of a normal gall bladder could be detected in all preparations: a single-layer, cuboid epithelium, isolated fold formations (creasing or wrinkling), and epithelium-coated ducts, which often terminated only inside the muscularis. No infiltration of the mucosa of any kind, and no gland-like (adenoid) neoplasms (new growth) were noted. (Fig. 1).

Figure 1

Normal Gall Bladder

Series II. Injection of Nonirradiated Ergosterol (Merck) in Linseed Oil, as a 0.2% Stock Solution.

In this series of tests, a reaction of the epithelium could already be noted in individual gall bladders. As an example, the histological findings in three gall bladders are reported.

Animal #21: Strong fold formation (creasing) of the mucous membrane. Deep in the recesses, the epithelium is preserved, and is cuboid and high, otherwise missing in part or macerated. No infiltration of the mucosa. At one spot, Luschka's crypt, terminating under the muscularis in a gland lobule-like structure.

TABLE 1

(1) Tier	(2) Operiert am	(3) Getötet am	Gewicht in g	(5) Sektionsbefund
18	29. IV. 1933	25. VI. 1933	500/350	a) Gefüllte Gallenblase. Wand unverändert
19	29. IV. 1933	15. V. 1933	600/450	b) Hämorrhag. Bronchopneumonie, Epithel erhalten, o. B.
21	29. IV. 1933	17. V. 1933	650/460	c) Gallenblase normal
21	29. VI. 1933	25. II. 1934	600/600	d) Epithel regelmäßig an einer Stelle ein bis unter die Serosa reichender Luschkascher Gang. Starke Schleimhautproduktion
22	29. VI. 1933	15. VII. 1933	500/400	e) Pneumonie. Gallenblase ohne Wandveränderung
23	29. IV. 1933	29. IV. 1934	500/450	f) Stark gedehnte Gallenblase. In der Wand vereinzelte Drüsenschläuche. Epithel erhalten
24	29. IV. 1933	12. V. 1933	500/400	g) Gallenblase unverändert
24b	11. V. 1933	20. II. 1934	600/350	h) Siehe unten

1--Animal
2--Operated On
3--Killed On
4--Weight in g
5--Autopsy findings
(Under 5--from top to bottom):
a--Gall bladder full, wall unchanged
b-- Hemorrhagic bronchopneumonia, epithelium preserved, No Pathological findings
c--Gall bladder normal
d--Epithelium regular; At one spot, one Luschka's crypt extending all the way under the serosa. strong mucous membrane formation
e--Pneumonia, gall bladder without any changes in the wall
f--Strongly dilated gall bladder. in the wall isolated gland tubes. epithelium preserved
g--Gall bladder unchanged
h--See below

II. February
IV. April
V. May
VI. June
VII. July

Animal #23: Epithelium stratified, well preserved, of cuboid structure. No fold formation (creasing). Isolated epithelium-coated ducts, extending partially into the muscularis. Small foreign body granuloma. Filament residue.

No pathological findings of any kind in the liver and other organs could be observed in any case.

Animal #24b: Gall bladder very strongly enlarged, cystic, containing three chambers (compartments). Microscopically, a very strong thickening of

the wall was noted, and the muscularis was partially replaced by callous connective tissue. The serosa showed a callous thickening, and in one spot deposits of yellowish substances were noted. Around these needle-shaped crystals, foreign body giant cells and foam cells were observed. The epithelium was preserved and showed one Luschka's crypt at one spot, as a depression extending into the muscularis.

Series III. Injection of Irradiated Ergosterol (Merck) in Linseed Oil, as a 0.2% Stock Solution.

The vitamin analysis listed in our record excerpts was carried out by us with arsenic trichloride, according to the directions of the Pharmacological Institute of the University, and in part at the above institute, which we wish to thank at this point for its extensive support of our work. In regard to this series of tests, we must state that here also no other organ findings of any kind could be observed. As the weight figures reported for Series II and III show, the weight was always different. At the beginning, we were able to note a definite weight loss in animals of Series III. In Series I, the weight figures remained unchanged and were, therefore, omitted.

A few additional explanations can be provided in connection with the figures (illustrations) given in this article.

Animal #13: Completely normal organ findings were obtained on autopsy of this animal, which received an injection of a 0.2% ergosterol solution. Only the gall bladder was enlarged up to the size of a cherry. The contents gave a negative vitamin analysis.

Histological picture: the epithelium of the gall bladder is partly missing and partly consists of granulation tissue. Fold formation (creasing) is noted in the major part of the circumference. Glandular proliferations, similar to Luschka's crypts, are present in exceptionally large numbers.

TABLE 2

(1) Tier	(2) Operiert am	(3) Getötet am	(4) Gewicht in g	(5) Sektionsbefund
10	24.(IV) 1933	7.(VI.) 1933 June	720/430 a)	Empyem der Gallenblase und angrenzenden Umgebung
11	24.(IV) 1933	20.(II.) 1934 Feb.	700/800 b)	Gallenblase groß, Inhalt schaliges Konkrement. Wand verdickt. Mikro: Granulome, Drüsenknospen, Drüsenwucherungen, Vitaminnachweis negativ
12	24.(IV) 1933	26.(IV.) 1934	unverändert c)	Keinerlei Reaktion
13	25.(IV) 1933	20.(II.) 1934	660/900 d)	Gallenblase zeigt baldige Cysten, in der Wand Wucherungen und Drüsenneubildungen, Pigmentzellen von wabiger Struktur, Gallengangsprosoma. Vitaminnachweis negativ
15	25.(IV) 1933	18.(II.) 1934	630/900 e)	Cystenbildung der Gallenblase. Die Wand zeigt lebhafte Drüsenbildung, teilweise durch die Muscularis gehend. Vitaminnachweis negativ
16	25.(IV) 1933	27.(V.) 1933 May	600/420 f)	Wand reaktionslos. Peritonitis als Todesursache. Vitaminnachweis negativ—positiv
25	9.(VI.) 1933	20.(II.) 1934 Feb.	590/900 g)	Laschkasche Gänge, bis weit unter die Muscularis reichend. Vitaminnachweis negativ
26	9.(VI.) 1933 June	10.(VII.) 1933 July	450/330 h)	Peritonitis. Wand [illegible]. Vitaminnachweis positiv
51	28.(II.) 1934 Feb.	15.(VI.) 1934 June	600/650 i)	Hohes Epithel, keine Falten, einzelne Epithelcysten und mit Epithel ausgekleidete Räume. Fadengranulom. Vitaminnachweis negativ
53	28.(III.) 1934 Mar.	16.(VI.) 1934	700/620 j)	Gallenblase klein, zusammengefallen. Epithel glatt, ohne Falten. Breiter infiltrativer Saum. Reichliche Drüsenneubildung, teilweise über die Wand hinausgehend. Vitaminnachweis negativ

1--Animal
2--Operated on
3--Killed on
4--Weight in g
5--Autopsy findings
(Under 5--from top to bottom):
a--Empyema of gall bladder and adjacent surrounding area
b--Large gall bladder, containing scaly concrements, with thickened wall. Microscopically, granulomas and gland buds are noted, as well as gland proliferations. Negative vitamin analysis
c--No reaction of any kind
d--Gall bladder shows sinuous cysts, proliferations in the wall and glandular new growth (neoplasms). Pigment cells have a honeycombed (alveolar) structure. Bile duct proliferations. Vitamin analysis: negative
e--Cyst formation in the gall bladder. The wall shows extensive gland formation, partially extending through the muscularis. Vitamin analysis: negative

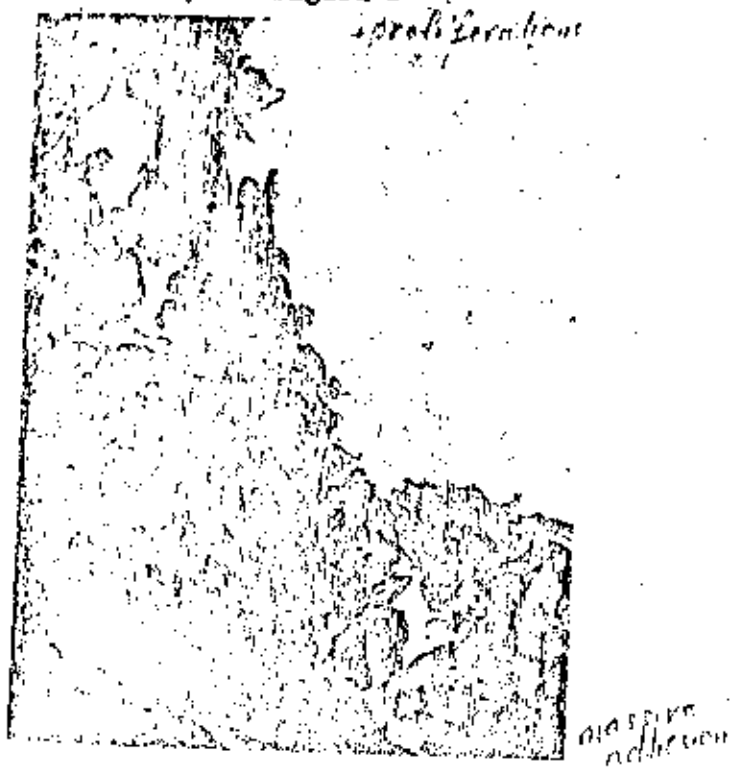
f--No reaction in the wall. Peritonitis as the cause of death. Vitamin analysis: negative-positive
g--Luschka's crypts, extending far under the muscularis. Vitamin analysis: negative
h--Peritonitis, no pathological findings in the wall. Vitamin analysis: positive
i--High epithelium, no folds, isolated epithelial cysts and epithelium-coated spaces. Fibrous granuloma. Vitamin analysis: negative
j--Small, collapsed gall bladder. Epithelium smooth, without folds. Wide infiltrative edge. Extensive glandular new growth (neoplasms), stretching in part over (across) the wall. Vitamin analysis: negative

This gland and cyst formation is particularly extensive inside the large granulation area (section). Enclosed in the granulation tissue are isolated filament residues with giant cells and fat absorption cells. In addition to these large glands and cysts, small buds are present in large numbers. However, a typical epithelium can be recognized everywhere.

Animal #15: This animal, similarly injected with irradiated ergosterol, exhibits the same course of recovery after the operation. On autopsy after nine months, individual massive adhesions of the liver and peritoneum can be detected. The gall bladder is large, embedded in the surrounding tissue, and its contents are crumbly. Vitamin analysis, performed on the contents with arsenic trichloride, is also negative here. Macroscopically, the wall of the gall bladder is thickened, but otherwise organ findings are normal. (Fig. 2).

Histological picture: the epithelium is partly well-preserved, but a strong fold formation (creasing) is noted. The mucosa is infiltrated with exudate cells of all kinds of the absorption giant cells. Epithelial proliferations, extending all the way up the subserosa and in part dilated (expanded) in a cyst-like fashion, are noted in the entire circumference. An acinous arrangement (grouping is found in part, and the subserosa also appears thickened and infiltrated.

Figure 2



Injection of Irradiated Ergosterol (Series III, Animal #15)

Animal #53: This animal belongs to an intermediate series, in which the concentration of the ergosterol solution was reduced to 0.1%. As can be seen from the record excerpt given above, the observation time extended only over a period of 3 1/2 months. On autopsy, normal organ findings were obtained. The gall bladder was of medium size, and its wall was thickened.

Histological picture: smooth epithelium, without fold formation (creasing), a large number of gland formations, which often extend far beyond the wall. (Fig. 3).

Series IV. Control Animals Under Identical External Conditions.

The animals of this series showed a completely normal behavior. Normal organ findings were obtained on autopsy after six and nine months. The gall bladder was unchanged, and its walls exhibited a normal histological picture.

FIGURE 3

Injection of Irradiated Ergosterol (series III, Animal #54)

The test results thus obtained suggested an expansion and continuation of these tests. In the meantime, the calcinose factor had been isolated from irradiated ergosterol by Holtz and prepared under the name AT 10.

In order to check whether the changes observed were caused by sterols. I wanted to continue these tests with a sterol derivative at the

Heidelberg Surgical University Clinic. Therefore, AT 10 (Merck) was used as the first derivative.

Series V. Injection of AT 10 Into Guinea Pigs.

In several series of tests, the gall bladder was exposed anf filled with AT 10 as described above. After the operation, the behavior of the animals was normal, and weight variations were noted in some animals. Some of the animals treated in this series were killed, and others died from intercurrent diseases. A total of 28 animals was tested, and 12 of these are still under observation at the present time. Briefly, some of the findings obtained in this series are reported.

Animal #85: Operated on 25 October, 1936, killed on 5 January, 1937. The gall bladder had a normal size, and no pathological organ findings were noted macroscopically.

Histologically, a fiber granuloma with large numbers of absorption cells, strongly proliferated Luschka's crypts in the wall of the gall bladder, and strong-fold formation (creasing) were noted. (Fig. 4).

Animal #88: Operated on 22 October, 1936, killed on 5 December, 1936. Slight weight loss, gall bladder enlarged and cystically altered. Histologically, large numbers of Luschka's crypts, giant cells, and strong-fold formation (creasing) were noted. The walls of the gall bladder were thickened.

FIGURE 4

Injection of AT 10 (Series V, Animal #85)

Animal #89: Operated on 9 July, killed on 31 October, 1936. No weight loss. On autopsy, the gall bladder was altered (enlarged) up to the size of a chicken egg. No pathological findings in the life ducts. On opening the gall bladder, a white-yellowish liquid (dropsy) was noted. The wall of the cysts consists of hard, fibrous connective tissue with diffused lymphocytic and leucocytic infiltration. In those sections where the cyst sac is adjacent to the liver, the latter is connected to the hepatic tissue by means of a hard, fibrous connective tissue. The epithelium of the gall bladder is well preserved in some spots. Large numbers of tubular structures are noted, which are coated with a high cylindrical epithelium. Large numbers of glandular ducts are observed in the innermost wall layers. Bundles of smooth muscles are noted, separated from each other by narrow connective tissue bands.

Animal #90: Operated on 22 October 1936, died from peritonitis (on 1 November 1936). Weight loss, bilateral pneumonia, gall bladder enlarged to the size of a cherry. The walls are thickened, and the contents of the gall bladder are absorbed (Figs. 5 and 6). Histologically, large numbers of Luschka's crypts, mitoses, and epithelial new growth (neoplasms) are noted. In some spots, the Luschka's crypts exhibit an adenoid proliferation, but no infiltrative growth.

FIGURE 5

Injection of AT 10 (Series V, Animal #90)

Similar changes were found in other animals subjected to a post-mortem examination. The average observation time was 3-4 months. The remaining animals will be examined after a longer observation time.

FIGURE 6

Injection of AT 10 (Series V, Animal #90)

Series VI. Injection of AT 10 Into Dogs.

In several dogs, the gall bladder was exposed by means of a pararectal incision of the costal arch and, after puncturing, 3 cc of AT 10 was injected. The observed blood calcium content (cf; in this connection, the studies of Holtz) had previously been determined. The animals are still being tested. A weight loss or a change in the general condition of the animals has not been noted so far. In multiple blood investigations, the blood calcium content was found to be normal so far.

C. Results

Sterile linseed oil does not cause any wall thickening or new growth (neoplasms) in the gall bladder of the guinea pig. The weight of the animals remains unchanged.

A slight reaction of the wall can be detected in some of the gall bladders tested under the effect of nonirradiated ergosterol in a 0.2% linseed oil

solution. Histologically, a partial fold formation (creasing) can be noted, and isolated epithelium-coated ducts can be detected. The weight of the animals shows a variable behavior.

Irradiated ergosterol in a 0.2% linseed oil solution causes a partially very strong fold formation (creasing) of the mucosa. Strong glandular proliferations, similar to Luschka's crypts are observed; in addition, the histological picture reveals the presence of foreign body giant cells and fat absorption cells. A weaker reaction is noted when the concentration of the solution is reduced to 0.1%. Vitamin analysis with arsenic trichloride, performed on the contents of the gall bladder during autopsy, gave negative results. In many animals, a weight loss was noted at first, but later the weight showed a variable behavior.

The tests carried out with AT 10 resulted in changes of the gall bladder in the sense of a partial cystic transformation of the bladder, and in part in the formation of wall thickenings with large numbers of epithelium-coated ducts, adenoma-like proliferations, foreign body giant cells, and mitoses. Infiltrative growth or metastase formation were not observed in any case. In dogs, no increase in the blood calcium content was noted after injection of AT 10 into the gall bladder. The observation time of animals treated with AT 10 was shorter than that of test animals treated with ergosterol. The tests are still in progress.

Since experience shows that foreign bodies exert a greater irritating effect on the tissue in guinea pigs than in other animals, I have also started carrying out tests on other test animals In a further series of tests, other sterol derivatives will be injected into the gall bladder and other hollow organs.This will make it possible to determine whether the walls undergo still more severe changes and thus allow a better evaluation of the results obtained so far.

Summary

Tests with irradiated ergosterol and AT 10 resulted in changes of the gall bladder wall, which were histologically identified as adenoidal proliferations. Control tests with nonirradiated ergosterol and pure linseed oil resulted in slight or no changes in the mucosa. No signs of malignant growth or metastase formation could be observed.

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for further literature, cf. ...

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(Aus dem Pathologischen Institut der Universität Tübingen [Direktor: Professor Dr. *Dietrich*] und der Chirurgischen Universitätsklinik Heidelberg [Direktor: Professor Dr. *Kirschner*].)

Die Wirkung bestrahlten Ergosterins und verwandter Stoffe auf Epithelgewebe.

Von

H. O. Schmid.

Mit 6 Textabbildungen.

(*Eingegangen am 9. Februar 1937.*)

In einer am Pathologischen Institut der Universität Tübingen 1932 von *Sander* durchgeführten Versuchsreihe war es durch Einwirkung von bestrahltem Ergosterin (Merck) gelungen, Epithelwucherungen am Kaninchenohr zu erzeugen. Diese Versuche wurden ergänzt durch eine aus dem gleichen Institut von *Nordmann* und *Hörger* mitgeteilte Beobachtung, nach der das Wachstum von Geweben durch Einwirkung von Vigantol in der Gewebskultur beschleunigt wurde. Die damals angestellten Kontrollen mit unbestrahltem Ergosterin waren negativ. Verschiedene andere Arbeiten haben in gleicher Weise die fördernde Wirkung des Vitamin D auf Epithelwachstum festgestellt (*Kemmler, Windaus, Genkin, Dessauer, Seyderhelm*) und die Verwendung vitaminhaltiger Salben zur rascheren Wundheilung (*Löhr*) geht auf diese Erfahrungen zurück. 1933 erschien eine Arbeit von *Matsudaira*, die sich mit der Wirkungsweise der Vitamine A bis D auf Grund von Tierversuchen befaßt. *Matsudaira* stellte hierin fest, daß Tumoren (Adenocarcinome) durch wechselnde Zufuhr von Vitaminen in ihrem Wachstum beeinflußt werden konnten. Er hatte diese Tumoren durch die Verbringung von Steinchen in die Gallenblase von Meerschweinchen erzeugt. Die von ihm als typisch abgebildeten Adenocarcinome sind jedoch auf Grund der von ihm beigegebenen Abbildungen nicht eindeutig als solche zu erkennen.

Auch die von *Askanazy* und *Bellow* mitgeteilten Beobachtungen, daß durch Tomatensaft maligne Tumoren erzeugt werden konnten, wurden anfangs vielfach auf eine Vitaminwirkung zurückgeführt. Später wurden Saprophyten (Subtilisgruppe) hierfür verantwortlich gemacht, auch wurden die Versuche nicht von allen bestätigt. *Caspari* und *Plonsdier* hatten gleichfalls echte Tumoren erhalten. Ich habe in einer Versuchsreihe von 100 Tieren, die fast ausschließlich von Tomatensaft ernährt wurden, keinerlei pathologischen Befund erheben können. In einer Serie von 100 Ratten, die mit Saft von teilweise vermehrtem Sonnenlicht ausgesetzten, teilweise künstlich bestrahlten Tomaten

intraperitoneal injiziert wurden, fanden wir nie mehr als gelegentlich Resorptionsgranulome. Nie wurde eine maligne Umwandlung eines derartigen Granuloms beobachtet.

Ich wandte mich daher nach diesen negativen Ergebnissen wieder den Versuchen mit reinem Vitamin D zu und suchte ein anderes, vielleicht geeigneteres Versuchsfeld als das Kaninchenohr (*Sander*) zu finden. Auf Rat von Prof. *Dietrich* wählte ich die Gallenblase des Meerschweinchens. Die von *Matsudaira* erwähnte Arbeit, die das gleiche Versuchsfeld beschreibt, war uns damals noch nicht bekannt und auch noch nicht erschienen.

Es wurde in den Versuchen mit Ergosterin (*Merck*) in bestrahlter und unbestrahlter Form gearbeitet, außerdem wurde AT 10 und zur Kontrolle reines Leinöl verwendet.

A. *Methodik.*

Wir verwendeten eine D-Vitaminlösung, die nach den Angaben von *Haffner* in folgender Weise hergestellt war: Zur Gewinnung der 0,2proz. Stammlösung werden 0,1 g Ergosterin. purissimum (Merck) im Quarzkolben in 50 ccm absolutem Alkohol gelöst, der Kolben verschlossen und nach Durchleitung von CO_2 paraffiniert. Das gelöste Ergosterin haben wir mit der Quarzlampe 2,5 Stunden in einer Entfernung von 15 cm bei mehrmaligem Drehen des Kolbens bestrahlt. Der Inhalt wird nach der Bestrahlung in einen Siedekolben verbracht, der Alkohol unter langsamem Erhitzen verdampft, wobei 40° nicht überschritten wurden. Es bilden sich gelbliche Krystalle, die in 5 ccm reinem Äther gelöst werden; der Äther wird im Exsiccator verdampft. Die Krystalle werden ausgekratzt, gewogen und in der entsprechenden Menge sterilem Leinöl gelöst. Die durch diesen Prozeß gewonnene Lösung ist von begrenzter Haltbarkeit und wurde daher von uns jeweils nach 14 Tagen frisch bereitet, außerdem im Dunkelschrank aufbewahrt.

B. *Tierversuche.*

Die Versuchstiere waren stets ausgewachsene Meerschweinchen beiderlei Geschlechtes. Nach Gewichtskontrolle wird in Äthernarkose durch bogenförmigen Schnitt die Gallenblase unter Druck auf den Thorax in das eröffnete Oberbauchgebiet gedrängt. Nach Erfassung der Spitze mit der Klemme wird der Ductus cysticus unterbunden, die Galle abpunktiert und unter Auswechslung der Spritze die gleiche Menge der Lösung injiziert. Nach Abdichten der Einstichstelle und Thyrodespülung des Operationsgebietes Naht. Die Operation wurde von den Tieren zumeist gut vertragen.

Es wurden insgesamt 6 Versuchsserien aufgestellt:

Serie I. Injektion mit reinem Leinöl.
Serie II. Injektion mit unbestrahltem, 0,2proz. Ergosterin.
Serie III. Injektion mit bestrahltem, 0,2proz. Ergosterin, gleichfalls in reinem Leinöl gelöst.
Serie IV. Kontrolltiere unter gleichen äußeren Bedingungen.
Serie V. Injektion von AT 10 Merck am Meerschweinchen.
Serie VI. Injektion von AT 10 Merck am Hund.

Um eine Kontrolle über die unbedingte Zuverlässigkeit der erfolgten Unterbindung des Ductus cysticus und damit die Gewähr einer über längere Zeit verteilten Wirkung des injizierten Stoffes zu haben, füllten wir bei einem Versuchs-

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tier die Gallenblase mit *Thorotrast*. Wir stellten jeweils in Abständen von 3 Wochen durch Röntgenaufnahme fest, wie lange der Inhalt nachweisbar war. Die nach 14 Monaten vorgenommene Sektion ergab noch geringe Mengen von Thorotrast in der Gallenblase, das Präparat war dauernd röntgenologisch nachweisbar gewesen. Es liegt uns fern, diese Resorption mit der des Ergosterins irgendwie vergleichen zu wollen, aber sie ist ein Beweis dafür, daß die unten beschriebenen Veränderungen der Gallenblase nicht etwa als rein durch den operativen oder auch chemischen Reiz entstanden anzusehen sind. Die mit Thorotrast gefüllte Gallenblase unterschied sich histologisch in nichts von dem Bild der noch zu beschreibenden normalen Gallenblase.

Abb. 1. Normale Gallenblase.

Wir geben im folgenden die Anordnung mit den groben Resultaten einiger Versuche, deren Einzelheiten an Hand ausgesuchter Beispiele unten noch besprochen werden.

Serie I: Injektion mit sterilem Leinöl.

Es wurden 8 Tiere injiziert, von denen eines an einer Wundinfektion starb. Bei der nach 12 Monaten vorgenommenen Sektion fand sich die Gallenblase gefüllt, keine Verdickung der Wand. In der Gallenblase feine Konkremente, in denen sich das injizierte Öl nachweisen ließ.

Mikroskopisch ist in allen Präparaten der Bau der normalen Gallenblase erkennbar: Ein einschichtiges kubisches Epithel, vereinzelte Faltenbildungen und mit Epithel ausgekleidete Gänge, die oft erst innerhalb der Muscularis endigen. Keinerlei Infiltration der Schleimhaut. Keine drüsenartigen Neubildungen (Abb. 1).

Serie II: Injektion mit Leinöl, *unbestrahltem Ergosterin (Merck)*, als 0,2proz. Stammlösung.

Tier	Operiert am	Getötet am	Gewichtsabnahme	Sektionsbefund
18	29. IV. 1933	25. VI. 1933	700/950	Gefüllte Gallenblase, Wand unverändert
19	29. IV. 1933	15. V. 1933	600/450	Hämorrhag. Bronchopneumonie, Epithel erhalten, o. B.
20	29. IV. 1933	17. V. 1933	650/400	Gallenblase normal
21	29. VI. 1933	25. II. 1934	600/900	Epithel regelmäßig an einer Stelle ein bis unter die Serosa reichender *Luschka*scher Gang. Starke Schleimhautproduktion
22	29. VI. 1933	15. VII. 1933	500/400	Pneumonie, Gallenblase ohne Wandveränderung
23	29. IV. 1933	29. IV. 1934	500/450	Stark gedehnte Gallenblase. In der Wand vereinzelte Drüsenschläuche. Epithel erhalten
24	29. IV. 1933	12. V. 1933	500/400	Gallenblase unverändert
24b	11. V. 1933	20. II. 1934	500/350	Siehe unten

Es ließ sich bei dieser Versuchsserie bereits bei einzelnen Gallenblasen eine Reaktion des Epithels feststellen. Als Beispiel sei kurz der histologische Befund dreier Gallenblasen angegeben.

Tier 21: Starke Faltenbildung der Schleimhaut. In der Tiefe der Nischen ist das Epithel erhalten, kubisch, hoch. Sonst teilweise fehlend oder maceriert. Keine Infiltrationen der Schleimhaut. An einer Stelle ein *Luschka*scher Gang, der unterhalb der Muscularis in einem drüsenläppchenähnlichen Gebilde endet.

Tier 23: Epithel eingeschichtet, gut erhalten, von kubischem Bau. Keine Faltenbildung. Vereinzelt mit Epithel ausgekleidete Gänge, teilweise in die Muscularis reichend. Kleines Fremdkörpergranulom. Fadenrest.

Irgendein pathologischer Befund an der Leber oder sonstigen Organen konnte in keinem Fall festgestellt werden.

Tier 24b. Gallenblase sehr stark vergrößert, cystisch, dreikammerig.

Mikroskopisch: Wand stark verdickt, Muscularis teilweise durch schwieliges Bindegewebe ersetzt. Serosa schwielig verdickt. In ihr an einer Stelle Ablagerungen gelblicher Substanzen. Um diese nadelförmigen Krystalle Fremdkörperriesenzellen und Schaumzellen. Das Epithel ist erhalten und zeigt an einer Stelle einen *Luschka*schen Gang, als Einsenkung bis in die Muscularis.

Serie III: Injektion mit Leinöl, *bestrahltem Ergosterin (Merck)*, als 0,2proz. Stammlösung (vgl. Tabelle auf Seite 339).

Der in den Protokollauszügen angegebene Vitaminnachweis wurde nach den Angaben des Pharmakologischen Instituts der Universität mit Arsentrichlorid von uns, teilweise auch in dem genannten Institut, dem wir an dieser Stelle für seine mannigfache Unterstützung danken, ausgeführt. Zu dieser Versuchsserie ist zu bemerken: Auch hier konnte irgendein anderer Organbefund nicht erhoben werden. Wie die bei

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Tier	Operiert am	Getötet am	Gewicht in g	Sektionsbefund
10	24. IV. 1933	7. VI. 1933	720/430	Empyem der Gallenblase und umgrenzenden Umgebung
11	21. IV. 1933	20. II. 1934	700/860	Gallenblase groß. Inhalt schaliges Konkrement. Wand verdickt. Mikr.: Granulome. Drüsenkomplex, Drüsenwucherungen. Vitaminnachweis negativ
12	21. IV. 1933	26. IV. 1934	unverand.	Keinerlei Reaktion
13	25. IV. 1933	20. II. 1934	660/900	Gallenblase zeigt buchtige Cysten, in der Wand Wucherungen und Drüsenneubildungen. Eigenartige Zellen von wabiger Struktur, Gallengangsprossen. Vitaminnachweis negativ
15	25. IV. 1933	18. II. 1934	630/900	Cystenbildung der Gallenblase. Die Wand zeigt lebhafte Drüsenbildung, teilweise durch die Muscularis gehend. Vitaminnachweis negativ
16	25. IV. 1933	27. V. 1933	600/420	Wand reaktionslos. Peritonitis als Todesursache. Vitaminnachweis negativ—positiv
25	9. VI. 1933	20. II. 1934	590/900	*Luschka*sche Gänge, bis weit unter die Muscularis reichend. Vitaminnachweis negativ
26	9. VI. 1933	10. VII. 1933	430/330	Peritonitis. Wand o. B. Vitaminnachweis positiv
51	28. II. 1934	15. VI. 1934	600/650	Hohes Epithel, keine Falten, einzelne Epithelcysten und mit Epithel ausgekleidete Räume. Fadengranulom. Vitaminnachweis negativ
53	28. III. 1934	15. VI. 1934	700/520	Gallenblase klein, zusammengefallen. Epithel glatt, ohne Falten. Breiter infiltrativer Saum. Reichliche Drüsenneubildung, teilweise über die Wand hinausgehend. Vitaminnachweis negativ

Serie II und III angegebenen Gewichtsziffern beweisen, war das Gewicht stets verschieden. Im Anfang konnten wir jeweils bei Serie III eine deutliche Abnahme beobachten. Bei Serie I waren die Gewichtsziffern nicht verändert und wurden daher weggelassen.

Zu den nebenstehenden Abbildungen seien noch einige Erläuterungen gegeben:

Tier 13: Das mit der 0,2proz. Ergosterinlösung injizierte Tier bietet bei der Sektion einen völlig normalen Organbefund. Nur die

Gallenblase ist bis auf Kirschgröße erweitert. Der Inhalt gibt einen negativen Vitaminnachweis.

Histologisches Bild: Epithel der Gallenblase zum Teil fehlend, zum Teil Granulationsgewebe. Im größten Teil des Umfanges Faltenbildung. Drüsige Aussprossungen nach Art der *Luschka*schen Gänge in außerordentlich großer Zahl. Besonders ausgedehnt ist diese Drüsen- und Cystenbildung in den großen Granulationsbezirk hinein. Eingeschlossen

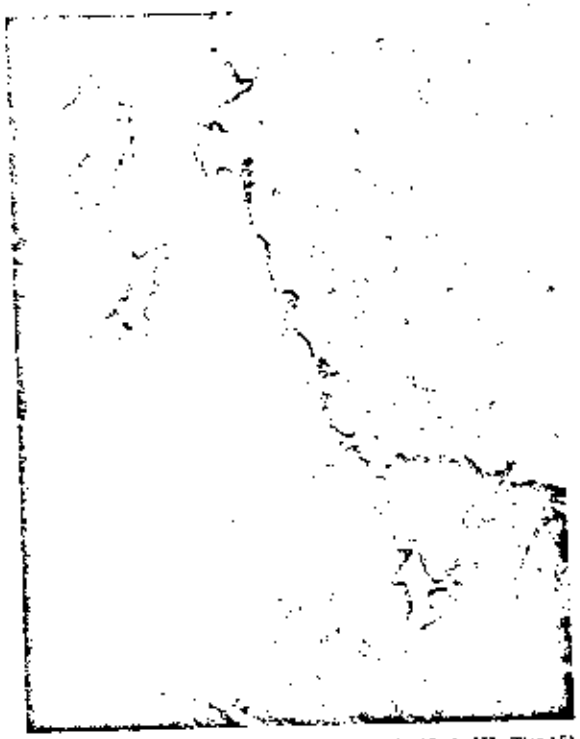

Abb. 2. Injektion mit bestrahltem Ergosterin (Serie III, Tier 15).

in Granulationsgewebe finden sich einzelne Fadenreste mit Riesenzellen und Fettresorptionszellen. Außer diesen großen Drüsen und Cysten vielerlei kleine Knospen. Das Epithel ist jedoch überall als typisch erkennbar.

Tier 15: Das gleichfalls mit bestrahltem Ergosterin injizierte Tier bietet denselben Heilungsverlauf nach der Operation. Die Sektion nach 9 Monaten [illegible] einzelne flächenhafte Verwachsungen von Leber und Bauchfell erkennen. Die Gallenblase ist groß, in umgebendes Gewebe eingebettet, der Inhalt krümelig. Der damit angestellte Vitaminnachweis [illegible] ist auch hier negativ. Makroskopisch ist die [illegible] verdickt, sonst normaler Organbefund (Abb. 2).

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Histologisches Bild: Epithel teilweise gut erhalten. Starke Faltenbildung. Die Mucosa ist infiltriert mit Exsudatzellen aller Art und Resorptionsriesenzellen. Im ganzen Umfange Epithelsprossen, die bis in die Subserosa reichen und zum Teil cystisch erweitert sind. Teilweise findet sich acinöse Anordnung, auch die Subserosa erscheint verdickt und infiltriert.

Abb. 3. Injektion von bestrahltem Ergosterin (Serie III, Tier 53).

Tier 53: Dieses Tier gehört einer Zwischenserie an, bei der wir die Konzentration auf 0,1% herabgesetzt haben. Wie aus dem oben gegebenen Protokollauszug ersichtlich ist, lief die Beobachtungszeit nur $3^1/_2$ Monate. Die Sektion ergab normalen Organbefund. Die Gallenblase zeigte mittlere Größe. Die Wand war verdickt.

Histologisches Bild: Epithel glatt, ohne Faltenbildung, reichliche Drüsenbildungen, die oft weit über die Wand hinausgehen (Abb. 3).

Serie IV: Kontrolltiere unter gleichen äußeren Bedingungen.

Die Tiere dieser Serie verhielten sich völlig normal. Bei der nach 6 und 9 Monaten vorgenommenen Sektion zeigte sich ein normaler

Organbefund. Die Gallenblase war nicht verändert. Die Wandung zeigte das normale histologische Bild.

Die so gewonnenen Resultate der Versuche legten eine Erweiterung und Fortsetzung nahe. Inzwischen war von *Holtz* aus dem bestrahlten Ergosterin der Calcinusefektor isoliert worden und wurde unter dem Namen AT 10 hergestellt.

Abb. 4. Injektion von AT 10 (Serie V, Tier 85).

Ich wollte daher zur Prüfung der Frage, ob die beobachteten Veränderungen durch Sterine hervorgerufen werden, die Versuche an der Chirurgischen Universitäts-Klinik Heidelberg mit einem Sterinderivat fortsetzen. Es wurde daher als erstes Derivat das AT 10 *Merck* verwendet.

Serie V: Injektion von AT 10 am Meerschweinchen.

Es wurde in mehreren Versuchsreihen in der oben beschriebenen Weise die Gallenblase freigelegt und mit AT 10 gefüllt. Die Tiere zeigten nach der Operation normales Verhalten. Bei einzelnen Tieren waren Schwankungen des Gewichts vorhanden. Von den in diesen Reihen

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behandelten Tieren haben wir einige getötet, andere sind an interkurrenten Krankheiten gestorben. Im Versuch waren insgesamt 28 Tiere, von denen 12 heute noch unter Beobachtung stehen.

Kurz seien einige Befunde wiedergegeben:

Tier 85: Operiert am 25. X. 1936, getötet 5. I. 1937. Gallenblase von normaler Größe. Makroskopisch kein krankhafter Organbefund.

Histologisch: Fadengranulom mit reichlich Resorptionszellen. Stark gewucherte *Luschka*sche Gänge in der Wand der Gallenblase. Starke Faltenbildung (Abb. 4).

Tier 88: Operiert am 22. X. 1936, getötet 3. XII. 1936. Leichte Gewichtsabnahme. Die Gallenblase vergrößert, cystisch verändert.

Histologisch: Reichlich *Luschka*sche Gänge. Riesenzellen, starke Faltenbildung. Wandung verdickt.

Tier 89: Operiert am 9. VII., getötet am 31. X. 1936. Keine Gewichtsabnahme. Bei der Sektion war die Gallenblase bis zu Hühnereigröße verändert. Gallengänge o. B. Bei der Eröffnung weißgelbliche Flüssigkeit (Hydrops). Die Wand der Cyste besteht aus derbfaserigem Bindegewebe mit diffuser lympho- und leukocytärer Durchsetzung. Die inneren Schichten zeigen stärkere Infiltration. In den Abschnitten, in denen der Cystensack der Leber anliegt, zeigt sich dieselbe verbunden mit dem Lebergewebe durch derbfaseriges Bindegewebe. Epithel der Gallenblase stellenweise gut erhalten. Reichlich schlauchförmige Gebilde, die mit einem hohen Zylinderepithel ausgekleidet sind. Reichliche Drüsengänge in den innersten Wandschichten. Bündel glatter Muskulatur, die durch schmale Bindegewebszüge voneinander getrennt sind.

Tier 90: Operiert am 22. X. 1936, † an Pneumonie 1. XI. 1936. Gewichtsabnahme. Pneumonie beiderseits. Gallenblase auf Kirschgröße vergrößert. Wandung verdickt. Inhalt resorbiert (Abb. 5 und 6, S. 344).

Histologisch: Reichliche *Luschka*sche Gänge. Mitosen, Epithelneubildungen. Die *Luschka*schen Gänge zeigen stellenweise eine adenoide Wucherung, aber kein infiltratives Wachstum (s. Abb. 6 rechts oben).

Ähnliche Veränderungen fanden sich bei den anderen, inzwischen obduzierten Tieren. Die Beobachtungszeit betrug durchschnittlich 3—4 Monate. Die übrigen Tiere werden nach einer längeren Beobachtungszeit untersucht.

Serie VI: Injektion von AT 10 am Hund.

Bei mehreren Hunden wurde von einem Rippenbogenparamedianschnitt aus die Gallenblase freigelegt und nach Abpunktierung 3 ccm

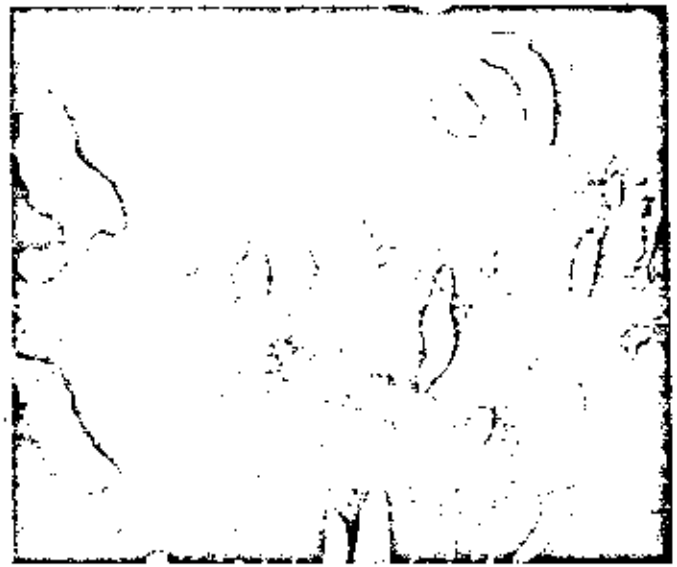

Abb. 5. Injektion von AT 10 (Serie V, Tier 90).

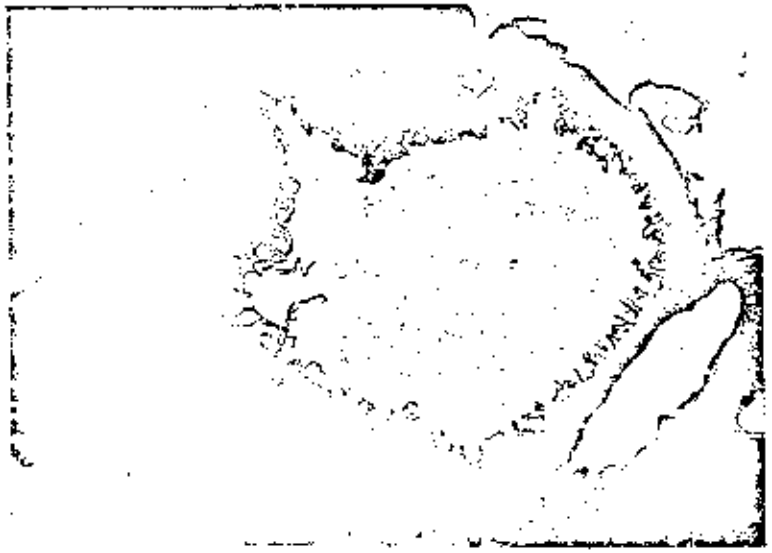

Abb. 6. Injektion von AT 10 (Serie V, Tier 90).

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AT 10 injiziert. Der beobachtete Blutcalciumgehalt (vgl. hierüber die Arbeiten von *Holtz*) war vorher festgestellt worden. Die Tiere stehen noch im Versuch. Eine Gewichtsabnahme oder eine Änderung des Allgemeinbefindens konnte bisher nicht festgestellt werden. Bei mehrfachen Blutuntersuchungen war der Blutcalciumgehalt bisher normal.

C. Ergebnisse.

Steriles Leinöl erweckt in der normalen Gallenblase des Meerschweinchens keine Wandverdickung oder Neubildung. Das Gewicht der Tiere war nicht verändert.

Unbestrahltes Ergosterin in einer 0,2proz. Leinölösung läßt in einzelnen der untersuchten Gallenblasen eine geringe Reaktion der Wand erkennen. Es findet sich histologisch teilweise Faltenbildung, einzelne mit Epithel ausgekleidete Gänge sind erkennbar. Das Gewicht der Tiere verhält sich unterschiedlich.

Bestrahltes Ergosterin in 0,2proz. Ergosterin-Leinölösung bewirkt eine teilweise sehr starke Faltenbildung der Schleimhaut. Es wurden starke drüsige Aussprossungen nach Art der *Luschka*schen Gänge beobachtet, außerdem finden sich im histologischen Bild Fremdkörperriesenzellen und Fettresorptionszellen. Eine Verringerung der Konzentration der Lösung auf 0,1% ließ eine schwächere Reaktion eintreten. Die bei der Sektion angestellte Untersuchung auf Vitamin mit Arsentrichlorid im Gallenblaseninhalt war negativ. Bei vielen Tieren wurde anfangs eine Gewichtsabnahme beobachtet, später verhielt sich das Gewicht unterschiedlich.

Die mit AT 10 angesetzten Versuche ergaben Veränderungen an der Gallenblase im Sinne einer teilweisen cystischen Umwandlung der Gallenblase, teilweise fanden sich Wandverdickungen mit reichlich epithelausgekleideten Gängen, adenomartigen Wucherungen, Fremdkörperriesenzellen und Mitosen. Ein infiltratives Wachstum oder Metastasierung wurde in keinem Falle beobachtet. Beim *Hunde* wurde keine Erhöhung des Blutcalciumgehaltes nach Injektion von AT 10 in die Gallenblase beobachtet. Die Beobachtungszeit der mit AT 10 behandelten Tiere war kürzer als diejenige der mit Ergosterin behandelten Versuchstiere. Die Versuche dauern an.

Da erfahrungsgemäß Fremdkörper beim Meerschweinchen das Gewebe stärker reizen als bei anderen Tieren, habe ich mit Versuchen auch an anderen Versuchstieren begonnen. Auch werden in einer weiteren Versuchsreihe andere Sterinderivate in die Gallenblase und in andere Hohlorgane injiziert werden. Hierdurch wird es gelingen, festzustellen, ob sich die Wände noch schwerer verändern und so die bisherigen Befunde besser zu bewerten.

Zusammenfassung.

Versuche mit bestrahltem Ergosterin und AT 10 ergaben Veränderungen der Gallenblasenwand, die sich histologisch als adenoide Wucherungen erwiesen. Kontrollversuche mit unbestrahltem Ergosterin und reinem Leinöl zeigten geringe oder keine Veränderungen der Schleimhaut. Zeichen von malignem Wachstum oder Metastasierung konnten nicht festgestellt werden.

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COMMENTARY

VITAMIN D DEPENDENCY

A CONDITION still quite unfamiliar to many physicians is discussed in this issue of PEDIATRICS.[1] The disease, called "Vitamin-D-dependent rickets" in the accompanying article, was first segregated from the array of "Vitamin-D resistant rickets" by Fraser and Salter[2] (type III, A, ii, in their classification), and by Prader and colleagues[3] (hereditary pseudo-vitamin-D-deficiency rickets, in their terminology). The condition is characterized by signs and symptoms which appear in the first year of life, and which are easily mistaken for those of severe (Stage III) vitamin D deficiency.[4] As revealed in typical case reports of the condition,[5-7] manifestations include muscular weakness, convulsions, severe rickets, growth failure, hypophosphatemia, low or normal total serum calcium, mild renal tubular acidosis, and hyperaminoaciduria. Vitamin D dependency responds dramatically to vitamin D_2 when given in the appropriate daily dose, which is about 100 times the normal requirement. A persistent need for this large amount of the vitamin has been observed throughout childhood, and it may persist for the patient's life-time; it is this nature which invokes the term "dependency."

The incidence of vitamin D dependency, which is inherited as a simple autosomal recessive trait,[7-9] is higher than once suspected, and two groups working in Toronto and Montreal each now have half a dozen patients under their care.[7] Therefore, it is all the more important that vitamin D dependency be distinguished from advanced vitamin D deficiency, an acquired condition which is still all too common.[4] This can be done most easily when the family history reveals other affected siblings with persistent rickets, and when there is a history of normal vitamin D intake in infancy (i.e., about 400 I.U. daily) prior to the onset of symptoms. In both conditions there is evidence for secondary hyperparathyroidism with attendant changes in bone and renal tubular function,[5-7] which serves to distinguish them from other forms of hypophosphatemic rickets caused by primary disorders of phosphate transport in the renal tubule. What then is common to the deficiency and dependency states, and yet why are they different; and what is the significance of the current paper in PEDIATRICS?

The understanding of these two conditions, and of other forms of vitamin D responsive rickets, lies in new information on the metabolism and action of vitamin D.[4,11] The lipid-soluble substances, known to most physicians as vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) are formed from provitamins by the opening of ring B under the influence of ultraviolet radiation. The vitamin is then bound to α_2 globulin and carried in plasma to the liver where it undergoes hydroxylation, to form the corresponding polar 25-hydroxy derivatives (25 HCC and 25 HCCC), which are the forms of the vitamin destined to carry out its biological

functions. After hepatic synthesis of its active form, the vitamin is again transported in plasma by α_2 globulin, until it is bound to the nuclear membrane of intestinal epithelial cells. Thereafter, by mechanisms still requiring elucidation, it initiates synthesis of a specific messenger RNA, by activating a portion of nuclear DNA (gene or genes). The m-RNA in its turn initiates synthesis of a cytoplasmic protein (or proteins) whose major site of action is in the brush border of intestinal epithelial cells. This protein which has been isolated and some of its properties characterized from chick intestinal mucosa,[12] mediates calcium transport from the brush border to the antiluminal border, and hence to the blood. The vitamin also stimulates a calcium-dependent ATPase in brush border which is important in augmenting initial rates of calcium uptake.[13] The 25-hydroxy derivative of vitamin D has a third important effect. It can act directly on bone in physiological doses to release calcium and phosphate ions;[14] this action is very similar to that of parathyroid hormone and it is inhibited by calcitonin.

It should be remembered when interpreting the clinical manifestations of the deficiency and dependency states, that parathyroid hormone can stimulate intestinal transport of calcium and mobilize calcium from bone only in the presence of vitamin D.[15] On the other hand, vitamin D, in any form, is not necessary for the inhibitory effect of parathyroid hormone on renal tubular conservation of phosphate and amino acids.[16,17] These complicated relationships between vitamin and hormone action on calcium metabolism have one important objective, namely, to maintain the extracellular concentration of calcium ion within the narrow range wherein it modulates membrane activity in many tissues of the body including the nervous system, muscle, and absorptive epithelia.

Dietary deficiency of vitamin D blocks the train of events which facilitate calcium absorption from the diet. The subsequent diminution of the extracellular calcium concentration sets in motion the protective parathyroid response in an attempt to maintain calcium levels in body fluids even at the expense of bone mineralization and renal tubular function. Any impairment in the conversion of vitamin D to the biologically important 25-hydroxy derivative, any inhibition of its binding to the nuclear membrane will have an effect on calcium nutrition similar to that of dietary deficiency of the vitamin.

At the present time, it is useful to consider vitamin D dependency as a Mendelian trait affecting the endogenous metabolism of vitamin D. This hypothesis can now be tested. The paper by the Toronto group[1] tells us that in vitamin D dependency there is a specific intestinal transport defect affecting calcium ion alone. Whereas this might be considered another "inborn error of membrane transport," we are now in a position to consider the trait as a potential inborn error of vitamin D metabolism. Two clinical features of the trait inform us further on the possibilities for productive research on this disease in the future. First, when sufficient vitamin D_2 or D_3 is given to vitamin-D dependent patients, an immediate regression of the fully expressed phenotype occurs; this indicates that normal calcium transport protein(s) can be synthesized endogenously under suitable conditions. Secondly, the vitamin D requirement is permanently 100 times greater than normal. This suggests that vitamin D dependency is either a "leaky" mutant in which high concentrations of vitamin precursor are necessary to initiate synthesis of a small amount of the 25-hydroxy derivative; or the trait represents a change in K_m of the binding site for 25-hydroxy derivative on the nuclear membrane which high concentrations of the 25-hydroxy derivative can offset.

Investigations into the distribution and synthesis of vitamin D metabolites in man could distinguish between these and other alternatives, and such investigations are now in progress as part of an international collaborative study. When they are completed it will be possible to add this knowledge of vitamin D dependency to the growing in-

functions. After hepatic synthesis of its active form, the vitamin is again transported in plasma by α_2 globulin, until it is bound to the nuclear membrane of intestinal epithelial cells. Thereafter, by mechanisms still requiring elucidation, it initiates synthesis of a specific messenger RNA, by activating a portion of nuclear DNA (gene or genes). The m-RNA in its turn initiates synthesis of a cytoplasmic protein (or proteins) whose major site of action is in the brush border of intestinal epithelial cells. This protein which has been isolated and some of its properties characterized from chick intestinal mucosa,[12] mediates calcium transport from the brush border to the antiluminal border, and hence to the blood. The vitamin also stimulates a calcium-dependent ATPase in brush border which is important in augmenting initial rates of calcium uptake.[13] The 25-hydroxy derivative of vitamin D has a third important effect. It can act directly on bone in physiological doses to release calcium and phosphate ions;[14] this action is very similar to that of parathyroid hormone and it is inhibited by calcitonin.

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Investigations into the distribution and synthesis of vitamin D metabolites in rickets could distinguish between these and other alternatives, and such investigations are now in progress as part of an international collaborative study. When they are completed it will be possible to add this knowledge of vitamin D dependency to the growing in

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mation on the treatable hereditary vitamin dependencies of man,[illegible] and to prescribe for such patients effectively and rationally.

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PSEUDOHYPOPHOSPHATASIA*

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DOROTHY CAMERON, R.T.

WE report herewith a condition, presumably hereditary, that resembles classic hypophosphatasia.[1] The new trait is characterized by an osteopathy of skull and long bones resembling that of typical hypophosphatasia, muscular hypotonia, hypercalcemia and phosphoethanolaminuria. One important feature distinguishes the new trait from the old, and that is the consistently normal total alkaline phosphatase activity in plasma, because of this, we have called it "pseudohypophosphatasia."[2]

CLINICAL FEATURES

L.W., a 3-month-old girl, born on April 20, 1964, attracted attention because of her irritable temperament, widened skull sutures, bulging anterior fontanelle and hypotonia. Investigation showed her to be below the 3d percentile for height and weight, although her birth weight had been normal. Roentgenographic findings in the skull and in the metaphyses of all long bones (Fig. 1) were typical of hypophosphatasia. Plasma analyses on several occasions revealed a total calcium of 10.8 to 13.0 mg per 100 ml (corrected to specific gravity of 1.0027),[3] inorganic phosphorus of 5.1 to 6.7 mg per 100 ml and total alkaline phosphatase activity of 15.7 to 22.4 King–Armstrong units (the latter assay remained normal on every examination over the subsequent 4 years of observation). The concentration of phosphoethanolamine, measured by elution chromatography on an ion-exchange resin column,[4] was slightly elevated in plasma (0.032 μmoles as compared with the normal value of less than 0.011 μmoles per milliliter[5]), whereas its excretion in urine was considerably elevated (1.05 μmoles per minute per 1.73 square meters, as compared with the normal value of less than 0.275 μmoles[5]), and readily visible at all times on 2-dimensional partition chromatograms developed in a phenol-lutidine system.[6]

The hallmarks of classic hypophosphatasia were therefore present with the exception of the normal alkaline phosphatase activity in plasma (Table 1). Dr. Donald Fraser, of Toronto, advised us that he had not yet seen a patient with the particular constellation of findings, but that we should proceed with management as if she had classic hypophosphatasia. A diet low in calcium was begun, and steroid medication (prednisone, 5 mg daily) was prescribed, nonetheless, hypercalcemia persisted during the next 4 months. The plasma calcium concentration then fell abruptly to the normal range, where it has remained ever since; steroid medication was stopped when this happened, and a normal diet was resumed. At that time (when she was 9 months of age), spontaneous healing of the bone lesions began, and by the 20th month, the bones were almost devoid of abnormalities; this status has been maintained ever since (Fig. 1).

During the 2d year of life intracranial pressure became elevated as a result of cranial synostosis; this required bicoronal and anterior squamosal craniectomies, which were performed by Dr. John Blundell in the 25th month of life. The subsequent clinical progress has been uneventful except that deciduous teeth have prematurely loosened, and severe

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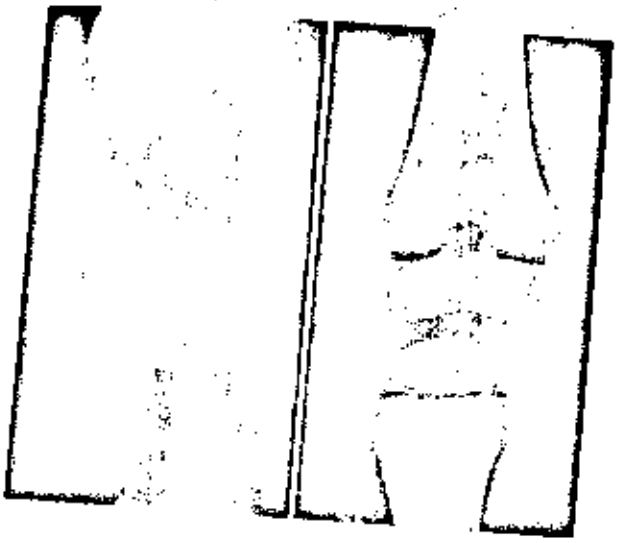

FIGURE 1. *X-Ray Films Showing the Abnormalities in the Metaphyses of the Tibia and Femur at Three Months (Left) and at Three and a Half Years (Right) of Age.*

Healing began spontaneously at nine months and reached its maximum at 20 months, remaining stable thereafter.

were shed or required extraction. When the patient was last seen at 4½ years of age, some catch-up growth had occurred and her height and weight are now just below the 3d percentile. Her intelligence, as measured by the Stanford–Binet language-memory test, is in the bright normal range. X-ray films show that the bone lesions remain almost healed. However, the phosphoethanolamine concentration remains elevated in urine.

The parents, who are not related to each other, both have normal total plasma alkaline phosphatase activity in their plasma (Table 1). Elution chromatography[4] of amino acids in their urine showed that the mother has a modest, but definitely elevated, phosphoethanolamine excretion (0.312 μmoles per minute per 1.73 square meters); the excretion of phosphoethanolamine by the father was not elevated on the occasion on which it was measured (0.220 μmoles per minute per 1.73 square meters). The patient has no sibs, and no relatives with bone disease, growth retardation or miscarriages were identified.

TABLE 1. *Phenotypic Features of Pseudohypophosphatasia and Classic Hypophosphatasia.*

Feature	Classic Hypophosphatasia	Pseudohypophosphatasia		
		Propband	Mother	Father
Osteopathy of long bones & skull	+	+	−	−
Premature loss of deciduous teeth	+	+	−	−
Hypotonia	+	+	−	−
Hypercalcemia	+	+	−	−
Phosphoethanolaminuria	+	−	+	−
Low plasma alkaline phosphatase	+	−	−	−

SPECIAL STUDIES

The nature of the patient's alkaline phosphatase activity in plasma was investigated (Table 2). The fractions of total activity derived from bone and intestine were normal, and the pH optimum for activity at various concentrations of substrate was normal. Furthermore, there was no difference between the patient's plasma and normal plasma when tested with the substrates β-glycerophosphate, phenylphosphate and phosphoethanolamine, each at 16 mM. However, the patient's plasma hydrolyzed low concentrations of phosphoethanolamine more slowly than normal plasma. The observed kinetics of hydrolysis suggest that her enzyme has less than the normal affinity for phosphoethanolamine, although the rate of cleavage under conditions approaching saturation is apparently normal.

Electrophoresis of the patient's plasma was performed on vertical starch gel (by Dr. Samuel Boyer, of Johns Hopkins University Medical School) and on acrylamide gel (by Dr. Sally Long, of Human Genetics Section, McGill University). These procedures did not reveal any detectable abnormality in the protein bands containing alkaline phosphatase activity as compared with four age-matched control subjects.

TABLE 2. *Characteristics of Alkaline Phosphatase Activity in Plasma of a Patient with "Pseudohypophosphatasia."*

Subject Test*	Total Alkaline Phosphatase Activity		Heat-Labile Fraction†	L-Phenylalanine-Inhibited Fraction‡	pH Optimum§					EAP/BGP Ratio‖	
	King–Armstrong units	Bodansky units	%	%	BGP 16 mM	EAP 16 mM	BGP 4 mM	EAP 4 mM	substrate at 16 mM, pH 9.8	substrate at 4 mM, pH 9.8	
Patient	18.0-24.3	5.2-10.8	11.5-21	44-46	9.8	9.8	9.6	9.6	0.87	0.[illegible]	
Normal	10.0-35.0	5.0-15.0	3.0-20	38-47	9.8	9.8	9.6	9.6	0.91	[illegible]	

*Each sample from patient was paired with sample drawn from normal subject, matched for age; analyses always performed on fresh samples, or c[illegible] equivalent times.

†Equivalent to "bone" isozyme; preincubated at 56°C for 30 min & measured at pH 9.8 with 16 mM β-glycerophosphate.

‡Equivalent to "intestinal" isozyme; incubated with 5 mM L-phenylalanine & measured at pH 9.8 with 16 mM β-glycerophosphate (patient [illegible] known).

§Measured in pH range 8.5-10.3, by Bodansky method; BGP = β-glycerophosphate; EAP = phosphoethanolamine.

‖Ratio reflects relative rate of orthophosphate released from substrate in 15 min under specified conditions; results average of 3 analyse[illegible]

¶P value <0.01 when compared with normal subjects.

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When the patient was two and a half years old, a test was performed to ascertain whether her serum contained an inhibitor of bone calcification; the method of Yendt et al.[9] was used to determine this. Rachitic rat tibia, taken from Holtzman rats deficient in vitamin D and depleted of calcium, calcified during incubation in the patient's serum as rapidly as in normal serum, suggesting that an inhibitor of mineralization did not account for the clinical trait.

Discussion

We conclude from these observations that a phenotype closely resembling that of classic hypophosphatasia can exist even in the presence of apparently normal total alkaline phosphatase activity in plasma. This enzyme is usually measured in the clinical laboratory at high substrate concentrations — far in excess of the physiologic conditions likely to be encountered by the enzyme. When alkaline phosphatase activity in the patient's plasma is measured at lower substrate concentrations, there is evidence for a qualitative difference in its ability to hydrolyze phosphoethanolamine. Whether this accounts for the accumulation of phosphoethanolamine in the trait is not known at present.

A rare mutant recessive autosomal allele, or possibly an X-linked allele, appears to account for the appearance of this new trait. On the basis of its phenotypic characteristics, the trait can be distinguished from classic hypophosphatasia; whether the pseudohypophosphatasia allele occurs at the same gene locus responsible for classic hypophosphatasia cannot be forecast on the basis of the present observations. The fact that bone lesions occur both in the classic and in the pseudohypophosphatasic traits might suggest that an abnormality of alkaline phosphatase activity in plasma is merely a remote phenotypic [illegible] and that the primary abnormal phenotype in [illegible] conditions is yet to be found.

We are indebted to Drs. Samuel Boyer, Donald [illegible] H. Harris and B. N. La Du for their advice in these [illegible] and to Drs. Jean-Paul Poirier and Julia Gulyas for [illegible] the patient and supervising her clinical care.

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VI. VITAMIN D AND CARDIOVASCULAR, RENAL, AND BRAIN DAMAGE IN INFANCY AND CHILDHOOD

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INTRODUCTION

There has been increasing notice taken in the literature of recent years of a presumed congenital abnormality characterized by supravalvular aortic stenosis, a peculiar facies and severe mental retardation. That an hereditary genetic defect may play a part in this syndrome has been suggested. The possibility that this syndrome may be induced by excess vitamin D during gestation has also been considered. This paper presents the possibility that this syndrome is an expression of hyper-reactivity to vitamin D that can be especially severe during early infancy. There is evidence that it may be the anatomic consequence of the disease known as hypercalcemia of infancy, which many investigators have found to develop in infants receiving doses of vitamin D that are only slightly to moderately above those considered prophylactic. This paper presents evidence that generalized arteriosclerosis of infancy may be the most severe manifestation of vitamin D toxicity in infants with marked susceptibility to vitamin D, and that renal acidosis may be a less severe expression of this sensitivity. There is evidence that recommended daily dosages of vitamin D may be toxic for some susceptible people, and that the potency of vitamin D is significantly greater in milk than in oil. As Taussig[1] has recently observed, the inborn "variant" in the metabolism of vitamin D that made some children less prone to rickets in the days before unlimited vitamin D supplementation of our foods is now the so-called "inborn error of metabolism" that may be responsible for vitamin D-induced injury to the cardiovascular, renal, and central nervous systems.

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THE SUPRAVALVULAR AORTIC STENOSIS SYNDROME

Williams *et al.*[2] first called attention (in New Zealand in 1961) to the association of severe mental retardation and a peculiar facies with supravalvular aortic stenosis in young patients with retarded growth (see FIGURE 1). Upon reviewing the literature on similar cases, they discovered that in two of the studies, the patients were found at autopsy to have had fibrous myocarditis and myocardial atrophy.[3,4] Since they suspected a myocardial abnormality in one of their four patients, they suggested the possibility that myocardial degeneration might be another facet of this new syndrome. It is of interest that the patient reported by Burry[4] had also been described as being congenitally mentally defective. The same year (1961), Perou[5] reported detailed histological studies of the supravalvular stenotic areas of the aortas of a 13-year-old boy and of a 24-year-old man who had been in a State Hospital for mental retardation. In the first case, the child's records indicated a history of malnutrition and congenital heart disease at one year of age; the facies of the second case seems to bear some relationship to that described by Williams *et al.*[2] The patient was described as having large ears, high and narrow palate, and irregular carious teeth, an appearance quite similar to that reported recently by Rosenthal and Doyle[6] in a child with supravalvular aortic stenosis. These characteristics are selected here for note because of their resemblance to those seen in survivors of infantile hypercalcemia[7] (see FIGURE 2). Friedman[8] has suggested that excess vitamin D during gestation may be responsible for the abnormalities of the craniofacial complex, characteristic of both the supravalvular aortic syndrome and infantile hypercalcemia.[8]

The histological characteristics of the circular ridge and constriction at the upper margin of the sinuses of Valsalva, seen in the two cases reported by Perou, included slight intimal thickening, hyalinized fibrous tissue, fragmentation and disorientation of the elastica, and foci of necrosis and calcification. The myocardium of Perou's second patient showed patchy areas of fibrosis, and the kidneys were contracted and granular. Histological changes of the aorta had also been reported earlier by other pathologists who sectioned the stenotic portion of the aorta.[3,4,9,10] It was Perou's conclusion that this type of supravalvular aortic stenosis is unquestionably congenital.

Since then, additional investigators have reported similar patients with the aortic lesions, mental retardation and peculiar facies, the entire complex often described as a congenital anomaly.[6,11-18] However, Eberle and Beuren,[19] who had reported three patients with characteristics similar to those described by Williams a year later, attempted to detect chromosomal abnormalities in their patients, without success. Merritt *et al.*[20] however, did find a chromosomal abnormality in one patient with the full-blown syndrome, among eight supravalvular aortic stenotic patients studied. In four of the eight families, only the proband was affected. The remaining four families, with only the supravalvular stenosis, had 25 probably affected individuals. In a more recent study by Kurlander *et al.*[21] of 27 cases of supravalvular aortic stenosis, nine also had mental retardation and peculiar facies. Chromosomal studies, performed in five of these cases, were normal.

In 1963, Black and Bonham-Carter,[7] one of whom was a co-worker in the early study by Schlesinger *et al.*[22] of an infant with hypercalcemia, noted the similarity of the appearance at the age of 11 of a survivor of that syndrome[23] to that of the children with the Williams' triad (see FIGURES 2 & 3). They

FIGURE 1. (top) Four patients with supravalvular aortic stenosis (courtesy of Williams). (bottom) Profiles of children in front row. (Reproduced by permission of Williams, J.C.P. *et al.* and Circulation.

FIGURE 2. Survivors of infantile hypercalcemia. (Reproduced by permission of Black, J.A. and R.E.B. Bonham-Carter and Lancet.

presented five additional cases, all with clear histories of feeding difficulties and developmental abnormalities very similar to those seen in infants diagnosed definitively as having infantile hypercalcemia. They recently reported confirmation[24] at autopsy of supravalvular aortic stenosis in the child reported earlier as hypercalcemic[23] and noticed as resembling Williams' triad at the age of ten.[7] The child died at 16 years of age with renal failure, hypertension, and left

ventricular failure. The necropsy findings were small granular kidneys with numerous calcified areas, left ventricular hypertrophy, and a supravalvular aortic stenosis that had reduced the diameter of the aorta to 1 cm.[24]

Direct association of the Williams' syndrome with biochemically diagnosed hypercalcemia of infancy, was reported by Garcia *et al.*[25] Their patient was a

FIGURE 3. Patient with infantile hypercalcemia at 21 months of age (top) and at 11 years of age (bottom). ((A) Reproduced by permission of Schlesinger, B.E. *et al.* and Brit. Med. J., and (B) by permission of Black, J.A. and R.F.B. Bonham-Carter and Lancet.)

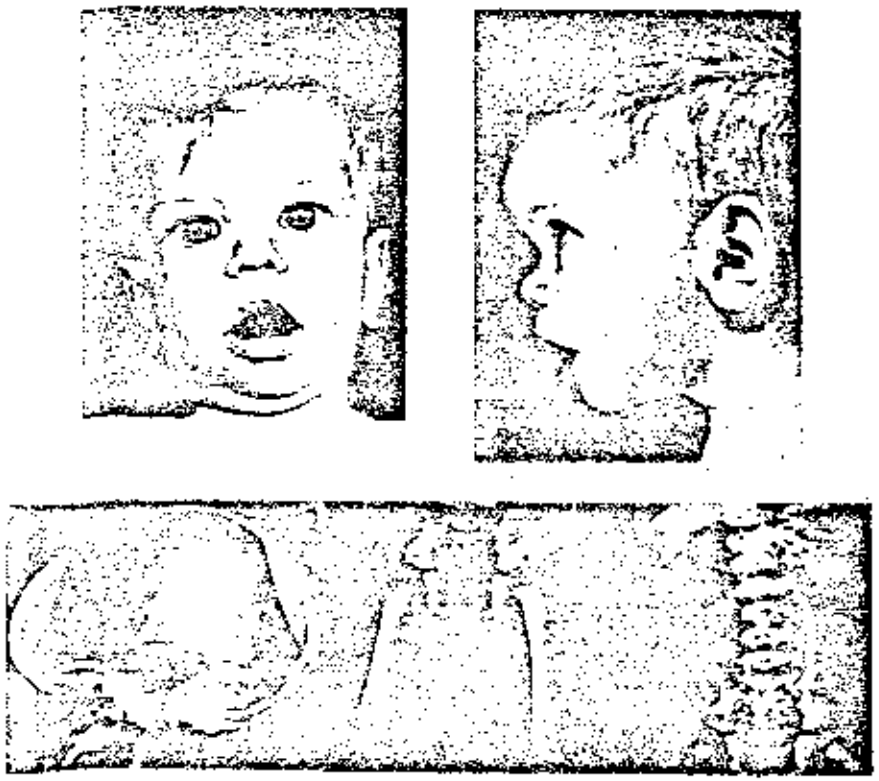

FIGURE 4. Hypercalcemic infant with supravalvular aortic stenosis. (top) Note elfin faces, high prominent forehead, epicanthal folds, underdeveloped bridge of nose and mandible, and overhanging upper lip. (bottom) Roentgenogram of skull, pelvis and long bones and spine showing diffuse osteosclerosis and bands of increased density at metaphyses and at epiphyseal margins of vertebrae. (Reproduced by permission of Garcia, R.E. *et al.* and New Eng. J. Med.)

nine-month-old infant, who had a history of a normal neonatal course. At five months, cardiomegaly and murmur had been detected; subsequent to a herniorrhaphy at six months, retarded development was noticed. The child exhibited an elfin facies. (see FIGURE 4) and on roentgenographic examination, showed considerable sclerosis of the calvaria and base of the skull, with diffuse osteosclerosis of the long bones and vertebrae. Cardiac catheterization and cineangiocardiography revealed supravalvular aortic stenosis and mild stenosis at the origin of the left pulmonary artery. This case is of particular interest in that the classic Williams' syndrome was detected early, and was correlated with both hypercalcemia and a markedly elevated vitamin D blood level (1,540 units/100 ml, as compared with the usual value range of 60-400 units). In a recent review of idiopathic hypercalcemia, Fraser *et al.*[26] supported the concept that supravalvular aortic stenosis, mental retardation, and peculiar facies is the late normocalcemic stage of infantile hypercalcemia.

Bauman and Bauman[27] reported two children with hypercalcemia and

cardiac damage. One was a child with a less severe form of the disorder during infancy, involving anorexia, failure to gain, developmental retardation, and congenital heart disease (see FIGURE 5-A,B), and who showed marked improvement, with disappearance of all symptoms, signs and abnormal laboratory findings five years later. (FIGURE 5–C) The only significant treatment had been curtailment of vitamin D intake. Their second patient was a child who wasn't seen by this group until she was 20 months of age, and whose growth and development had been normal until she was one year old. This patient developed a harsh systolic murmur, renal damage, and mental retardation that was not reversible. Beuren *et al.*[28] reported nine additional children with the supravalvular aortic stenosis, most of whom also had pulmonic stenosis, and one of whom had a definite history of infantile hypercalcemia. (see FIGURE 6) More recently, Beuren *et al.*[29,30] reported a total of 55 children with supravalvular aortic stenosis in Germany, 40 of whom also had pulmonary stenoses. In this series, 23 had the typical facies of hypercalcemia of infancy and mental retardation. In many of these cases, clear histories of marked overdosage with vitamin D during infancy could be elicited; the mothers had also been given vitamin D prenatally. In the 14 children with supravalvular aortic stenosis reported by Antia and associates,[31] there was a wide spread in the range of their I.Q.'s, many of them showing only slight retardation, and others with normal I.Q.'s showing curious variations in responses to mental states.[1] One patient with normal facies had abnormal dentition suggestive of the syndrome in which aortic stenosis and mental retardation co-exist. Myers and Willis[18] have also reported the broad clinical spectrum of this disease. They have found that the supravalvular aortic lesion occurs in the absence of intellectual impairment and elfin facies almost twice as commonly as in the classic syndrome and tends to occur in families.

With the similarity in the two syndromes having been established, many investigators have commented on the possibility of a direct relationship in their reports of cases of supravalvular aortic stenosis associated with the "hypercalcemic facies" and mental retardation.[1,7,11,13,18,26,29–37] Taussig[1] has suggested that the two types of supravalvular aortic stenosis syndrome—that associated with, and that without, the characteristic facies and severe mental retardation—may represent different responses to the same underlying condition. Bonham-Carter and Sutcliffe[34] have called attention to a concomitant syndrome in which multiple arterial stenoses occur at the bifurcation of arteries, both in the systemic and pulmonary circulation. Since in infantile hypercalcemia the blood cholesterol is also high, they have postulated that atheromatous change at the stress points of bifurcation might be responsible for the stenotic changes. Friedman[35] reported that the Williams syndrome is relatively common, and that its association with infantile hypercalcemia may be missed. Beuren *et al.*[29,30] have commented on the frequency of this syndrome in Germany, where supplementation with high dosage of vitamin D is common. In a personal communication to Taussig,[1] Beuren voiced his suspicion that patients with supravalvular aortic stenosis respond abnormally to vitamin D. Friedman[35] has stressed the importance of early diagnosis because of the possibility of arresting, or possibly even reversing, the lesions by instituting appropriate therapy. Taussig[36] has published a provocative article, discussing the possibility that some relationship exists between the two conditions. She suggested that the inborn variation in man's ability to metabolize vitamin D may be responsible for the valvular changes seen in these conditions. The increase in blood cholesterol often seen in hypervitaminosis D, further suggests that such variation in

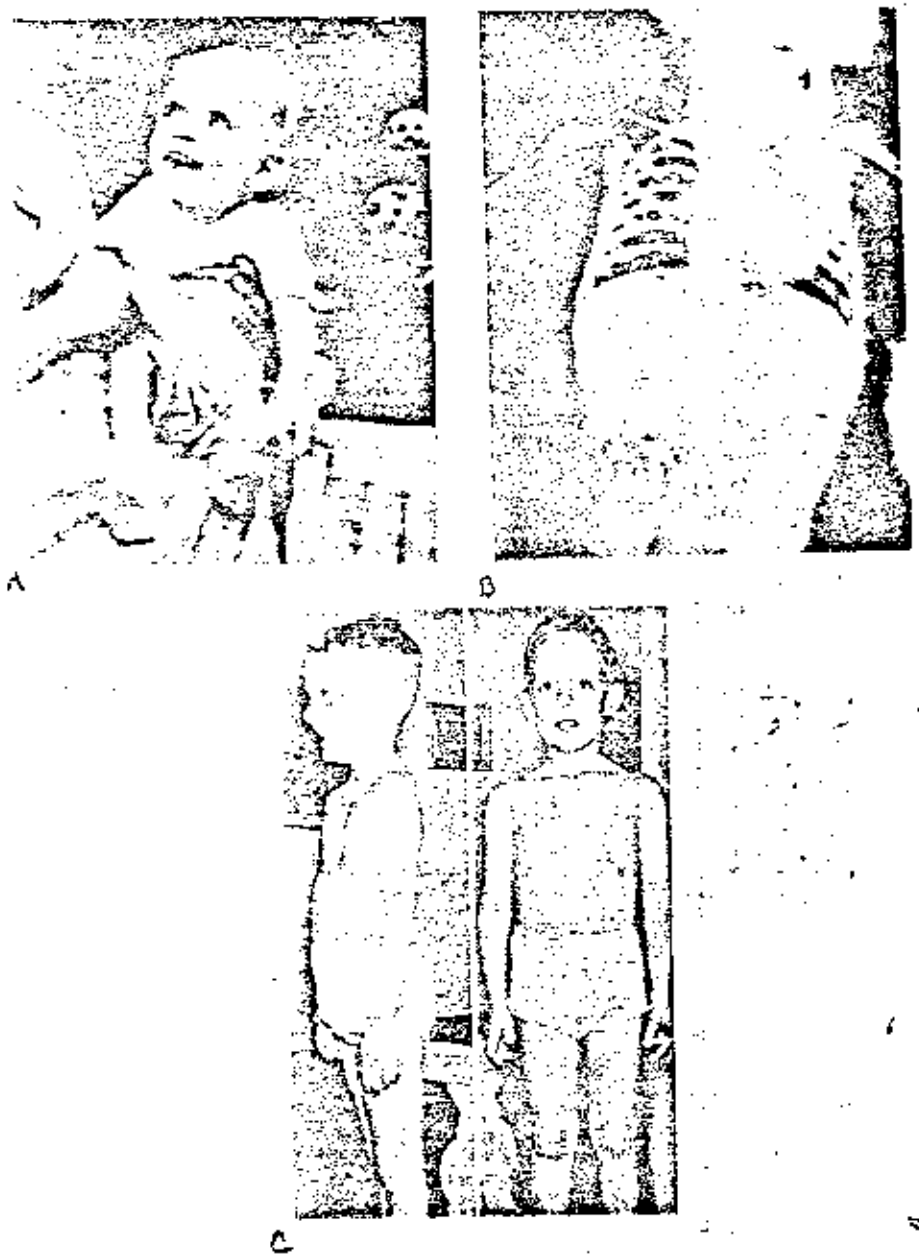

FIGURE 5. Hypercalcemic patient, who recovered on curtailment of intake of vitamin D.

A. Hypercalcemic infant: note malnutrition, poor development; low position of pinnas.
B. Roentgenogram, showing cardiac enlargement; "coeur en sabot" configuration.
C. Patient, five years later, demonstrating alert appearance, good muscular development.
(Reproduced by permission of Bauman, A. & C.G. Bauman and N.Y. State J. Med.)

FIGURE 6. Nine patients with supravalvular aortic stenosis. (Reproduced by permission of Beuren, A.J., C. Schulze, P. Eberle, D. Harmjanz & J. Apitz and Circulation Am. J. Cardiol.)

susceptibility to vitamin D may possibly be related to the far commoner vascular lesions associated with abnormal cholesterol metabolism. Her most recent article on the subject[1] presents a review of several of the published papers on this syndrome and includes comments on the similarity in the appearance of the children with supravalvular aortic stenosis to those with idiopathic hypercalcemia.

IDIOPATHIC HYPERCALCEMIA, RENAL ACIDOSIS, AND VITAMIN D

Hypercalcemia of Infancy and Vitamin D

The similarity of the syndrome comprising supravalvular aortic stenosis, mental retardation, and peculiar facies to that seen in survivors of hypercalcemia of infancy calls for a careful examination of not only the pathological picture,

but also the epidemiological background and the personal histories of the patients reported, to the extent that retrospective evaluation of the published data is possible. Reference should be made to Black's review[38] of the relationship of vitamin D to idiopathic hypercalcemia, and to the similarities of the clinical pathology caused by overdosage with vitamin D to that of the hypercalcemia syndrome.

The full complex of chronic hypercalcemia was first described in 1952 by Schlesinger and colleagues[22] and reported briefly by Lightwood[39] in England, and in detail by Fanconi and Girardet[40] in Switzerland. Each report dealt with a patient whose history indicated an intake of vitamin D that was not considered excessive (approximately 1,400 and 2,000 units after the first month of life.) Feeding difficulties, persistent vomiting, constipation, and failure to thrive or gain in weight characterized the early signs, with hypotonia and renal impairment seen in all three infants, Schlesinger's and Fanconi's patients also exhibited early electrocardiographic changes, and Schlesinger's patient had a harsh systolic murmur at six weeks. The patient reported by Lightwood had been referred to him because the symptoms suggested renal acidosis, a diagnosis that he excluded because of the normal plasma chloride, bicarbonate and inorganic phosphorus and because of the acidity of the urine. Each child had hypercalcemia and urea retention. Schlesinger's report included a follow-up to the age of three. The child's failure to develop normally was manifested by the age of five months. The retardation, associated with hypercholesterolism, at first suggested the possibility of hypothyroidism, and treatment with thyroid extract was attempted, without success. By two years of age, there was definite renal impairment, hypertension, and sclerosis of bone. There appeared to be no further progression of the disease from the age of two, but at three the picture was of a mentally retarded dwarf, with defective renal function, hypertension, and a systolic murmur. The similarity of the clinical picture, at the age of two, to hypervitaminosis D led the investigators to omit vitamin D from the diet from that time on. The failure of this deletion to change the biochemical picture or to alter the clinical status led the investigators to question their original impression that the condition may have been due to hypersensitivity to vitamin D.

Analysis of the many case reports on hypercalcemia of infancy reveals that the characteristics can be divided into early, or mild and apparently reversible signs, and the later irreversible changes of the severe form of the disease. Both the mild and irreversible forms start in the early weeks or months of life with symptoms of fretfulness, anorexia, vomiting, constipation, polydipsia, polyuria, hypotonia, and cessation or marked slowing of growth and of weight gain. At this stage, blood chemistry changes include hypercalcemia (usually over 12 mg/100 ml), azotemia, and often elevated blood cholesterol (primarily the free form). Blood inorganic phosphorus may be normal or slightly elevated, and alkaline phosphatase readings are generally low. Hypercalciuria and white cells in the urine are often detected. Recurrent infection is common, the disease often becoming overt after an acute severe infection.

Several infants, detected by blood chemical studies at that stage, have been put on a low calcium diet, delivering no vitamin D.[26-27,41-50] They exhibited fairly prompt relief of vomiting and anorexia, relief of constipation somewhat later, but persistence of elevated calcium and NPN levels for several months. Weight gain, and improvement in disposition and in muscle tone, have also been seen on such corrective diets. Corticosteroids have proven useful in inducing clinical improvement in infants with more severe manifestations.[26,42,50-58] Hypercalcemia and decreased renal function improved,[41,43,44,48,50]

[54–56,59] – as did cardiac function[26,27,41,45,46] in a few cases. The mental retardation, however, was irreversible. Apparently the longer the delay in diagnosis, the more severe was the physical damage. Hypertension and systolic murmur (often shown to have been due to supravalvular aortic stenosis) were seen, and renal damage became more severe with fixed gravity of urine, albuminuria, casts, hematuria, and signs of increasing renal insufficiency. Increased bone density, detectable on x-ray, developed. Craniostenosis and osteosclerosis, especially of the base of the skull, and, to a lesser extent, of the long bones and vertebral column, were frequently seen. In addition to retardation of growth, severe mental retardation became manifest in these infants. The infants are characterized by an "elfin" facies that is considered typical of the severe hypercalcemia syndrome. (see FIGURES 3–A, 4, 5–A, 7-9)[24,25,27,31,61,62] In later childhood, their appearance is characterized by a wide slack mouth, severe malocclusion and caries, prominent upper lip, underdeveloped mandible, depressed bridge of nose, widely set eyes, sometimes a convergent strabismus, epicanthic folds and low-set, prominent ears (FIGURES 2, 3–B). [7,23,24] This facies is indistinguishable from that seen in the "congenital" supravalvular aortic stenosis syndrome (FIGURES 1,6).[2,28]

FIGURE 7. "Elfin" facies in idiopathic hypercalcemia. Note thin delicate ears, depressed nasal bridge, small mandible and high prominent forehead. (Reproduced by permission of David, N.J. *et al.* and Am. J. Med.)

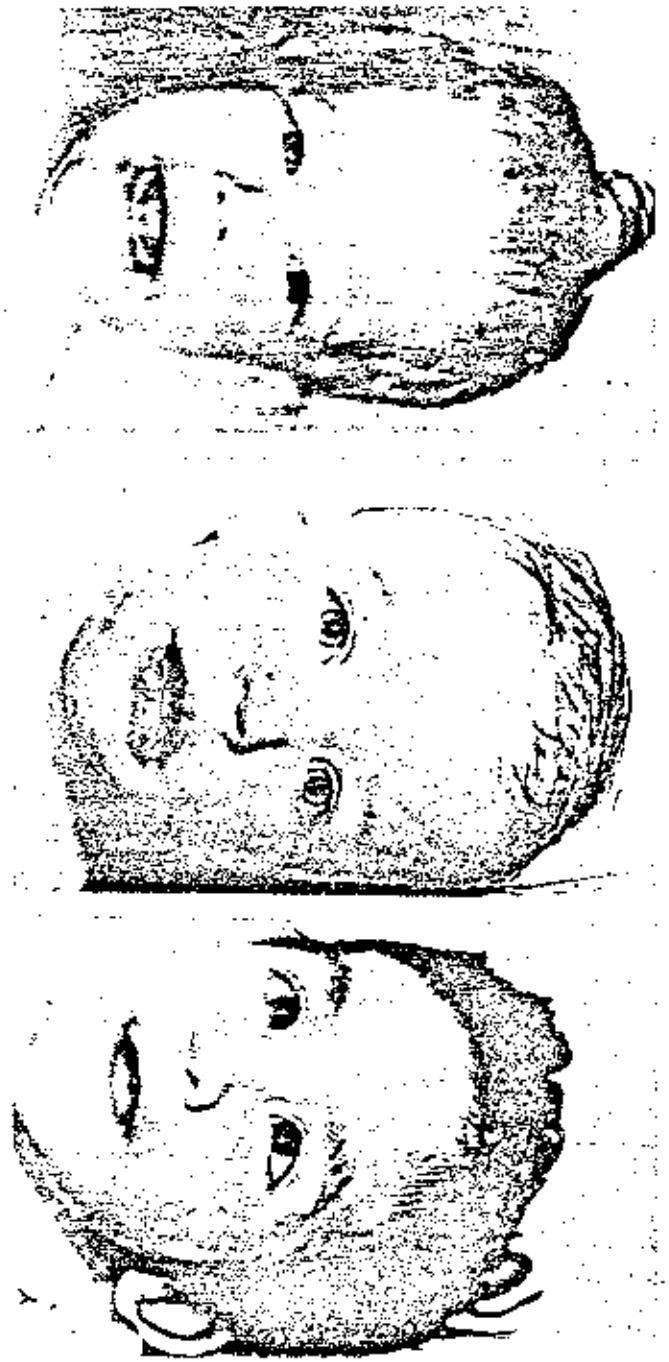

FIGURE 8. Typical facies of infantile hypercalcemia. (Reproduced by permission of O'Brien, *et al.* and J. Am. Med. Assoc.)

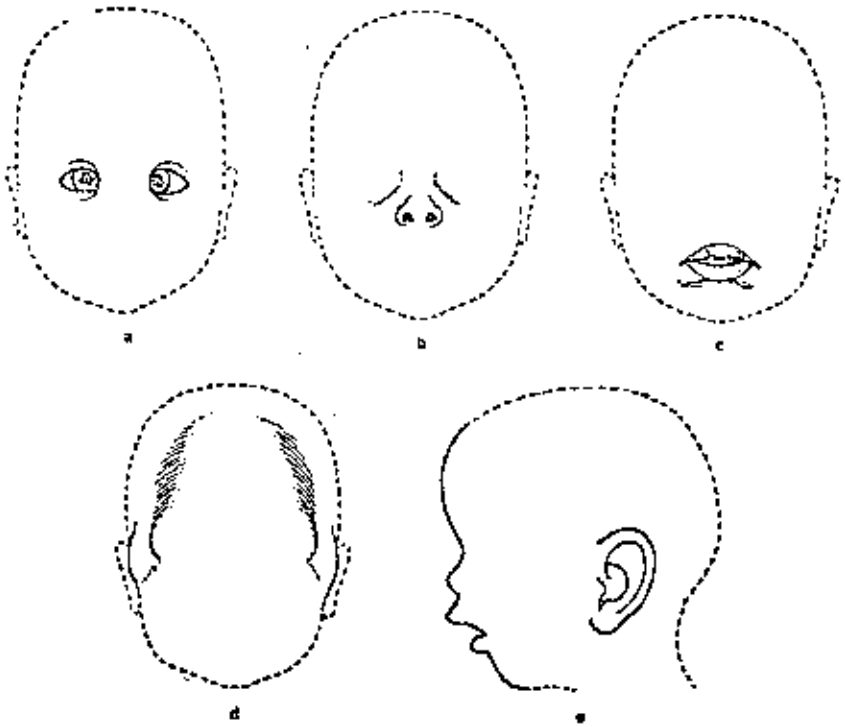

FIGURE 9. Components of the facies of infantile hypercalcemia. (Reproduced by permission of Joseph, M.C., & D. Parrott and Arch. of Diseases in Childhood.)

In order to determine whether the mild or simple form of infantile hypercalcemia does, as has been said, result in complete recovery once the infant is taken off vitamin D and put on low-calcium diet, Mitchell[61] followed the courses of 18 infants, diagnosed as having simple hypercalcemia in Scotland from 1953 through 1956, for three to six years. Four of these infants died, and autopsy disclosed nephrocalcinosis.[63] One was lost to follow-up at eight months, four months after having been put on a low-calcium milk preparation. Of the remaining 13 children, most showed slight delay in sitting up and walking, but all were sitting without aid by 12 months, and all had taken the first unaided step by 21 months. All but one seemed to have average mental development; the three who had had cardiac murmurs during the acute phase had no detectable cardiac abnormality at their last examination. Tests of renal function revealed no significant abnormality, and satisfactory growth progress was made. In all cases, the abnormal biochemical findings originally present, including hypercalcemia, depressed alkaline phosphatase, and elevated plasma cholesterol, had become essentially normal. On the other hand, Coleman,[64] who expressed concern at the persistence of infantile hypercalcemia in Scotland (13 patients admitted to hospital in 1962-1963), despite the lowered vitamin D allowance as food additives, reported electrocardiographic changes in 12 of those 13 infants. These changes, which he had reported earlier in 14 out of 24 children

admitted to a medical unit in Glasgow,[65] are characterized by an abnormal shape of the ST-T complex. These changes outlasted the hypercalcemic phase of the disease and suggested to this investigator that they may reflect left ventricular myocardial lesions.

Correlation of the extremely high incidence of "idiopathic" hypercalcemia of infancy in England in the years 1953-1957 with the excessive fortification with vitamin D of National Dried Milk and other Welfare and proprietary foods suggests that Schlesinger and colleagues'[22] original impression that hypersensitivity to vitamin D may have been an etiological factor may have been correct. Search of the literature has elicited over 25 articles dealing with case reports on over 80 infants who developed the syndrome during that period of time.[7,23,24,38,39,41,43,46,49,52,56,57,60,63-78] That probably few infants so affected have been reported in print was clearly indicated by the 1956 report of the British Pediatric Association that 204 cases of hypercalcemia were reported by June, 1955 to 196 consultant pediatricians in the United Kingdom.[73] When the feeding difficulties, failure to thrive, the hypotonia, and susceptibility to infection characteristic of the early stage of this syndrome are taken into account, one wonders what the true incidence of this condition might have been. How many biochemical diagnoses are made among infants not brought to the attention of consultant pediatricians? How many such infants fail to survive the early months of life? How many may survive a difficult infancy, without having had biochemical tests performed, and appear later with the triad of supravalvular aortic stenosis, mental retardation, and facies — with renal impairment often also present? (see also, discussion of generalized arterial sclerosis of infancy, as resembling the acute hypervitaminosis D syndrome elicited in the laboratory, *vide infra*).

Despite the high frequency of infantile hypercalcemia seen in Great Britain at a time when the vitamin D intake was very high, this was not, even then, a common disease. Although it was estimated in 1955, that at least 55% of British infants were consuming excessive quantities of vitamin D[79] (FIGURE 10) only 5% of admissions of infants six to 12 months of age to the Dundee Royal Infirmary the following year were due to hypercalcemia of infancy.[79] The attention of British physicians was called to the correlation of this syndrome with excessive vitamin D in milk and cereal, as well as in cod liver oil, as early as 1954,[80-83] the year after manufacturers were instructed to increase the fortification of National Dried Milk from 280 I.U. to 500 I.U. of vitamin D to assure delivery when marketed of the full recommended minimum of 280 I.U./dry ounce (allowing for a maximum deterioration of 44%).[83] One physician (Ball)[82] wrote a letter to the editor of Lancet cautioning that as little as 1,000 units per day might give rise to subjective symptoms of hypervitaminosis D, and a physician (Woodcock)[81] on the staff of one of the milk companies, in another letter to the editor, protested the amount of vitamin D that had to be added to milk. He referred to the early work by Jeans,[84] that demonstrated that at a daily intake of 1,800-2,000 units, growth of infants was retarded as compared with that of infants on 340-500 units in cod liver oil or even 135 units of vitamin D in milk. Creery and Neill[80] of Belfast concurred, the same year, that providing optimum or higher vitamin D intakes from both milk and cod liver oil might be related to the syndrome of hypercalcemia in infants with hypersusceptibility to the vitamin. The British Pediatric Association issued a report dated July 1955,[73, 83] in which they referred to their 1943 recommendation of daily intake (in cod liver oil) of 500-700 I.U. of vitamin D.[85] In view of the high incidence of hypercalcemia and the high vitamin D.

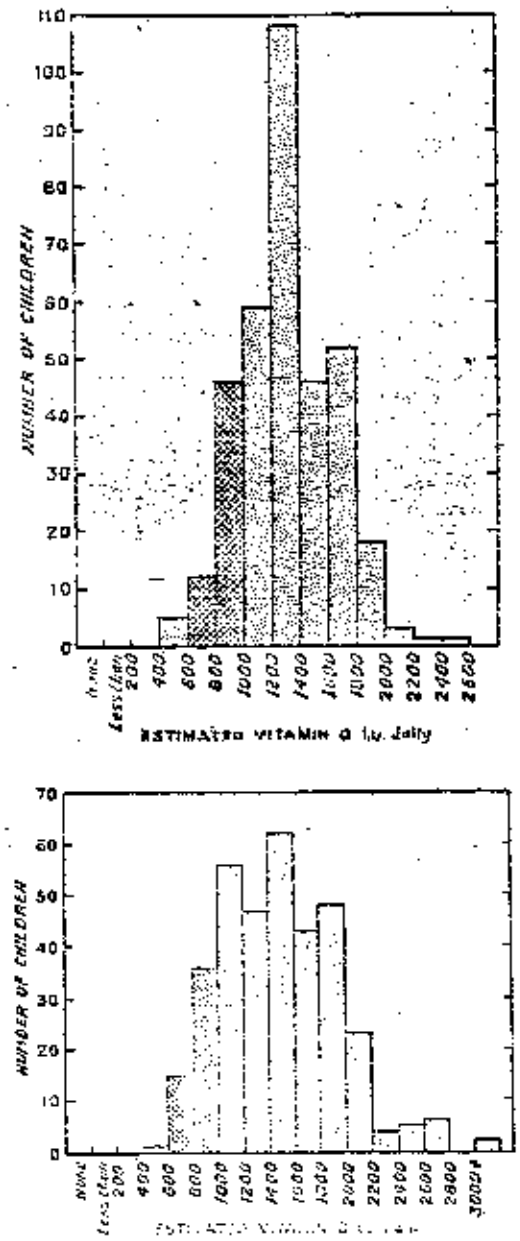

FIGURE 10. (top) Of 351 babies fed on national dried milk, 249 (70.9%) had vitamin D supplements: 208 had cod-liver-oil compound; 30 had adexolin; four had halibut-liver oil; and seven had other preparations. (bottom) Of 348 babies fed on proprietary brands of dried milk, 263 (75.6%) had vitamin D supplements: 172 had cod-liver-oil compound; 65 had adexolin; 16 had halibut-liver oil; and ten had other preparations. (Reproduced by permission of Creery, R.D.G. & D.W. Neill and Lancet.)

content of Welfare and proprietary infant foods (see TABLES 1A and 1B), they recommended that infant requirements of vitamin D be reconsidered, and that the amount added to food be lowered. An editorial, in 1956, speculated whether a hypercalcemic infant who was hypersensitive to calciferol (added to dried milk, cereals, and many concentrates) would be similarly affected by the natural vitamin D found in fish-liver oils.[86] Hubble,[87] in 1956, raised the question whether addition of vitamin D to milk and cereals might cause maximal absorption of calcium and phosphorus (a point which is discussed later in this paper). In 1957, the Ministry of Health published a report[88] on Welfare food supplementation, in which they recommended reducing (1) the vitamin D content of National Dried Milk to a minimum of 90-100 I.U./dry ounce from the minimum of 280 I.U./dry ounce; (2) the vitamin D content of infant cereals from 1,000 I.U. to 300 I.U./dry ounce; and (3) the vitamin D content of cod liver oil from 800 to 400 I.U./teaspoonful. The solution, thus, was to reduce the content of vitamin D in each of the foods to a level that would protect against rickets, should only one source be consumed. Of necessity, this means that an infant taking all three will be consuming at least three times the anti-rachitic dose. In view of the individual variation in sensitivity to vitamin D, and the demonstrated increase in potency of vitamin D when it is given in milk, (*vide infra*) it is dubious whether this provides a sufficient safety factor against causing infantile hypercalcemia in susceptible children.

In North America, for example, where the Council on Foods and Nutrition of the American Medical Association, since 1941,[89] has considered only milk containing 400 U.S.P. units of vitamin D acceptable, there have been many published reports on infants with hypercalcemia.[25-27,42,44,45,47,51,54,55,61,62,90-103] It must be noted, however, that despite the official recommendation that 400 units of vitamin D from all sources meet the full daily requirement, the common fortification of foods (in addition to vitamin supplements) may lead to daily intakes that may reach as much as 2,000 units/day, or more.[104] Whether the occurrence of hypercalcemia of infancy in Continental Europe and Israel[28-30,46,50,53,59,105-112,121] may also be

TABLE 1A

FORTIFICATION OF NATIONAL DRIED MILK

Date	I.U. Vitamin D per Dry Ounce	Recommendation by
1945	280	Ministry of Health[88]
1953	500	Ministry of Food[83]
1957	90–100	Ministry of Health[88]

TABLE 1B

CALCULATED DAILY INTAKE OF VITAMIN D (1956)[83]

1½ pints dried milk (460 U/dry oz.):	1,725 I.U.
1 ounce cereal (1,000–1,500/dry oz.):	1,000 – 1,500 I.U.
1 tsp. cod liver oil	700–800 I.U.
Total:	3,525 – 4,025 I.U.

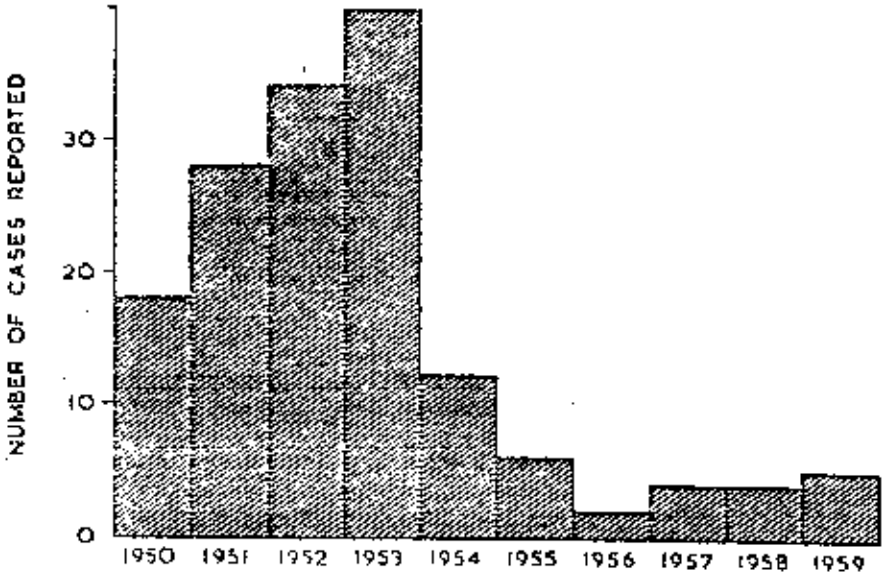

FIGURE 11. Infantile renal tubular acidosis: 153 cases by year of occurrence (14 centers). Information is lacking for 1950 for Liverpool. (Reproduced by permission of Lightwood, R & N. Butler and Brit. Med. J.)

correlated with such moderately high intakes of vitamin D by susceptible infants cannot be averred from available data. It must be noted, however, that in their recent reports of 55 cases of supravalvular aortic stenosis, 23 of whom had clear manifestations of hypercalcemia, Beuren *et al.*[28-30] did attribute the high incidence of these disorders in Germany to high intakes of vitamin D during infancy, and to high susceptibility to vitamin D of affected children.

Renal Acidosis and Vitamin D

Another disease of infancy that has been correlated with vitamin D intake and that is possibly related to hypercalcemia of infancy is renal acidosis. Lightwood and Butler[113] observed that there was a steady increase in the incidence of this disease from 1950 (the first date in the reported tabulation) through 1953, at which time there was a sharp drop in incidence (see FIGURE 11). Since infants with this syndrome often exhibit elevated serum calcium levels, it was reasoned that there was clearly a biochemical overlap between infantile hypercalcemia and infantile renal tubular acidoses. It was concluded, however, that vitamin D was probably not the common etiological denominator, since the drop in incidence occurred in 1954, three years preceding the correction of the excess vitamin D milk content. One point that was not considered, however, was that, from 1945 to 1953, the calciferol content of milk was still excessive (280 I.U./dry ounce), cod liver oil enriched with extra vitamin D was commonly administered in addition, and during that period of time supplementation of infant cereals with vitamin D was also coming into vogue. The drop in incidence of infantile renal acidosis in 1954 coincided with the sharp rise in incidence of hypercalcemia of infancy. The biochemical overlap

between the two conditions suggests that those infants who developed the full-blown syndrome of hypercalcemia of infancy, which includes renal impairment and renal calcinosis, may well have developed renal tubular acidosis on half the amount of vitamin D. The symptoms common to infantile renal acidosis include failure to thrive, vomiting, and growth retardation, symptoms that are characteristic also of hypercalcemia of infancy.[38,67,114,115]

The association of renal acidosis with infantile hypercalcemia has been pointed out by Graham,[49] who published a summary report on 38 infants with hypercalcemia, seen at the hospital of the University of Glasgow, and on nine additional cases of hypercalcemia complicating renal acidosis during the same period. Except for the low carbon dioxide content of the plasma, the findings of all 47 infants were essentially the same. Renal calcinosis is common to both diseases. The author commented that in this hospital, renal acidosis uncomplicated by hypercalcemia was rarely seen. In a study of 60 cases of nephrocalcinosis infantum, detected at autopsy, Shanks and MacDonald[116] observed that only 12 had belonged to the "renal acidosis" group: four were diagnosed as hyperchloremic renal acidosis, seven as idiopathic hypercalcemia, and one child had both. It was apparent that only a few of the children were recognized in life to have been suffering from one of the known causes of nephrocalcinosis. On going back over the records, vomiting was found to have been reported in 40, and constipation in 21. Prolonged administration of fortified milk and/or vitamin supplements were reported in the histories of 52. The authors presented the theory that symptomless hypercalcemia is a more wide-spread condition than has been appreciated, and that the resultant renal lesions may be detected only when the child happens to die of some other condition. It is noteworthy that in the Table giving the ante-mortem diagnoses of these patients (see TABLE 2), congenital cardiovascular abnormalities were listed in six instances, congenital central nervous system abnormalities in ten, and other renal abnormalities in three.

TABLE 2

DIAGNOSES OF 60 CASES OF NEPHROCALCINOSIS SEEN DURING 1948-57

	Congenital Abnormality	Infection	Miscellaneous	Total	Percentage Total Cases
System:					
Alimentary	5	14	8	27	45
Respiratory	–	16	–	16	26
Cardiovascular	6	–	–	6	10
Central nervous....	10	4	4	18	30
Genito-urinary (excl. nephrocalcinosis)	1	1	1	3	5
Miscellaneous Conditions:					
Renal acidosis			5		
Hypercalcemia			8		
Hypophosphatasia..			1		
Others			6		

From SHANKS R.A. & A.M. MACDONALD. 1959. Arch. Dis. Child. 34: 115–119.

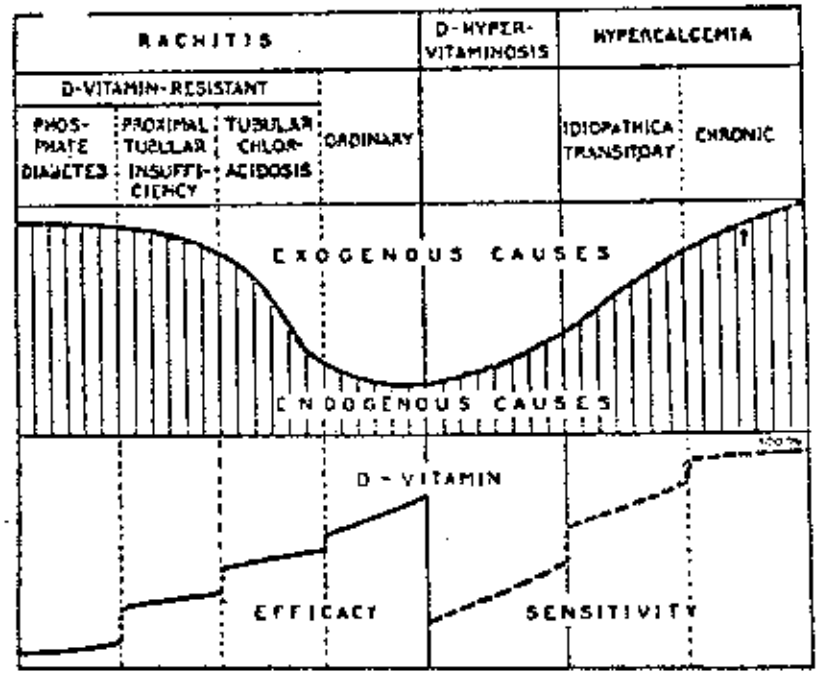

FIGURE 12. Spectrum of varying reactions to vitamin D. (Reproduced by permission of Fanconi, G. and Little, Brown & Co.)

DIFFERENCES IN SUSCEPTIBILITY TO VITAMIN D

Susceptibility to vitamin D ranges from that of children who require very large doses to prevent rickets to that of infants who respond to doses of vitamin D that are normally prophylactic by developing hypercalcemia. The diagram (FIGURE 12) from Fanconi[117] depicts the spectrum of response to vitamin D. In referring to vitamin D-resistant renal rickets, it is important to note that vitamin D is contra-indicated in the hyperphosphatemic form, and in that, associated with renal hyperchloremic acidosis. In the progressive form, in which the serum phosphate may be normal or low, nephrocalcinosis develops. As has been mentioned, the similarity of the findings in infantile hypercalcemia to those of hypervitaminosis D, in the face of intakes that were not excessive, has led to consideration of the possibility that the afflicted children are hyperreactive to vitamin D.[1,22,23,36,38,41-43,45,47,51,52,56,58,63,66,67,77,86,90-92,95-99,102,103,105,117-119,121]

Positive proof of vitamin intakes at periods of life earlier than that at which diagnosis is made is difficult to obtain retrospectively. Only a few infants, diagnosed as hypercalcemic during the first four months of life, had their intakes of vitamin D reported by the investigators. The infant reported by Stapleton *et al.*[46] who was diagnosed at four months of age with the early characteristics of vomiting, constipation, cessation of weight gain, azotemia, systolic murmur, and hypertension, had had no vitamin D for the first half month other than that in National Dried Milk which was used to supplement breast feeding. From then

on, the total daily intake of vitamin D from the milk formula was about 600 units. Once hypercalcemia was detected at four months, he was put on a low-calcium formula that also lacked vitamin D. The systolic murmur disappeared in one month; at 11 months he seemed normal. It was noted that the mother of this infant had received no pre-natal vitamin supplementation. The three infants reported by Michael *et al.*[97] similarly recovered on discontinuing vitamin D and being put on a low calcium diet. In one instance, the infant had received only 130 units daily for the first month, and 640 units of vitamin D daily for ten days until the diagnosis was made at six weeks. Another infant received a total of 375 units daily for the first month, and 1,000 units a day for the next three months, and then 2,000 units a day for one week until the diagnosis was made. The third infant in this series received a total daily dosage of 650 units for two months before diagnosis. The infant reported by Wilkerson,[98] who died at 15 weeks of age, had never received more than 500 units daily. The condition of this infant, who had marked arterio-venous, renal, and soft tissue calcinosis, as well as hypercalcemia, may well be classified as generalized arteriosclerosis of infancy, which is compared later in this paper to the acute form of experimental hypervitaminosis D.

Among the infants diagnosed as hypercalcemic at six to 20 months of age, the early vitamin D intakes were estimated at having been probably not over 2,000 units daily in over 50 cases. One of the patients reported early by Lowe *et al.*,[66] who was diagnosed at eleven months, but whose onset of clinically manifest disease was estimated from the history to have been at five months, had been breast-fed to three weeks of age, and had then been on cow's milk (probably not fortified). The intake of vitamin D from other supplemented foods was not taken into consideration. Another infant in this series had been breast-fed for four months, and was then given National Dried Milk formula until seven months, at which time clinical signs developed that led to the diagnosis of hypercalcemia at ten months of age. The third infant, who had been breast-fed for 11 weeks before being put on National Dried Milk formula, showed the first signs of the hypercalcemic syndrome at four and a half months, and was diagnosed at seven months. The remaining three infants in this series of cases had been on the fortified milk from birth. Only one of these six infants was also given cod liver oil supplements. Five survived and showed some clinical improvement on hospital diets lacking vitamin D. In this group, it is probable that after three to four months of age, all but one had received somewhat over 1,400 units of vitamin D, when all sources are considered. Total daily vitamin D intakes ranging from 500–1,400 units were reported for five infants by Forfar *et al.*,[67] who three years later reported[77] that among 20 hypercalcemic infants (possibly including the five children reported previously), only rarely had any received more vitamin D than that considered optimal. Schlesinger *et al.*[23] reported that among the ten hypercalcemic infants seen by them, they were unable to find any evidence of abnormally high intakes of vitamin D by the infants, or by their mothers, prenatally, in the four cases they were able to investigate fully. However, four of the infants had received National Dried Milk formula and vitamin supplements, and thus may have received as much as 2,000 units daily, from all sources. One, who had had a history of milk intolerance, was described as having received only 750 units in cod liver oil. In this instance, it is possible that the "milk intolerance" may have been an early manifestation of vitamin D toxicity from the National Dried Milk formula. One of the infants had been breast-fed and received "vitamin supplements" until six months of age. In this instance, the diagnosis was made at 14 months. Among the 38 infants

with hypercalcemia reported by Graham,[49] ten had received under 1,000 units daily, and two had no history of vitamin D supplementation. The infant reported by Bonham-Carter *et al.*[43] whose condition was diagnosed as hypercalcemia at nine months, had been breast-fed for three months before being placed on a fortified milk formula. There was no history elicited of excessive fortification, although this infant developed his syndrome during the period of excessive addition of vitamin D to infant foods and dried milk in England. Other infants reported from Great Britain during that time as not having received undue amounts of vitamin D include those reported by Stapleton *et al.*,[56] who, from their experience, postulated that there may be some humans who need no exogenous vitamin D, whereas others need much more than normal amounts.

Infantile hypercalcemia in American infants who had received total daily intakes of 1,200–1,400 units were reported by Bongiovanni *et al.*[95] in two cases, and by Schwartz,[42] Snyder,[45] Garcia *et al.*,[25] and Rushkind *et al.*[96] in a total of four more. Histories of somewhat lower vitamin D intakes were reported by Fellers and Schwartz[91] in three infants, who were shown to have very high blood levels of vitamin D (20–30 times normal values). David *et al.*'s[61] patient had no record of excessive vitamin D, although the exact intake had not been elicited. Daeschner and Daeschner's[92] patient, who died at 32 months, had had a daily vitamin intake of 1,000 units. The three hypercalcemic infants diagnosed at eight months by Fraser *et al.*[26] in Canada, had received no more than 800–1,000 units of vitamin D daily. In two instances, the mothers were known to have received 1,000 units daily of vitamin D during pregnancy.

Histories of supplementation early in infancy, although not very reliable when obtained from families of children diagnosed in later childhood, seem to indicate that in at least 11 instances, excessively high intakes were unlikely. Black and Bonham-Carter[7] obtained data that indicated that among their five cases diagnosed in late childhood, two had had "usual" supplementation, and three had been breast-fed for one and a half to six months, before receiving "usual" care. In two of the instances, the mothers reported that they had received the "usual" vitamin supplements prenatally. Seven such patients reported in the United States had histories of irregular vitamin supplementation,[54] no more than "usual prophylactic doses,"[93,102] 1,000–1,500 units daily for five years,[58] and less than 1,000 units daily.[103] The last two patients, twins reported by Wiltse *et al.*[103] were born to a mother who had received less than 1,000 units of vitamin D/day during pregnancy. The patient reported by Gribetz and Wolf[102] is of interest in that hypercalcemia, renal damage, abnormal electrocardiographic tracings and apical systolic murmur and osteosclerosis were diagnosed at ten years of age. His history suggested possible mild hypercalcemia during infancy, at which time he had had polyuria, polydypsia, and retarded growth, but no apparent mental retardation. Exploration parathyroid surgery at the age of 11 revealed normal parathyroids; follow-up three years later revealed a bright child, who had undergone a marked adolescent growth spurt. In retrospect, the investigators concluded that the history of this boy, and his blood laboratory findings, which included not only hypercalcemia, but also slightly elevated serum cholesterol, phospholipids and lipoproteins, suggested an intermediate form of idiopathic hypercalcemia.

In one of the early studies, Bonham-Carter *et al.*[43] found that loading of a hypercalcemic baby in remission with high doses of calciferol led to deterioration of his condition, concomitant with a rise in plasma calcium. On verification of the role of vitamin D, his intake of vitamin D and calcium was reduced with

subsequent improvement. Hubble,[87] however, gave large doses of vitamin D to two children with the mild form of the syndrome, without producing recurrence of untoward symptoms. Forfar[120] has administered large doses of calciferol (50,000–100,000 units) for about a week to three infants with hypercalcemia. In two instances, after a few days, the serum calcium rose significantly. It took about three weeks for the serum calcium to fall to pre-calciferol levels. In one case the serum calcium level fell, following the administration of calciferol, and rose again to the pre-calciferol level within a day of stopping treatment. Although hypercalcemia is an essential feature of the syndrome under discussion, the response to vitamin D loads and the degree of elevation of serum calcium apparently differ from case to case. Fraser *et al.*[26] have recently commented on the wide fluctuations in serum calcium which they have observed to occur spontaneously during the acute phase of the disease. Using reproducible methods for measuring serum calcium, they have seen variations of 2 to 3 mg per 100 ml within a few days in a single hypercalcemic patient. Girardet[112] has suggested that in certain individuals, even large doses of vitamin D may produce toxic effects without causing a substantial or sustained rise in serum calcium. His impression was based on the development of transitory albuminuria and cylindruria, with elevated NPN, after administration of single doses of vitamin D as large as 600,000 units, despite rapid return to normal of blood calcium levels that did not exceed 11 mg%.

Metabolic balance studies, showing the high percentage of dietary calcium that is absorbed (60%), has suggested to investigators that either an overaction of vitamin D,[43,82] or defective inactivation of vitamin D[47] might cause the syndrome. Assays of vitamin D in the plasma of hypercalcemic infants have led to determination of insignificant differences from normal values in two reports,[119,121] and to clear demonstration of markedly higher than normal values in others.[25,58,90,91] Black[38] has tabulated the serum levels of vitamin D in published reports of different degrees of infantile hypercalcemia and has commented on the conflicting results. He called attention to the failure of the currently used rat line test to demonstrate the vitamin D equivalence (in man) of dihydrotachysterol (Thomas *et al.*[100]). He has cautioned that it should not be assumed that the rat test for vitamin D activity necessarily will detect every agent in the serum which is capable of raising the serum calcium level in the human infant.

Garcia *et al.*[25] reported a serum value of 1,540 units in a child whose supravalvular aortic stenosis was correlated with hypercalcemia, and whose daily vitamin D intake was 400 units from milk and 1,000 units from a supplement. (The normal range of serum vitamin D in this institution was 60–400 units.) Fellers and Schwartz,[90,91] reported serum vitamin D activity of three patients with hypercalcemia to be 20 to 30 times that seen in normal infants, even in the absence of exogenous vitamin D. Two of a set of triplets, whose vitamin D intake during their first half year of life far exceeded the amounts now considered advisable, were diagnosed by Manios *et al.*[122] as hypercalcemic by their 23rd month, at which time all vitamin D was removed from their diets. Manios and Antener[105] then, three months later, demonstrated that serum vitamin D levels rose twofold from pretreatment values of 520 and 580 (as compared with the normal mean of 133 units, and the average range of 50 to 200 units), following administration of 700 I.U./d vitamin D in oil for four days.

These observations suggest that a defect in the metabolism of this vitamin plays a role in this condition. Forfar and his co-workers[67,77,120] observed a marked increase in the free cholesterol, as well as in the calcium levels in

hypercalcemic infants. In view of the vitamin D-like effect of some cholesterol derivatives, these investigators have suggested that this disease may be related primarily to a disorder of cholesterol metabolism. In his doctoral thesis,[120] Forfar has postulated that calciferol may act as a chemical mediator in this disease by activating endogenous vitamin D precursors present in excess in susceptible infants. This concept may explain the relatively small intake of vitamin D associated with hypercalcemia of infancy, and the prolonged vitamin D activity in such infants after withdrawal of all exogenous sources. Black[38] has reviewed several theories involving vitamin D indirectly in the pathogenesis of infantile hypercalcemia. These include (1) deficiency of unsaturated fatty acids, (2) adrenal insufficiency, and (3) hypothyroidism, as well as the disordered cholesterol metabolism already referred to.

Reactivity to Vitamin D by Race

Variations in endogenous precursors may explain the marked variations in susceptibility to rickets seen in different racial groups. In a study in Toronto, reported in 1937 by Drake,[123] of 244 infants of British or Northern European stock, who had received no vitamin D, only 13% had rickets of a moderate or marked degree. At the end of a five-month observation period, 21% had active advancing rickets. The preceding year, Lewis[124] included a control group of 22 infants on no vitamin D in a New York City study of vitamin requirements. Of these infants, observed during a five-month period from December through April, eight or 36.3%, developed rickets. None of the white children in this control group was affected, whereas eight of the 13 Negro children developed roentgenographic evidence of rickets. Thus, of the Negro infants, rickets developed in 62% of those receiving no vitamin D. Of the 27 infants who received crystalline vitamin D in various doses and who developed rickets, 21, or 78%, were Negro. (In this study 55% of the infants were Negro, and 45% Puerto Rican or Italian.) Follis *et al.*[125,126] reported that in a series of 1,303 subjects coming to autopsy during the first two years of life, about 50% of the infants who had definite histories of receiving no vitamin D had rickets. In this group, also, it was observed that the rachitic lesions were more prevalent and severe in Negro children than in whites.

The amount of vitamin D required by the two racial groups to heal rickets or to prevent rickets was also found to vary markedly. In Drake's study,[123] as little as 300 USP units of vitamin D, administered as irradiated ergosterol, were sufficient to heal rapidly rickets of moderate or marked degree. As little as 95 units daily in irradiated milk, or 150 units of vitamin D as cod liver oil or viosterol supplements, prevented the development of rickets. No evidence was obtained that this small amount was less effective in preventing rickets, as measured by roentgenograms, than were the larger amounts usually administered. In the population group reported by Lewis,[124] larger amounts of vitamin D were required to prevent rickets. His data showed that about 14% of 58 infants given 145 USP units in oil or propylene glycol developed moderate to severe rickets, but only 5% showed mild rachitic changes if given the same dose of vitamin D in milk. When the dose of vitamin D in oil or propylene glycol was increased to 290 units, 10% of 51 infants developed rickets; 2% showed early changes on 290 units in milk. On increasing the dose of crystalline vitamin D in corn oil to 1,450 USP units, one Negro child developed a mild form of the disease. Since no hazard was envisioned from such a dose, he recommended addition of 332 USP units to milk to achieve at least this potency. A later paper

by Glaser *et al.*[127] presented evidence that all preparations of vitamin D in an oily vehicle, given as vitamin D_2 (prepared by electron activation of ergosterol), viosterol (ultraviolet-irradiated ergosterol), crystalline D_2, or vitamin D_3 (ultraviolet-irradiated 7-dehydrotachysterol), were effective in daily doses of 100 units in preventing clinical rickets even in premature and in Negro infants. However, because nine infants developed roentgenographic evidence, or suspicion of rickets, among the 166 who were followed for the full observation period of eight months, the authors recommended the use of 400–800 units (in oil) as a prophylactic dose.

Some insight into the difference in vitamin D requirements by racial groups may be gained by examining the findings of Warkany and Mabon.[128] They tested blood samples from 34 white children and 26 Negro children (see TABLE 3) and found that the average value of 122.6 units for the white children was about 15% higher than that for the Negro children (106.5 units). Perhaps more significant in considering factors that may play a role in diseases that may be caused by either a hypersusceptibility or a hyposusceptibility to vitamin D is the distribution of values. Six of the white children had levels twice as high as those of nine of the black children (165 and 83 units, respectively). The possibility that the generally higher anti-rachitic blood levels and greater response to vitamin D in white than in Negro children may be a consequence of the screening of ultra-violet rays by the skin pigment must be considered.

TABLE 3

FREQUENCY DISTRIBUTION OF PERSONS ACCORDING TO LEVELS OF VITAMIN D IN BLOOD SERUM

U.S.P. Units per 100 cc. of Blood Serum	Total No. Tested	White Adults	White Children	Negro Children
165	9	2	6	1
132	27	10	11	6
110	31	14	10	7
94	8	2	4	2
83	13	1	3	9
66	1	1	0	0
Total	89	30	34	25
Average vitamin D level, units per 100 cc	116.4	117.6	122.6	106.5

From J. Warkany & H.E. Mabon. 1940. American J. Dis. Children.

The work demonstrating the ability of melanin or keratin in the skin to screen out ultra-violet light, thereby allowing for much greater exposure to sun without formation of excessive vitamin D, has recently been summarized by Loomis.[129] On the basis of the observation by Bekemeier[130] that 1 sq cm of white human skin synthesizes up to 18 units of vitamin D in three hours, Loomis[129] calculated that exposure to the skin of the cheeks of fair, thin-skinned children provides about 400 units daily. He postulated that as man moved north, and as he was exposed less and less to the rays of the sun, the fairer-skinned individuals had a survival advantage over those with darker skins,

who w[illegible]ore subject to developing rickets.

It is noteworthy that with only one exception, all of the children with infantile hypercalcemia reported in the literature reviewed in this paper were white. Similarly, generalized infantile arteriosclerosis, a disease not often correlated in the literature with vitamin D, presents a pathological picture that strongly resembles that seen in experimental vitamin D toxicity (*vide infra*), and is also found predominantly in white infants,[131] only two colored infants having been reported with this condition.[98,132] Daeschner *et al.*[114] reported that in a clinic with a significant proportion of Negro or Latin American children, there was a much higher incidence of urinary tract calculi in the white children. Conversely, since the lowering of vitamin D in infant foods and supplements in Great Britain, the incidence of rickets was not found to have increased in white children, but did in colored immigrants.[133]* As for the postulated late normocalcemic stage of infantile hypercalcemia, the Williams' syndrome, that too has been reported predominantly in white children. Several colored children with supravalvular stenosis, however, have been reported.[18,31]

Although the trait responsible for hyper-reactivity to vitamin D predominates in fair-skinned children, it is apparently not limited to that racial group. It seems unlikely that difference in skin pigment, alone, can account for the extremes in reactivity to vitamin D. Additional metabolic differences in handling vitamin D must also exist.

EFFECT OF VEHICLE ON VITAMIN D POTENCY

The greater efficacy of vitamin D in milk, in preventing rickets, as compared with vitamin D in oil or in propylene glycol,[123,124] was foreshadowed in 1935 by Stearns and Jeans[84,134,135] who calibrated the effect of different doses of vitamin D against the growth of infants. They showed that infants given 300–400 units of vitamin D in cod liver oil grew at faster than average rates, whereas infants getting all of their vitamin D from cod liver oil emulsified in milk required only 135 units per quart for the same rate of growth. Their demonstration that daily vitamin D intakes of 1,800–4,000 units caused a gradual slowing of growth, which immediately increased after the intake had been reduced to 400 units in cod liver oil, has since been criticized as not standing up to modern tests of significance.[26]

The clinical reports by Drake,[123] Lewis,[124] and Stearns and Jeans[84,135] indicated an approximately threefold increase in potency of vitamin D when it was given in milk. An even greater enhancement effect by milk of the potency of vitamin D was indicated by Lewis'[136] clinical experience with the anti-rachitic effect of crystalline ergosterol in oil or in milk (see TABLE 4). He demonstrated that the therapeutic effect of 90 units of crystalline vitamin D in milk healed all nine rachitic infants in his study, as demonstrated by roentgenogram at four, six, eight, and ten weeks. Only 45 units in milk were curative in six out of eight rachitic infants. Of the nine infants who received 90 units in oil, three showed no healing at six weeks, and the rachitic process became worse at the end of eight weeks in two. One of the infants in this group, who showed advancement of the rachitic process at four weeks, was then given 90 units in milk with resultant definite healing in four weeks, and calcification in the rachitic zone

*A few cases of florid rickets have since been reported among white children, in cases of neglect (Editorial, Brit. Med. J. 1956. 558:

TABLE 4

COMPARISON OF HEALING, AMONG 36 RACHITIC INFANTS, BROUGHT ABOUT BY CRYSTALLINE VITAMIN D INCORPORATED IN MILK OR IN CORN OIL

Case	Age (Mo.)	Date Begun	Menstruum in which Crystalline Vitamin D was Incorporated	No. of Rat Units (Steenbock) Given	Roentgenologic Rickets: At Onset	Healing after 4 Wk.	Healing after 6 Wk.	Healing after 8 Wk.	Healing after 10 Wk.	Comment
C.M.	8	1/23	Milk†	45	Moderate	+	++	+++	++++	Porto Rican
B.G.	6	1/25			Moderate	+	+±	++	++±	Porto Rican
F.S.	13	1/26			Moderate	0	0			Negro
E.R.	6	2/7			Slight	+	++	+++±		Italian
G.N.	8	2/18			Moderate	+	++±	++	+++	Porto Rican
C.W.	22	2/17			Marked	++	++	++±		Negro
G.B.	8	2/21			Moderate	++	++±	+++±		Negro
J.N.	6	2/24			Mild	0	0	0 (worse)		Negro
V.L.	6	1/13	Milk‡	90	Slight	++	+++	Normal		Italian
S.Q.	4	1/13			Moderate	++±	++±	+++±	++++	Porto Rican
J.A.	5	1/13			Slight	+++	++++	Normal		Negro
R.L.	5	1/19			Moderate	+++	Normal	Normal	Normal	Negro
C.H.	20	1/25			Marked	+++	+++±	++++	Normal	Negro—1/25: calcium, 9.8 mg.; phosphorus, 3 mg. 2/23: calcium, 10.6 mg.; phosphorus, 5 mg.
K.S.	4	2/22			Slight	++	+++	Normal		Negro
B.C.	7	3/2			Moderate	+				Porto Rican
F.S.	15	3/17			Moderate	+	+±			Negro
B.R.	6	3/17			Moderate	+±	+++			This infant had received 90 units of crystalline vitamin D in oil for one month, and no healing resulted

TABLE 4 (cont)

Case	Age (Mo.)	Date Begun	Menstruum in which Crystalline Vitamin D was Incorporated	No. of Rat Units (Steenbock) Given	Roentgenologic Rickets: At Onset	Healing After 4 Wk.	Healing After 6 Wk.	Healing After 8 Wk.	Healing After 10 Wk.	Comment
T.R.	5	2/4	Oil†	90	Moderate	+±	++±	+++	++++	Porto Rican
S.L.	7	2/12			Slight	?	+	+±		
B.M.	4	2/14			Slight	+	++		++	Negro
B.R.	5	2/16			Slight	0 (worse)				Negro
V.B.	14	2/16			Marked	+	+±	++	+++	Negro
D.R.	7	2/21			Moderate	0	0	0 (worse)	0	Negro
C.O.	5	2/23			Slight	?	+	+±	++±	Porto Rican
C.B.	8	2/28			Moderate	0	0	0 (worse)		Negro
T.B.	8	3/19			Moderate	0	0			Negro
J.B.	3	2/16	Oil§	900	Slight	++		Normal	Normal	Negro
P.G.	6	2/9			Slight	++	++++			Italian
J.B.	6	2/16			Slight	++	++±	+++	+++±	Negro
T.R.	6	2/16			Slight	++	++++	Normal		Negro
C.M.	6	2/16			Moderate	++				Negro
R.P.	17	2/17			Marked	+				Negro
H.B.	4	2/17			Slight	++	+++	+++±	Normal	Negro
J.D.	7	2/17			Moderate	++	++±	+++	+++±	Negro
N.E.	3	2/17			Slight	+	++	++±		2/15: calcium, 7.2 mg.; phosphorus, 5.3 mg. 3/13: calcium, 10.3 mg.; phosphorus, 6.1 mg.
J.D.	24	1/30			Marked	+	+	+	++	Negro; at 12 weeks healing ++

*X-ray picture taken at 13 weeks.
†One liter contained 60 units.
‡One liter contained 120 units.
‖One drop contained 15 units.
§One drop contained 150 units.
+Denotes slight healing; ++, moderate healing; +++, marked healing; ++++, healed rickets.

from LEWIS, J.M. 1935. J. Pediatrics 6:362-375.

two weeks later. Administration of 900 units of vitamin D in oil to ten rachitic infants resulted in beginning improvement within four weeks in all. One Negro baby, however, who had marked rickets, showed only slight to moderate healing by the end of 12 weeks. Lewis concluded that the 900 units in oil was a good therapeutic dose for mild or moderate cases of rickets, but might not be adequate for severe forms. He commented that the therapeutic results obtained from 90 units in milk were superior to those obtained with 900 units in oil.

Supplee *et al.*,[137] intrigued by the evidence that vitamin D in milk was a more effective anti-rachitic agent than vitamin D in oil, undertook an evaluation of the factors in milk that might have accounted for this enhancement effect. Using the rachitic rat assay technic, they found marked differences in the anti-rachitic response, depending on the vehicle in which the vitamin D had been delivered. Milk and even an isolated milk constituent, lactalbumin, were shown to have enhanced the potency of vitamin D. The combination of the lipid-free lactalbumin with the vitamin was found to have exhibited a greater anti-rachitic potential than the lactalbumin-vitamin in combination with lipids. They concluded that vitamin D administered in the water phase was more effective clinically than when administered in an oil vehicle.

Since vitamin D in milk is much more active than when it is administered in oil, the addition of 400 units to a quart of milk may be the equivalent of several times that quantity administered in oil. For the children with a high intake of milk, it can be presumed that the daily intake of vitamin D is undoubtedly higher. Were there no risk in providing therapeutic dosages of vitamin D to the entire population instead of prophylactic doses, this excess over that considered sufficient by the Committee on Nutrition of the American Pediatric Association (400 units from all sources)[104] could be safely disregarded. There is evidence, however, that infantile hypercalcemia, renal acidosis, and nephrocalcinosis may well be related to moderately high intakes of vitamin D by children with high susceptibility to this vitamin.

HISTOPATHOLOGIC FINDINGS IN ANIMALS WITH EVIDENCE OF VITAMIN D-TOXICITY: CORRELATION WITH CLINICAL SYNDROMES

It is clear from the foregoing that there is a wide range of response to vitamin D. A dose little more than that usually necessary to prevent rickets may produce severe toxicity in some children even though it is well tolerated by most. The lesions produced in such susceptible children are very similar to those produced in experimental animals by toxic doses of vitamin D.

Experimental Hypervitaminosis D

As early as 1927–1928, Pfannenstiel[138] and Kreitmair and Moll[139] reported pathological changes in animals given large doses of irradiated ergosterol. Kreitmair and Moll[139] described the pathological effects of 0.2 to 10.0 mg/day in mice, rats, guinea pigs, rabbits, cats, and dogs. Death ensued after six days to three months; postmortem changes included massive deposits of calcium in arteries, kidneys, heart, and other organs. In 1929 Harris and Moore[140] showed that the toxic effects are specifically attributable to the vitamin. Duguid[141] observed, in 1930, that there was a considerable variation in susceptibility of their rats to 10 mg of irradiated ergosterol in olive oil fed for a maximum of 34 days. Of 120 rats fed the supplement for 25 days, only 71 showed aortic lesions, and 92 showed lesions in the kidneys. The earliest signs of vitamin D toxicity

developed in the kidneys, which showed calcareous casts in the tubules and, occasionally, calcification of the interstitial tissue. One of the aortic lesions found chiefly in the media consisted of muscular degeneration. Calcification of the elastic lamina seemed to have occurred early, with calcification in the muscular element of the media occurring only following the degenerative changes. Such calcification was seen first and most frequently in the ascending portion of the aorta, including the sinus of Valsalva. The intimal changes, which were minimal, were considered to have been secondary to the medial damage. A year later Vanderveer[142] injected young rabbits subcutaneously with 5 cc/kg of irradiated ergosterol twice weekly until the deaths of the animals. He, too, found the earliest calcareous lesions to have been intimately related to the elastic fibers of the aorta. In these experiments, the calcium was deposited over the elastic fibers and muscle cells before either showed degenerative changes. Focal areas of muscle degeneration were seen in the aorta. In some animals, extensive calcium deposition was seen in the myocardium, as was some sclerosis of the coronary arteries. Generally there were calcium casts in the tubules of the renal cortex, and in some animals there were also parenchymal calcareous deposits. Interested in the sequence of development of the deforming, sclerotic changes of the ascending and transverse portions of the aortas of animals that died of poisoning with vitamin D, Spies[143] studied the effect of sublethal dosage on young rabbits. They were sacrificed 97–102 days after the final dose of viosterol was given. The walls of the ascending aorta and transverse arch were calcified, irregular, and tortuous. There was aneurysmal dilatation in some areas and reduction in caliber of the vessels in others (caused by protrusion inward of the arterial lesions). The internal elastic lamina of the media was most severely affected. Comparison of the lesions of this group of animals with those of rabbits given massive doses that caused death[144] led Spies to speculate that degeneration of the affected cells and reabsorption of some of the calcium may have taken place after ceasing vitamin D administration. Similarly, the kidneys of these rabbits had strikingly fewer calcium deposits than those of the acutely poisoned animals, suggesting to the investigator that the calcium deposits may have been reduced.

To determine whether the calcifications caused by vitamin D toxicity were degenerative changes, or merely due to precipitation of calcium from the blood, Ham[145] administered enormous single doses of irradiated ergosterol to one series of rats, and daily doses to another. Twenty-four hours after the single dose, no lesions were seen; within 48 hours massive calcifications were seen in the aorta, coronary arteries, and cardiac musculature, around which was a marked inflammatory cell infiltration. He concluded that, among other factors, the precipitation of the calcium depended on the saturation of the serum with diffusible calcium. On the other hand, Steck *et al.*[146] found evidence, in a study of 64 dogs given 15,000–500,000 units of vitamin D/kg daily, that cellular injury occurs first and that calcium deposition then takes place at the site of injury. The influence of vitamin D on the calcium and phosphorus content of tissues of dogs was studied by Reed *et al.*[147] in 1933. Dogs on high dosage irradiated ergosterol showed higher concentrations of calcium in all tissues analyzed (see TABLE 5). The greatest increments were seen in the heart and kidneys. The increase in the content of calcium in the brain is of particular interest, since it provides a clue to the cerebral damage that is probably associated with the mental retardation seen in children with infantile hypercalcemia. There was no direct correlation between the calcium content of the tissues and the total amount of viosterol injected per kilogram of body weight or

TABLE 5

THE CALCIFICATION OF TISSUES BY EXCESSIVE DOSES OF IRRADIATED ERGOSTEROL

Tissue	Normal mean a	Ergosterolized mean b	$E_\Delta = \sqrt{a^2+b^2}$ *	Δ/E_Δ †	Probability
Calcium, mg per 100 g					
Adrenal	37.6 ± 3.24	203.1± 81.9	81.9	2.02	0.04
Aorta	64.23± 6.84	175.3± 35.7	35.9	>3	<0.01
Brain	55.4 ±14	125 ± 16.7	21.8	>3	<0.01
R. ventricle	2.27± 4.43	149.6± 37.69	37.9	2.9	<0.01
L. ventricle	18.3 ± 1.64	220.4± 63	64	>3	<0.01
Kidney	55.6 ±15.9	671.6±236.3	236.8	2.6	<0.01
Liver	17.9 ± 1.22	122.8± 45.1	45.5	2.33	0.02
Lung	88.6 ±13.2	338.4±160.2	165	1.514	0.13
Muscle	17.8 ± 1.16	64.8± 13.3	13.4	>3	<0.01
Skin	23.6 ± 1.95	69.8± 16.1	16.2	2.85	<0.01
Spleen	14.3 ± 2.19	100.8± 21.26	21.4	>3	<0.01
Thyroid	101.3 ±22.9	509.3±144.66	147	2.77	<0.01
Phosphorus, mg per 100 g					
Adrenal	595.8 ±30	648 ±109.18	113.3	<1	0.64
Aorta	272.2 ±18.4	315.4± 13.1	28.07	<1	0.12
Brain	1317.3 ±31.5	1018.3±130	164	1.82	0.07
R. ventricle	708.1 ±37.7	736.9± 42.36	50.6	<1	0.61
L. ventricle	767.3 ±50.3	694.5± 49.2	70.4	1.34	0.27
Kidney	815.8 ±74.3	994 ±126.5	146.7	1.21	0.23
Liver	790 ±24	681.5±104.1	106.8	1.02	0.30
Lung	865 ±53.3	977.6±163.6	172	<1	0.51
Muscle	699.3 ±43.6	536.7± 47.7	60.4	2.19	0.03
Skin	152.3 ±37.1	280 ± 50.5	62.5	2.05	0.04
Spleen	925.1 ±40.8	901.3± 59.4	72.12	<1	0.74
Thyroid	554 ±57	712.6±119	132	1.2	0.23

*$E_\Delta = \sqrt{a^2+b^2}$, a=standard deviation of normal series.
b=standard deviation of experimental series.
†Δ/E_Δ Δ=difference between means of the two series

From Reed, C.I., E.A. Dillman, E.A. Thacker, & R.I. Klein. 1933. J. Nutr. 6: 374.

the size of the individual injections. There were wide variations in the amounts of vitamin D that were toxic, but there was a tendency towards a comparable degree of calcium deposition in most of the tissues of any given animal.

Because the arterial sclerosis produced by vitamin D in the experimental animals seemed to have involved primarily the media, whereas that seen in man was usually correlated with intimal damage associated with hypercholesterolemia, Harrison[148] studied the combined effect of sequentially administered cholesterol and vitamin D in rabbits. In those cases in which the vitamin D was given after challenge with cholesterol, medial sclerosis was produced in aortas that were already the seat of severe cholesterol sclerosis.

although the two different types of lesions were found in different areas and were not superimposed. Both types of lesions favored the proximal half of the aorta including the arch. It is of interest, in this regard, to recall that children with hypercalcemia of infancy have not only elevated calcium levels but also elevated blood cholesterol.

Gillman and Gilbert[149] investigated factors that might explain the greater incidence of cardiovascular disease among the white population, as compared with that among Africans (who consumed diets low in calcium and cholesterol). Differences in metabolism of calcium and phosphorus were considered factors that might play a role in the variations in susceptibility to arteriosclerosis. Since hyperlipemia may exist even when the diet is low in fat, and the deposition of lipids in tissues is dependent upon complex biochemical processes, they considered the possibility that such injury to the arterial wall as was caused by vitamin D might have predisposed to atheromatous plaque formation. In their intensive studies of 300 rats, ranging in age from three to six months, fed one to five daily doses of 25,000 units of vitamin D as crystallized calciferol in oil, they observed a wide range of reactions. It was apparent that not all rats developed toxic reactions to the same dose of the vitamin. One of the commonest reactions seen was mild damage to the arterial endothelium with migration of monocytoid elements and polymorphs into the superficial part of the media and lifting of the overlying endothelium. Necrosis of the intima was associated with metachromasia and disruption of the elastica. Damage to the endothelium and subendothelial tissues in the aorta was confined to the sinus of Valsalva and the first part of the aortic arch. Mild subendothelial edema was accompanied by a variable degree of metachromasia and accumulation of Schiff-positive material. Depending on the degree of involvement, there was lesser or greater infiltration with white cells. The mildest form of medial injury was edema, with presence of Schiff-positive material and metachromasia, changes which were usually preceded by cellular swelling or even endothelial degeneration at the site. Calcification of the elastic lamina, followed by degeneration of the muscle cells, preceded replacement of the degenerated fibers by large, clear, fluid-filled spaces that widely separated the elastic fibers. At this stage, the inner part of the wall was thrown into folds. Intense subendothelial and medial calcification, in some instances, led to the formation of solid plates, which were often progressively covered by multiple layers of endothelial cells. The calcium plate gradually became reduced and more deeply embedded in the much thickened wall of the aorta. Animals sacrificed 150 days after the last dose of vitamin D showed aortas with the original calcified portions replaced by a thick, cartilage-like material with calcified tissues forming irregular bands in the deeper layers of the aortas. By graded administrations of vitamin D, Gillman and Gilbert[149] were able to demonstrate that myocardial injury and calcification were preceded by swelling and intense eosinophilia of the muscle cells. After discontinuation of vitamin D, repair of the degenerated myocardial area took place, with hypertrophy of the neighboring muscle cells and reestablishment of the capillary networks. Valvular lesions, the earliest changes in which were subendothelial edema and intense metachromasia similar to that seen in the aorta, were also common after toxic doses of vitamin D. Calcification of the valves was uncommon.

Recently Coleman,[64,65] who demonstrated electrocardiographic changes in patients with infantile hypercalcemia and considered the changes as probably indicative of left ventricular myocardial damage, administered vitamin D in oil orally to 18 young rabbits (40-420 units D2; 100-770 units D3).[65] Aortic lesions were found in every rabbit given vitamin D, but in none of the controls. The inner half of the media was affected, and in some instances there was

intimal thickening. Where there was distortion or disruption of the elastic fibers, and muscle cells were destroyed, there was calcium deposition. Two of the rabbits died in congestive heart failure; four others had focal myocardial lesions, consisting in three of periarterial muscle necrosis, a cellular reaction, and staining reaction suggesting calcium deposition. Ten of the rabbits given vitamin D had lesions immediately subjacent to the endocardium. Serum calcium levels higher than those of control animals were found in the experimental rabbits for 32-38 weeks after discontinuing administration of vitamin D.

It is worthy to note that in the series of experiments with rats reported by Gillman and Gilbert,[149] there was another similarity to the syndrome of infantile hypercalcemia, which includes failure to gain weight, atony, and muscle wasting. Toward the end of the five-day period, during which very high doses of vitamin D were given, there was a precipitous loss of weight, with obvious wasting, and severe frank degeneration of the skeletal muscles.

More recent work has emphasized the role of the mucopolysaccharides in the lesions caused by vitamin D.[150-153] Eisenstein and Groff[150] reported that rats given massive doses of vitamin D2 showed not only elevated serum calcium levels but also increases in seromucoid. The deposition of calcium in the soft tissues (renal tubular casts, in myocardium at the site of inflammation, and in the aorta), took place in a matrix composed at least partially of an unidentified polysaccharide. Gillman and Hathorn[151] found that the initial aortic lesions (in rats) appeared six to 15 days after the first dose of calciferol, and were localized around the medial vascular elastic membranes just deep to the intima. Calcification occurred early and was associated with local accumulation of nonsulphated P.A.S.-positive polysaccharide. If the dose of vitamin D was not very high, the initial mineralization sometimes resolved in the next six to ten days, after which the tissue between the injured elastic membranes became laden with metachromatic mucopolysaccharide. In such instances, regeneration of the original vascular architecture took place. With more severe or prolonged injury, fibrosis and scar formation occurred, preceded by localized accumulation of metachromatic, sulphated mucopolysaccharides. In their later studies of the histochemical and chemical changes associated with the soft tissue calcification following acute intoxication with vitamin D, Gillman *et al.*[152] identified the tissue polysaccharides. They, too, found a rise in serum mucopolysaccharides, which was prolonged for about two-thirds of the entire observation period of 160 days in contrast to the hypercalcemia, which persisted for only ten days. The levels of aortic hexose and hexosamine approximately doubled; these increases were not quantitatively related to the calcium content. The laying down of calcium was, however, closely paralleled by large increases in the phosphorus content of the aortas. The total aortic collagen appeared to have increased toward the end of the experimental period, coinciding with the histological appearance of aortic and coronary sclerosis. This group of investigators found that the serum mucoprotein changes of calciferol-intoxicated rats seemed to be more closely associated with necrosis and the subsequent healing process in the heart, kidney, and other soft tissues than with calcification.[153]

Most recently, on the assumption that the supravalvular aortic stenotic lesion may result from intrauterine damage caused by administration of vitamin D to the pregnant mother,[99] Friedman and Roberts[154] studied the young of pregnant rabbits given 1.5-4.5 million units of vitamin D. Since all rabbits on 2.5-4.5 million units died within 65 days after their first injection, the results of the study pertain only to the rabbits given 1.5 million units. The blood levels of

the pregnant rabbits and of their offspring were seven and nine times greater than those of the control rabbits and of their young, indicating that transplacental passage had occurred. All of the mothers showed the classic vitamin D-induced changes in the aortas. Fourteen of the 18 newborn rabbits had normal aortas, but 11 of these 14 showed an exaggeration of the supravalvular plica that was similar to that seen in five of the 23 control neonates. Four of the vitamin D-rabbit neonates, each from a different litter, had abnormal aortas. In each there was a prominent annular protrusion at the superior margin of the sinus of Valsalva, with resultant narrowing of the lumen. The protrusion was caused by medial proliferation; the intima was normal. Offspring of vitamin D-mothers that died at two to 20 days showed lesions of the aortas ranging from early medial degeneration and thickening at seven days, to triangular-shaped protrusions into the lumen of the aorta, caused by localized thickening of the aortic media. The six rabbits sacrificed at three months showed changes in their aortas quite similar to those found in the mothers given the vitamin. It should be noted that these changes were very similar to those reported by Gilfman and Gilbert[149] late in their 150-day rat study, and by Coleman[64] in young rabbits given very much smaller doses of vitamin D by mouth. Apparently the late results of acute vitamin D toxicity are similar, whether induced *in utero*, or in the young or adult experimental animal.

Note should be taken here of an inadvertent demonstration that administration of large daily doses of vitamin D early during gestation to a mother of a child with infantile hypercalcemia is not necessarily causative of infantile hypercalcemia. To ascertain whether the mother of a four-year-old patient with the supravalvular aortic stenosis-peculiar, facies-mental retardation syndrome was hypersensitive to vitamin D, Forbes *et al.*[155] gave her 10,000 units daily. To their dismay, after two and one-half weeks, the mother reported that she was pregnant. The vitamin D was immediately discontinued, and the milk intake of the mother was restricted to one pint of nonfortified, skim milk daily for the duration of her pregnancy. A normal child was born at term, who has remained normal throughout 18 months of follow-up.

Histopathology of Clinical Conditions Resembling Experimental Hypervitaminosis D

The histopathology described by Peron in clinical supravalvular aortic stenosis,[5] which included hyalinized fibrous tissue, as well as foci of necrosis and calcification, often associated with degenerated elastica within the thickened media of the aortas, bears a striking resemblance to the late lesions reported in the foregoing section. Infants dying early in the course of hypercalcemia had histological aortic changes bearing a significant resemblance to the earlier experimental lesions caused by vitamin D toxicity. Intimal and subendothelial thickenings with fragmentation, degeneration and calcification of the elastica, as well as medial and myocardial degenerative changes have been reported.[22,23,40,52,62,66,96,98]

At the autopsies of infants with generalized arterial calcification, changes resembling even more closely the acute vitamin D toxicity damage seen in laboratory animals have been observed. Stryker,[132] in 1947, reported four infants who died at two to seven months of age, and whose autopsies revealed calcification of the coronary and other arteries, in relationship to the internal elastic lamina. A fifth infant, stillborn to a 14-year-old girl, had similar arterial lesions, as well as focal calcification in the first portion of the aorta, myocardial necrosis and calcification, and multiple developmental anomalies. Foci of recent

myocardial infarction were found in two of the infants who survived the neonatal period; renal damage ranging from cloudy swelling of the parenchyma to marked calcification of the renal tubules was seen in two. Considering the possibility that hypervitaminosis D may be a causal factor in generalized arterial calcification of infancy, Stryker recorded the available information on vitamin D intake in his series of cases, as well as in the 15 cases he tabulated from the literature. In the case of the infant who died at three months, the family physician had suspected hypervitaminosis D but could elicit no history of unusual vitamin intake by either the mother or by the infant. It is of interest that the sibling of this infant was reported by the physician to have died with a similar clinical picture, but no autopsy had been done. The infant who died at seven months had been breast-fed, and had received two drops of a concentrated cod-liver oil twice daily during the first month. No history regarding vitamin D intake was obtained in the remaining three cases. Among the patients tabulated from the literature was one reported by Lightwood[156] of a child who died at 27 months. She had been found to have hypercalcemia (11 mg%) and albuminuria, and at autopsy showed fibrotic and calcified glomeruli, interstitial fibrosis and degeneration, fibrosis, and calcification of glomeruli and tubules. She had osteoporosis and osteosclerosis and was described as mentally retarded and dwarfed. Having been reported as having received only "physiological" amounts of vitamin D, at a time (1932) when there was no vitamin supplementation of food, this may be the first recorded case of infantile hypercalcemia caused by hyper-reactivity to vitamin D. Her mother had taken no vitamin D supplements during pregnancy. Among the remaining 14 infants, tabulated by Stryker, were two reported by Anderson and Schlesinger,[157] who had had moderate hypercalcemia and renal damage but who were reported to have had hyperparathyroidism; one of these two infants however, had received about four times the usual amount of vitamin D. All others had been given a single dose of 300,000 units. Menten and Fetterman[158] reported an infant who died of generalized arteriosclerosis and myocardial infarction, born to a mother who had taken no vitamin D during pregnancy, but who had taken two capsules of dicalcium phosphate daily. He had received five drops of oleum percomorph daily starting at two weeks. At one month he began to vomit; at 54 days he seemed cranky and upset—both symptoms that are now commonly considered characteristic of infantile hypercalcemia. At autopsy, all of the superficial coronary branches were found to be thickened, sclerotic and calcified, and the aorta was diffusely thickened. Histological examination revealed coarsening of the internal and medial elastica fibers of the coronaries, with calcification, and degeneration of elastic fibers of the media of the aorta, with calcareous thickening. Foci of myocardial infarction and fibrosis were present. Hyalinization of about 2% of the glomeruli was noted, as was calcific degeneration of occasional glomeruli and collecting tubules. The remaining two infants reported by Menten and Fetterman[158] were siblings, who died at six and seven weeks, respectively, and who showed generalized arteriosclerosis, myocardial infarction, and parenchymatous degeneration of the kidneys. The commonest change seen in the aorta was swelling and degeneration of irregularly distributed elastic fibers; there were also atheromatous thickenings, with thinning of the underlying media. The infant reported by Wilkerson[98] as having hypercalcemia of infancy, who had severe calcinosis of arteries, kidneys, and soft tissues on autopsy at 17 weeks, and who had never received more than 500 units daily of vitamin D, may also be grouped with the infants described above. Hunt and Leys,[159] who compared the pathological changes seen in two siblings who died at four weeks

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of age with those seen in infantile hypercalcemia,[22, 40] reported a marked predilection for calcification of the internal elastic lamina in the arteries of his patients and of others reported in the literature. In addition to calcification of the peripheral and pulmonary vessels, the infants also had fibrous intimal proliferation to the extent of obliteration of the lumen in some sites, calcium incrustation of the elastica throughout the aortic media, as well as renal calcification. One of these infants showed clear evidence of myocardial infarction, as did an eight-week-old girl reported by Traisman *et al.*[160] and another infant (one of two more siblings with this syndrome) reported by Moran and Becker.[131] The latter report included a summary of 44 published cases of arterial calcification in infancy. In all but two of these case, calcific material was reported in the coronary arteries, and fibrous intimal proliferation was seen in many other arteries in most of the cases.

Specific attention was called to the renal damage seen in the picture of generalized arteriosclerosis of infancy by Stryker,[132] and by Moran and Becker[131] who reviewed the published cases to date in 1947 and 1959, respectively. Moran and Becker suggested that metastatic arterial calcification may occur secondarily to renal damage. Their series of cases excluded any in which there was clear evidence of primary renal disease, although concomitant renal lesions, ranging from "local glomerulonephritis" or "changes" to calcification of renal arteries and/or tissue, was reported in most of the cases. The authors commented that the arterial lesions were indistinguishable from those seen in arterial calcification secondary to advanced renal disease, or associated with anomalies of the heart or great vessels, or known to be caused by hypervitaminosis D.

DISCUSSION

Among the diseases seen in infancy and childhood are several that seem to be relatively new, at least as regards frequency of reporting in the literature during the past 20 to 25 years. It is difficult to be certain whether supravalvular aortic stenosis (Williams' syndrome), hypercalcemia of infancy, renal acidosis, and generalized arteriosclerosis of infancy are detected more frequently because of the greater sophistication of the clinician, or whether they have actually increased in incidence during the past quarter of a century. That all are characterized by lesions seen in experimental hypervitaminosis D, and that during the same period vitamin D supplementation has become prevalent in those countries from which most of the cases have been reported, has been demonstrated.

The cardiac changes seen in the clinical conditions described bear a striking resemblance to those seen in rats and rabbits on high doses of vitamin D. About a quarter of the infants who died of generalized arteriosclerosis were found to have had myocardial lesions, described as focal necrosis, infarction, fibrosis, and areas of calcification.[131,132,157-160] Such lesions have also been reported in infants who died of infantile hypercalcemia, but far less frequently. In his review of the cardiac lesions of infantile hypercalcemia reported in the literature, and seen in his own clinic, Coleman[64] listed left ventricular or biventricular myocardial hypertrophy, myocardial fibrosis or calcification, and valvular endocardial lesions including fibrosis and calcification. Suspecting a myocardial lesion in one of the children with the supravalvular aortic stenosis syndrome, Williams *et al.*,[2] found that Denie and Verheugt[3] and Burry[4] had reported fibrous myocarditis and myocardial atrophy in similar patients. They suggested

that myocardial degeneration might be another facet of the syndrome. Perou[5] also found evidence of myocardial fibrosis in one of his cases of supravalvular stenosis. If, indeed, these three conditions represent increasingly severe manifestations of excessive response to vitamin D, it is not surprising that in the form characterized by infantile arteriosclerosis, there is focal myocardial necrosis or infarction and calcification, as has been reported in acute vitamin D toxicity studies in rats[145,149] and in rabbits.[65,142] The less acute myocardial lesions seen in older infants bear a resemblance to the later lesions reported in rats.[149] The myocardial fibrosis and atrophy seen in the children or young adults coming to autopsy with the supravalvular aortic stenosis syndrome may possibly represent the final cardiac phase of the disease. It is not known whether overdosage with vitamin D may contribute to renal calcinosis and cardiovascular disease in later life.

The stigmata of the Williams' syndrome include supravalvular aortic stenosis, mental retardation and a characteristic facies. Children with this disease resemble survivors of hypercalcemia of infancy, a disease associated with hyper-reactivity to vitamin D. The pathologic lesions of infants with acute hypercalcemia seen at autopsy[23,52,62,66,96,98] resemble those seen during the acute or subacute phase of experimental hypervitaminosis D in the laboratory,[141-154] and those reported in infants with generalized arteriosclerosis.[131,132,155-160] Arterial changes include intimal and/or subintimal thickening, with fragmentation and degeneration of the internal elastic lamina, and medial degenerative changes, thickening, and metastatic calcification, particularly in the aorta. In the kidneys are seen glomerular ischemia, with endothelial proliferation, periglomerular fibrosis, reduction in numbers of tubules, flattened tubular epithelium, hyaline, granular, or calcareous casts, and calcium deposits in lining cells and in basement membranes. Survivors of the acute phase of the disease, who had the peculiar facies characteristic of the Williams' syndrome, and who exhibited cardiac damage, hypertension, and supravalvular stenosis, at autopsy[7,24,34,78] were found to have had lesions that resembled the supravalvular aortic lesions reported by Perou in "congenital" aortic stenosis,[5] and by Gillman and Gilbert[149] late in the course of vitamin D toxicity in rats. It is noteworthy that the aortic stenotic lesions described by Friedman and Roberts[154] in three-month-old rabbits, whose mothers had been given an enormous dose of vitamin D during gestation, were similar both to those reported by Perou[5] and by others[3,11,33] in clinical supravalvular stenosis and to the lesions in rats after loading with vitamin D.[149] The renal changes seen in such children either at autopsy or after biopsy included contracted, granular kidneys with numerous areas of calcification[7,24,33,74,101] and were similar to those seen as a late expression of vitamin D toxicity in the experimental animal.[141,144]

From the available evidence, it seems unlikely that the pathologic findings in these severe clinical syndromes, which are so similar to those produced experimentally by toxic doses of vitamin D, can be merely coincidental. Great variations in the responses to vitamin D in animals and in man have been recognized. It has long been accepted that Negro infants are unduly susceptible to rickets, and because of relative resistance to the anti-rachitic activity of vitamin D, often require higher prophylactic doses of vitamin D than do white infants, as well as much higher therapeutic doses. That at the other end of the spectrum are children (predominantly white) who need very little vitamin D, who are very susceptible to this vitamin, and who cannot tolerate even slightly elevated doses over minimal amounts, has not been realized. From the data reported in 1937 by Drake,[123] it appears that a significant number of children

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of British Northern European origin require little or no more vitamin D than is obtained from nonfortified foods and from sunshine. Confirmation of the lesser daily vitamin D requirements of white children, as compared with those of Negroes, was reported by Lewis.[124] He found that none of the white children observed over a five-month (winter) period of no vitamin D supplementation developed rickets, whereas 62% of the Negro children without vitamin D during the same time did. The significance of the ability of some children to remain free of rickets in the absence of outside sources of vitamin D has not been analyzed. One may speculate that it may be the result of a genetic mutation, which allowed for thriving of children who live in areas where exposure to sunlight is uncertain and where other sources of vitamin D are unreliable. With the coming of the public health era, with its emphasis on supplying all infants with such supplemental amounts of vitamin D as have been found necessary for those most susceptible to rickets, this presumed metabolic advantage may have become a liability. It must be noted that this genetic variant is apparently not limited only to the white race, despite the far greater frequency of the disease resembling hypervitaminosis D in fair-skinned children. Two Negro infants with generalized arterial calcification,[98,136] which may be the most extreme expression of high susceptibility to vitamin D, and several Negro children with supravalvular aortic stenosis have been reported.[19,31] On the basis of the available evidence, it seems likely that the genetic variant that results in poor responsiveness to vitamin D, thereby protecting against vitamin D toxicity, but increasing susceptibility to rickets, is more common in Negroes. On the other hand, the variant associated with markedly high responsiveness to vitamin D that protects against rickets and that increases susceptibility to vitamin D toxicity seems to be more common in fair-skinned children.

The amount of vitamin D recommended as a daily supplement was selected on the basis that it should be sufficient to protect against rickets in at least 98% of the total population. The data used were probably obtained from Lewis's[124] study, which showed that even at 1,450 units/day, one Negro child still developed rickets. On that account, he recommended addition of 332 units of vitamin D to milk to achieve at least the protective value of 1,450 units in oil. That 332 units in milk may exert an even greater anti-rachitic effect than 1,450 units in oil is suggested by Lewis' work[130] indicating that 90 units of vitamin D in milk was therapeutically more effective than 900 units in oil. In fact, he found 45 units in milk to be curative in six of eight rachitic infants; the remaining two, who required higher doses, were Negroes.

Warkany and Mabon's[132] demonstration of significantly higher blood levels of vitamin D in white than in Negro children was the first bit of direct laboratory evidence that there are wide variations in serum anti-rachitic values. Fellers and Schwartz,[91] who reported exceedingly high levels of anti-rachitic activity in the blood of hypercalcemic children, even when given no exogenous vitamin D and when protected from sunlight, provided the second clue to the pathogenic role of high serum anti-rachitic values. Forfar's[65,77,120] observation that cholesterol levels are markedly elevated in hypercalcemic infants, and that some cholesterol derivatives have vitamin D-like activity, is an important piece of evidence that anti-rachitic factors in the serum are responsible for the disease, hypercalcemia of infancy. He has postulated that susceptible infants have vitamin D percursors that are activated by synthetic vitamin D.[120] This may prove to be true. Another possibility that available evidence suggests is that the addition of crystalline vitamin D to milk, by enhancing its potency three-to tenfold, may be causing vitamin D toxicity directly in such children, whose

vitamin D requirements are very low.

The possibility that the syndrome of infantile hypercalcemia may be caused by overdosage with vitamin D during pregnancy has been suggested.[8,99,154] That vitamin D overdosage, especially to pregnant women who may be unduly susceptible to vitamin D, may cause fetal damage seems probable. Thus far, however, search through the literature for verified instances of overdosage with vitamin D during pregnancy of mothers of infants with the syndromes described in this paper has failed to reveal any such cases. In fact, a normal infant was born to a mother who had accidentally taken high daily doses of vitamin D for two weeks early in her gestation period.[155] Since she had an older child with the Williams syndrome, the failure of the vitamin load to cause infantile hypercalcemia is quite significant. Earlier reports of questioning of mothers of children with infantile hypercalcemia revealed that in the ten from whom data were obtained, there were intakes no higher than 1,000 units daily during their pregnancy.[7,23,26,46,103,156] In generalized arteriosclerosis of infancy, resulting in death by two months of age, which would appear to be more likely to have been caused by prenatal insult, evidence that vitamin D had not been taken during pregnancy was given in two instances.[132,158] Since infants are known to have immature enzyme systems, it seems more likely that administration of vitamin D during early infancy to hyper-reactive infants is the cause of these pediatric diseases than is its administration during pregnancy.

These observations raise the question: Just what are the best means of assuring optimum protection against rickets in children with high vitamin D requirements, without at the same time precipitating cardiovascular, renal, and possibly mental, disease in children with very much lower requirements, who may be hyper-susceptible to vitamin D? In the case of a substance to which there is such a wide variation in response, the amount unavoidably taken by those on a recommended diet should not exceed that found to be anti-rachitic in the group most susceptible to the action of vitamin D. The larger requirements of children more susceptible to rickets should be provided by specific supplements. Whether there should be addition of vitamin D to foods, including milk, should be reconsidered. The demonstrated increased potency of vitamin D in milk and the variability in the amount ingested make it difficult to guard against ingestion of doses that are potentially harmful.

SUMMARY

1. The striking histopathological similarity of several serious diseases of infancy and childhood that are characterized by cardiovascular, renal, and brain damage, to the changes seen in experimental vitamin D toxicity, points to vitamin D as an important etiological factor in these diseases.

2. There is strong evidence that hypercalcemia of infancy is caused by hyper-reactivity to vitamin D, as indicated by (a) the increased incidence of the disease in England during a period of overdosage with vitamin D; (b) its occurrence in infants given massive doses of vitamin D; (c) demonstration of increased anti-rachitic activity of the blood of affected infants; and (d) metabolic evidence of increased response to vitamin D by such infants.

3. Supravalvular aortic stenosis, mental retardation, and characteristic facies constitute a syndrome that is strikingly similar to that seen in survivors of hypercalcemia of infancy, and to that which has been reported in children given massive doses of vitamin D during infancy and early childhood.

4. Ev / is presented that renal tubular acidosis and nephrocalcinosis infantum may be less severe manifestations of hyper-susceptibility to vitamin D, and that generalized infantile arteriosclerosis may be a more severe manifestation.

5. The racial difference in susceptibility to vitamin D may account not only for the higher susceptibility of Negro infants to contract rickets, but also for the apparently greater susceptibility of fair-skinned children to develop conditions such as infantile renal acidosis, hypercalcemia, generalized arterial calcification in infancy, and the supravalvular aortic stenosis syndrome.

6. The enhanced activity of vitamin D in milk, three-to tenfold, as compared to the same unitage in oil, delivers excessive quantities of vitamin D to those drinking milk freely. Four hundred units in oil have been shown to be prophylactic even in the group most susceptible to rickets, and to exceed considerably that required by those least susceptible to rickets.

7. The question of the amount of vitamin D to be used as a prophylactic supplement should be re-investigated. The daily prophylactic dose should not exceed that shown to be safe for children most reactive to vitamin D. Larger amounts should be prescribed for those more susceptible to rickets.

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Examination of Calculations and Colorimetric Procedures in the Chemical Evaluation of Vitamin D

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Examination of data obtained from the 1963 collaborative study of a proposed chemical assay of vitamin D shows the following: the vitamin D content of the sample may be calculated by using a reading at 550 mμ as the blank, with an accuracy and precision equal to or better than that of the proposed calculation; an internal standard is not necessary.

These findings suggest a simplified colorimetric procedure which requires less than 5 μg (200 units). Data demonstrate a marked improvement in the color reagent resulting from reduction of the concentration of antimony trichloride.

Preliminary data indicate that the method can be applied to high potency irradiated yeast.

A chemical method for the estimation of vitamin D has been used for some time for the analysis of USP products (1). This method is applicable to other pharmaceutical preparations and products of relatively high potency. It has been the hope of the AOAC to adapt this method to the analysis of food, feeds, feeding oils, and milk. In 1963 a collaborative study (2) was carried out in which the USP procedure as modified by Osaden and DeRitter (3) was used. Results of this study showed that this modification gives an estimate essentially the same as the biological method. However, the sample requirement of 25 μg (1000 units) is still quite large. It is the purpose of this presentation to examine the data of this study with the intent of further reducing the sample size. The present method requires the final assay solution to be made to a volume of 5 ml. Of this solution, three 1 ml aliquots are used for the colorimetric evaluation: an internal standard, the sample, and an inhibited sample. This leaves 2 ml, which is generally unused. If the colorimetric evaluation could be performed upon the entire sample in a single tube, the initial sample requirements could be reduced by a factor of 5, to under 5 μg (200 units). This could be accomplished if the internal standard and inhibitor were not required.

Methods of Calculation

To examine these possibilities the results from the 1963 collaborative study have been recalculated by using the inhibited value at 500 mμ as a blank and substituting the 550 mμ value for the 500 mμ inhibitor value. The approach is suggested by the observation that the A^I_{550} is very similar to the correction $A^I_{500} - 2/3\,(A^I_{500} - A^I_{550})$. The formula for the three calculations is as follows:

USP

$$\frac{\text{Vit. D}}{\text{Aliquot}} = \frac{C_s}{A^1_{500} - A^4_{500}\,[A^1_{500} - A^I_{500} - 2/3\,(A^I_{500} - A^I_{550})]}$$

Inhibitor

$$\frac{\text{Vit. D}}{\text{Aliquot}} = \frac{C_s}{A^1_{500} - A^4_{500}\,[A^1_{500} - A^I_{500}]}$$

"550"

$$\frac{\text{Vit. D}}{\text{Aliquot}} = \frac{C_s}{A^1_{500} - A^4_{550}\,[A^1_{500} - A^I_{550}]}$$

A^1 = absorbance of sample plus standard.

A^4 = absorbance of sample plus 1,2-dichloroethane.

A^I = absorbance of sample plus color inhibitor.

500 and 550 designate wavelength at which readings are obtained.

C_s = concentration of standard.

The "Inhibitor" calculation assumes that this A^I_{500} value is a true blank and that there is essentially no inhibitory effect on the color development of other materials. The "550" calculation is based upon the fact that there is no vitamin D color absorption at this wavelength, and it assumes that interfering materials have essentially the same color development at 500 mμ. Neither assumption is quite true, but the error seems to be relatively small if the interfering materials are not present in very large amounts.

Of the reporting laboratories in the 1963 collaborative study eight submitted sufficient data to recalculate the values by the three methods. The potency values obtained, expressed as per cent of biological potency, and the coefficients of variation (relative standard deviation) are shown in Table 1. There are no real differences; therefore any of the three calculations gives essentially the same answer. If the three values for each of the four samples are arranged according to the magnitude of the difference from the biological, the "550" calculation is closest to the biological in three of the four, and the "USP" is second in three of the four. If the coefficients of variation are arranged according to magnitude, the "550" calculation results in smaller errors in three of four samples, the "USP" ranks second in three samples, and the "Inhibitor" calculation has the greatest variation in all samples. Therefore, it appears that an estimate of vitamin D can be obtained from a single determination by using the absorbance at 550 mμ as a blank. This conclusion is in agreement with the original proposal of Nickl, *et al.* in 1940 (4).

Internal Standard

The A^1_{500} and A^4_{500} differences, which represent the color developed by the standard in the presence of the sample, were ranked according to Youden's (5) procedure. In this procedure the numbers 1–4 are assigned to the responses for the four samples according to their magnitudes. This is repeated for each set of data from each laboratory. These numbers are then totaled for each sample. We had 14 sets of data, equivalent to 14 determinations under four conditions. To demonstrate a significant effect due to the sample the total rank must be outside of the limits 24 and 46. The samples ranked as follows: 31; 40.5; 32.5; 36. The lack of significant differences among rank totals indicates that the samples had no influence upon the color developed by the standard in the presence of the sample. This finding indicates that an internal standard is not necessary. This is also the opinion of Osaden and DeRitter, who use an external standard.

If these two modifications are used, i.e., omitting the internal standard and using the reading at 550 mμ as a blank, the entire sample preparation can be used in a single tube color development, so that the sample requirement is reduced to well under 5 μg (200 units).

Table 1. Comparison of calculation procedures[a]

Method	Sample 1 Potency[b]	Sample 1 RSD[c]	Sample 2 Potency	Sample 2 RSD	Sample 3 Potency	Sample 3 RSD	Sample 4 Potency	Sample 4 RSD
"USP"	102	28.9	86	21.6	76	13.5	103	30.7
"Inhib"	111	38.0	84	21.4	79	15.2	115	40.6
"550"	99	26.0	87	28.2	77	10.1	107	18.0

[a] Samples units × 10[1]: (1) Fortified fish oil, about 200; (2) Fortified evaporated milk, about 4000; (3) Gelatin coated D concentrate, about 20,000; (4) Vit. A + D fortified oil, about 300.
[b] Potency as percent of biological value.
[c] Coefficient of variation (relative standard deviation).

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Colorimetry

During a study of alternative techniques in which these changes could be used, it was found that adding a diluted reagent directly to the dry residue of vitamin D gave a linear response and color in every way exactly the same as when the vitamin D was in solution. Application of these conclusions and techniques suggests the following procedure:

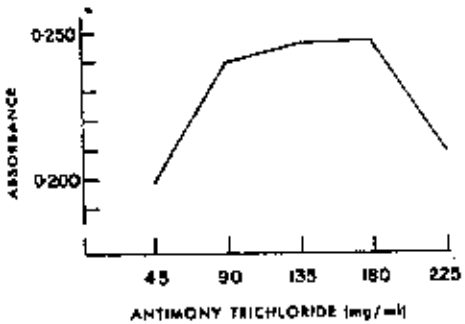

***Fig. 1*—Color development versus antimony trichloride concentrations, 4 μg vitamin D per tube.**

Table 2. Effect of $SbCl_3$ concentration on the color development with Vitamin D[a]

$SbCl_3$, g/ml	Absorbance 500 mμ	Absorbance 550 mμ	Vitamin D units	% Rec.
0.180	0.125	0.070	76	310
0.135	0.086	0.048	44	176
0.090	0.070	0.041	28	112
Biological assay			25	-

[a] All determinations are on 1 ml aliquots of a sample solution obtained from evaporated milk.
[b] All determinations were made with acetyl chloride present at 0.01 ml/ml of color reagent.

The final solution eluate is brought to dryness and the quantity of diluted reagent necessary for the colorimeter is added and mixed, the colored solution is transferred to the cuvette and read at 500 mμ, 45 seconds after the reagent addition, and the 550 mμ reading is obtained 20 seconds later.

It must be emphasized that the reagent should be diluted if it is to be added to a dry residue. Figure 1 shows the color development with respect to the antimony trichloride concentration. It is evident that the concentration must lie between 90 and 180 mg/ml for maximum color to develop. This is equivalent to an addition of 2–4 ml of the reagent per 5 ml of final colored solution. The acetyl chloride present in the reagent at 0.05 ml/ml was found to give maximal color when present as low as 0.005 ml/ml. The original reagent of Nield, *et al.* contained approximately 135 mg antimony trichloride per ml, while the concentration of the present reagent as used is about 160 mg/ml.

The effect of different concentrations of antimony trichloride has been examined on a sample eluate obtained from evaporated milk. This sample was chosen because it contains the greatest quality of contaminating sterol, with a ratio of cholesterol to vitamin D of about 10,000:1. The result of this work is shown in Table 2. These determinations were made with less than 1 μg (25 units) per tube, representing less than 2 oz. of milk. It is quite evident that the lower concentration of antimony trichloride gives the more accurate estimate, which indicates that considerably less color is developed by the contaminating cholesterol. The values were duplicated by using the acetic anhydride inhibitor.

Table 3. Chemical evaluation of vitamin D in irradiated yeast

Calculation Method	% Biological Potency Lab A	Lab B	Lab C	Lab D*	Av.	RSD
"USP"	113	93	96	125	101	10.9
"Inhib"	115	93	98		102	11.7
"550"	118	119	100		112	9.6

Biological Potency 107,000 units/g

* Laboratory D—insufficient data for recalculation, not included in average or RSD. [illegible]

Irradiated Yeast

Last year a sample of irradiated dry yeast was sent to several laboratories in addition to the collaborative samples. The data from four laboratories have been evaluated and are presented in Table 3. Three of these laboratories supplied sufficient data to allow the recalculations. The over-all accuracy is good; no value, regardless of calculation, differs from the biopotency by more than 20%.

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IDIOPATHIC HYPERCALCEMIA

A Case Report with Assays of Vitamin D in the Serum

By David W. Smith, M.D., Robert M. Blizzard, M.D., and Harold E. Harrison, M.D.

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In 1952, Lightwood[1] reported a case of idiopathic hypercalcemia of infancy with failure to thrive. By October, 1956, over 200 cases had been documented, chiefly from the United Kingdom.[1-17] Relatively few reports have appeared in the United States: Deutch and Senturia[18] and Daeschner and Daeschner[19] described individual cases, and Bongiovanni *et al.*[20] reported three cases with a review of the literature. With few exceptions most infants with unexplained hypercalcemia have been receiving preparations of cow's milk with a goodly intake of vitamin D, usually more than 1,500 units/day. Many infants in the United Kingdom receive such dosage. Creery and Neill[21] reviewed the vitamin D intake of 1,087 normal infants in Belfast, Ireland, and found that 51% received between 1,000 and 2,000 units/day. Therefore, it is readily evident that the incidence of the disease among infants taking 1,000 to 2,000 units/day is quite low. The basic etiology of the disease has remained obscure, although the predominant feeling is that the condition is secondary to an exaggerated response to a moderate dosage of vitamin D.[5, 10, 22, 23]

The purpose of this paper is to report a case of prolonged hypercalcemia in which assays of vitamin D in the serum were performed.

METHODS

The initial assay for content of vitamin D in the serum was performed by Dr. Bernard Spur of the Milk Research Laboratory of the Children's Hospital of Philadelphia. The two subsequent assays were performed by Dr. C. E. Bills, Dr. William Thomas and Dr. Gemmell Morgan utilizing the method of Bills.[24]

The methods used for analysis of blood and urine were as follows: concentration of calcium in serum was determined by the method of Harrison and Harrison;[25] concentration of phosphorus in the serum by the method of Fiske and Subbarow;[26] and that of citrate by the method of Natelson *et al.*[27] Concentration of calcium in the urine was determined by the method of Clark and Collip.[28]

CASE REPORT

History

S.H. was admitted to the Harriet Lane Home at 5 years of age (September 15, 1954) with the chief complaints of unilateral facial weakness, intermittent abdominal pain, and poor weight gain.

The patient was born on July 25, 1949, of a full-term pregnancy during which the mother received supplemental calcium. The birth weight was 2,610 gm. Formula consisted of evaporated milk (400 units of vitamin D per 13 oz can). At 15 months of age homogenized milk (400 units of vitamin D per quart) was substituted for evaporated milk. The intake was about one quart daily after 2½ years of age. Supplemental water-soluble vitamin D was taken in the following dosages: 1,000 units/day until 18 months of age, 1,500 units/day from 18 months until 5 years of age, and 4,400 units/day during the month before admission. Developmentally, she sat at 7 months of age and walked and said small sentences by 2 years

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of age. From birth she had frequent vomiting, slow weight gain, anorexia, relative constipation, and frequent upper respiratory illnesses.

At 10 months of age she was first admitted to Harriet Lane Home weighing 6.65 kg. She was a slim, undernourished infant with fretful disposition and tendency to vomit.

Concentrations of calcium in the serum ranged from 12.2 to 16.2 mg/100 ml; phosphorus, 4.0 to 5.8 mg/100 ml; alkaline phosphatase, 9 to 15 Bodanski units/100 ml. The concentration of nonprotein nitrogen in the serum was 32-49 mg/100 ml and the content of carbon dioxide 23.4 mmole/l. Urinary excretion of phenolsulfonphthalein after intravenous injection was 60% in 2 hours.

An intravenous pyelogram was normal with no evidence of nephrocalcinosis. Roentgenograms of the long bones disclosed slight osteoporosis with relative increase in density of the epiphyseal plate.

During a 4-month period of hospitalization, while receiving milk and 1,000 units of vitamin D per day, she continued to vomit and was discharged weighing the same as at the time of admission.

Between 14 and 26 months of age vomiting ceased and she gained to 8.86 kg. The concentration of calcium in the serum during this period ranged from 13.7 to 16.4 mg/100 ml. She was not seen at the Harriet Lane Home from 26 months of age until 5 years of age.

At 3 years of age anorexia except for milk was a problem, and there was gradual onset of polyuria, polydipsia and occasional headaches. Three months before the second admission she became listless and intermittently complained of abdominal pain and vague pains in the extremities. Two months before admission an episode of severe abdominal pain occurred with fever and leukocytosis (30,000/mm³). Because of these findings and a rigid abdomen, exploratory laparotomy was performed at another hospital. White plaques were noted over the peritoneal surfaces, which on biopsy proved to contain calcium. She remained listless and suffered from generalized pruritus. A right facial palsy developed which resolved partially before admission.

Physical Findings

At the time of admission to the Harriet Lane Home at 5 years of age the weight was 11.5 kg, height-age of 3½ years. She was slim and undernourished with an apathetic appearance. She preferred to lie down, whimpering when disturbed. The blood pressure was 120/90 mm Hg. The skin over the abdomen and lateral aspects of the trunk showed a faint *café au lait* discoloration. There was redness of the left conjunctivae. Slit-lamp examination revealed fine crystalline deposits beneath the conjunctival basement membrane. There was brownish discoloration at the base of the teeth. Abdominal palpation disclosed variable tenderness with muscular guarding, most marked in the left upper quadrant. No mass was palpable. Pressure exerted over the wrists and knees caused pain, though no redness or swelling was discernible. There was slight facial weakness on the right.

Laboratory Findings

In the serum the concentration of calcium was 17.1 mg/100 ml; that of phosphorus, 4.8 mg/100 ml; alkaline phosphatase, 15.4 Bodansky units; nonprotein nitrogen, 60 mg/100 ml; and total protein, 7.9 gm/100 ml. The content of carbon dioxide was 23.3 mmole/l and the concentration of citrate was 5.3 mg/100 ml (normal 2.5 mg/100 ml). The concentration of hemoglobin was 11.0 gm/100 ml with a leukocyte count of 10,700/mm³. Urinalysis showed a trace of albumin, no cellular elements, maximal specific gravity of 1.014, and a 4+ Sulkowitch test for calcium. Urinary excretion of phenolsulfonphthalein was 40% in 2 hours.

Roentgenographic Findings

Roentgenograms of the abdomen disclosed nephrocalcinosis in addition to mottled density in the perirenal areas. The intravenous pyelogram showed poor concentration with delayed clearance. Roentgenograms of the skull revealed calcification in the falx cerebri, tentorium cerebri, petroclinoid ligaments and internal carotid arteries (Fig. 1). The base of the skull did not appear abnormally dense. Roentgenograms of the long bones showed relative osteoporosis (Fig. 2) with prominent trabecular pattern, periosteal elevations along the shafts and a band of radiolucency in the subepiphyseal portion of the long bones. The epiphyseal plates appeared relatively dense.

Initial Assay of Vitamin D

Determination of vitamin D in serum obtained in September, 1954, was performed by

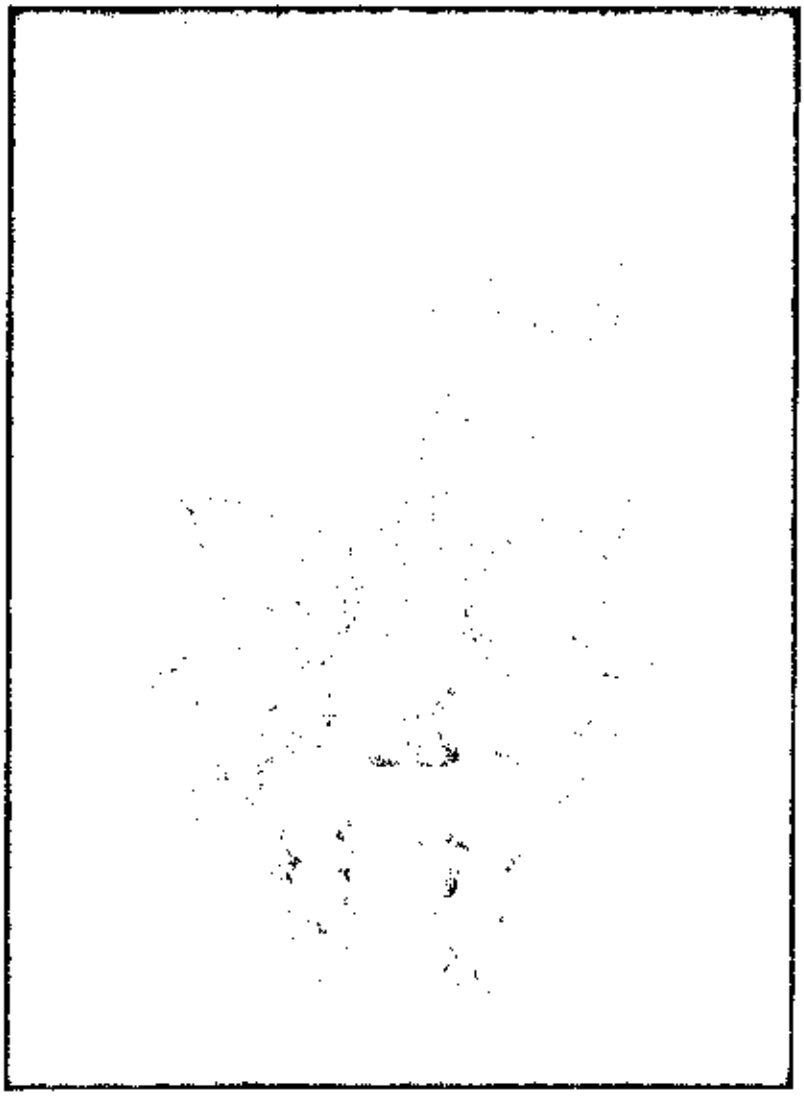

FIG. 1A. Anteroposterior projection showing calcification in the falx cerebri and the carotid arteries (arrow).

Dr. Bernard Spur, Director of the Milk Research Laboratory of the Children's Hospital of Philadelphia, and showed a concentration of 23 units of vitamin D per milliliter of plasma, as compared to a normal value of 0.66-1.65 units/ml noted by Warkany and Mahon.[29] Because of an insufficient amount of serum, this test was performed on only four rachitic rats instead of the usual 8 or 10 animals. The result must therefore be taken with some reservation.

Course (Fig. 3)

EXCRETION OF CALCIUM IN URINE: During the hospitalization of 2 months the patient was weak, listless and complained of occasional pain in the abdomen and extremities. Initially, while receiving a milk diet, the 24-hour excretion of calcium in the urine was 135 mg. After 2 weeks of receiving a milk-free diet, the 24-hour urinary excretion of calcium on two occasions was less than 40 mg.

HYPERCALCEMIC "CRISIS": On September 23 after 24 hours of poor fluid intake, she complained of severe abdominal pain; the abdomen was noted to be rigid, the leukocyte count was 30,000/mm³ and the blood pressure rose from 128/90 to 210/160 mm Hg. She vomited blood-stained material, had a generalized con-

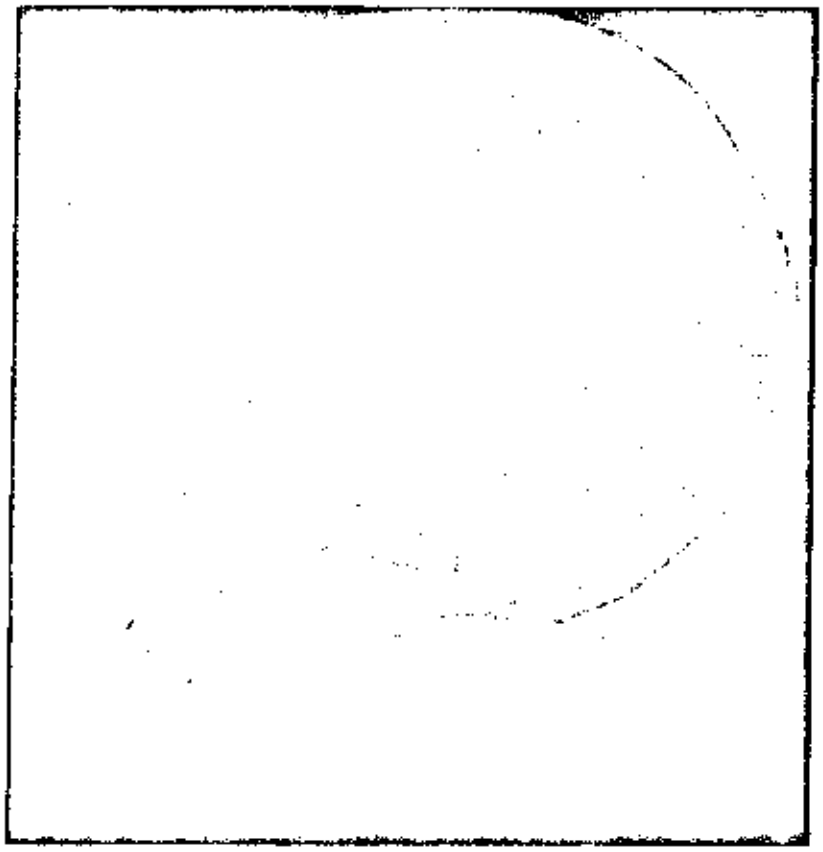

FIG. 1B. Lateral projection showing calcification of petroclinoid ligament. The base of the skull does not appear sclerotic.

vulsion, and lapsed into coma. The concentration of calcium in the serum was 16.0 mg/100 ml, and that of phosphorus was 6.8 mg/100 ml. Sodium EDTA* (sodium Versenate®) was given intravenously, 200 mg over the first hour and 800 mg over the next 16 hours. Within 30 minutes she regained consciousness and 6 hours thereafter was able to retain fluids orally. After 8 hours of therapy the oxalate-precipitable calcium in the serum was 12.8 mg/100 ml, with a total ashed calcium of 15.7 mg/100 ml.

Subsequently, the blood pressure remained elevated between 130/90 and 210/160 mm Hg, being usually 160/110. There was no response to administration of Randixin® or intramuscular administration of magnesium sulfate. The concentration of non-protein nitrogen remained elevated between 35 and 60 mg/100 ml.

In order to exclude the possibility of parathyroid adenoma, an exploration of the neck and anterior portion of the mediastinum was performed and no adenoma was found.

On October 1, the patient had a second "crisis," though symptomatically not as severe as the first. On this occasion the concentration of calcium in the serum was 18.2 mg/100 ml. She was again given sodium Versenate® intravenously with improvement.

CORTISONE THERAPY: On October 2, oral administration of cortisone, 100 mg/day, was begun with a gratifying fall in the concentration of calcium. Before cortisone therapy, the concentration of calcium was 14.7 to 18.2 mg/100 ml, and the alkaline phosphatase activity was

* Sodium ethylenediaminotetraacetate.

Lamb soup "recipe":[20]

1½ cups strained lamb (prepared baby food)
2½ tbs. sugar
½ tsp. salt
2 tsp. olive oil
2½ tbs. rice flour
32 oz. water

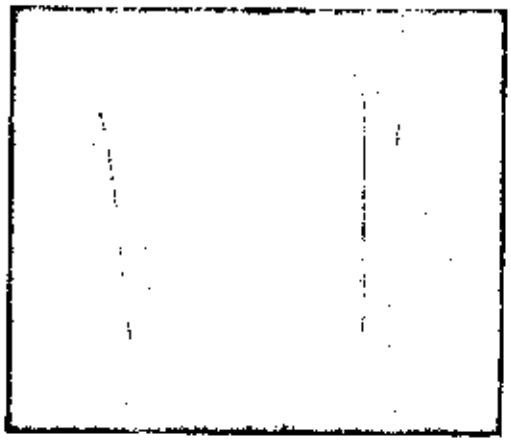

FIG. 2A Roentgenogram at 11 months of age (June 1950) showing slight osteoporosis and relative density of the epiphyseal lines.

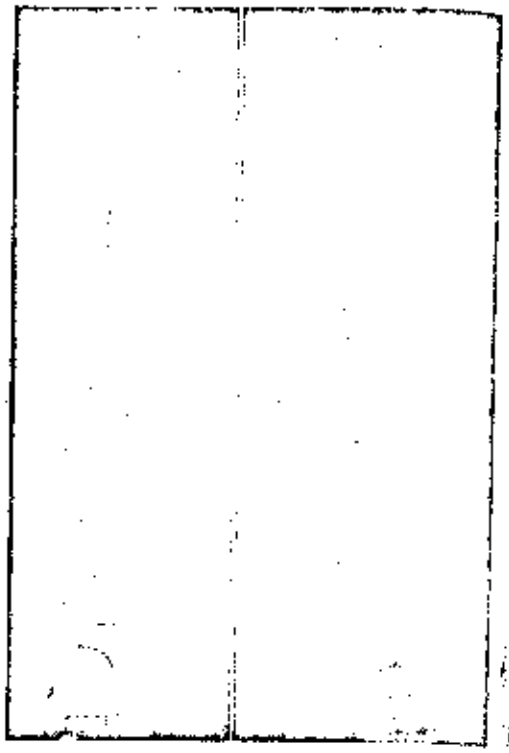

FIG. 2B. Roentgenogram at 5½ years (September 1954) (second admission to the hospital) showing relative osteoporosis with prominent trabecular pattern, periosteal elevations along the shafts, relative density of the epiphyses, and a band of radiolucency in the subepiphyseal area.

12.0 to 17.3 Bodansky units; the concentration of citrate in the serum was 5.3 mg/100 ml. While receiving cortisone therapy the concentration of calcium in the serum was 11.8 to 13.7 mg/100 ml and the alkaline phosphatase activity was 3.2 to 11.1 Bodansky units. The concentration of citrate dropped to levels of 1.8 and 2.8 mg/100 ml. She continued to refuse most foods other than milk, which she was allowed to take. While on this regimen the 24-hour urinary excretion of calcium was less than 40 mg/24 hours on two occasions.

On November 3, 1954, multiple petechiae were noted over the lower extremities but these disappeared in 3 days; the platelet count was 388,000/mm³.

The dosage of cortisone was gradually decreased and, finally, administration was discontinued. On November 11, she was discharged from the hospital with a mandatory fluid intake of 1,000 ml/day, with milk as desired. No vitamin D was given after admission or subsequently.

Five days after administration of cortisone was discontinued, the concentration of calcium in the serum had risen to 14.6 mg/100 ml, and after 2½ weeks it was 16.4 mg/100 ml. On November 30, the oral administration of 25 mg of cortisone daily was again begun and the concentration of calcium in the serum fell to 13.4 mg/100 ml. In January 1955, the dosage of cortisone was reduced to 12.5 mg/day, and 3 months later the concentration of calcium had risen to 15.6 mg/100 ml. Henceforth, 18.5 mg of cortisone was given per day until November 1955. The dosage of cortisone was then gradually reduced and administration was discontinued without a subsequent rise in concentration of calcium in the serum.

At the time of discharge from the hospital a firm abdominal mass was palpable in the left upper quadrant. This enlarged further during the subsequent week and was associated with moderate pain. Roentgenograms of the abdomen disclosed a mottled radio-opaque mass in the left upper quadrant. One month later the mass was no longer palpable, nor was it demonstrable by roentgenograms. This was presumed to be spleen which became infarcted and receded, with probable atrophy.

Low Calcium Diets: In February 1955, because of persistent hypercalcemia while receiving cortisone and ad libitum ingestion of milk, she was given a diet low in calcium with elimi-

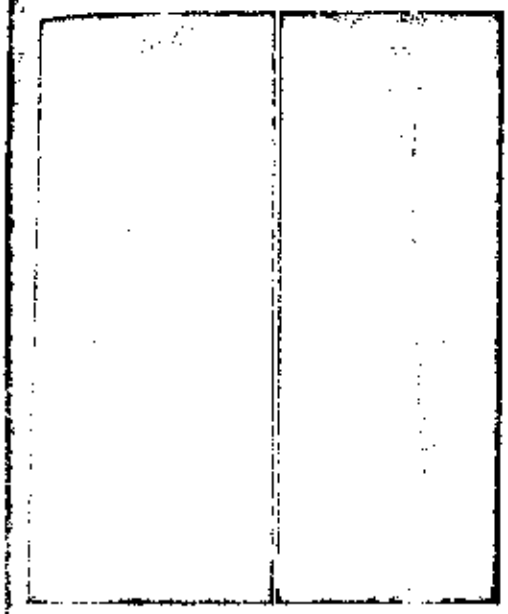

FIG. 2C. Roentgenogram at 5⅔ years (March 1955) discloses increase in the width of the subepiphyseal rarefaction with minute trabecular fractures noted at the ends of the long bones. The relative density of the epiphyses is again noted.

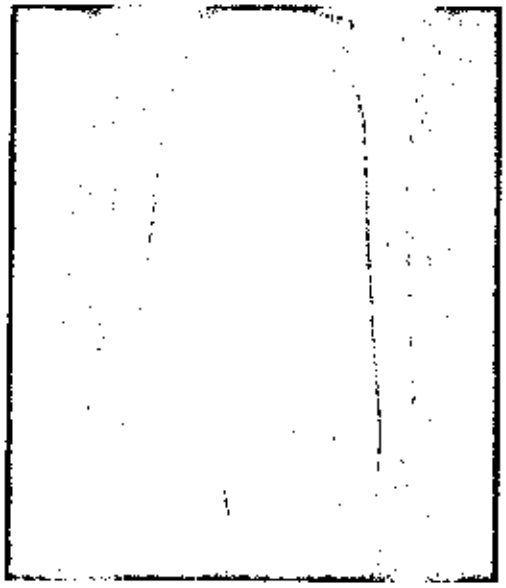

FIG. 2D. Roentgenogram at 6½ years (February 1956) discloses re-mineralization of the radiolucent areas with healing of the minute trabecular fractures, calcification in the periosteal elevations, and decrease in the relative density of the epiphyses.

ination of foods of high-calcium content and substitution of a "lamb soup"* for milk. In spite of the unpleasant taste of the lamb soup, she took 600 ml/day, which contained 27 mg of calcium. The total daily diet was analyzed on two occasions and contained 58 mg/day and 60 mg/day of calcium, respectively.

From the time the diet low in calcium was introduced there was gradual and remarkable improvement in personality and activity. From a listless, fretful child she became active and pleasant.

Concentration of Vitamin D in Serum: Six months after admission and discontinuation of administration of vitamin D, a vitamin D assay of serum revealed a level of 5 units/ml, which is above the normal of 1 ± 0.5 units/ml. A vitamin D assay performed in June 1956 revealed a vitamin D level in the serum of 2.0 units/ml. This was after 21 months of a regimen without added vitamin D.

Citrate Therapy: The concentration of calcium in the serum continued to be at the upper limits of normal in spite of the low intake of calcium, and the urinary excretion of calcium was high (108 to 208 mg/24 hr). In September 1955, in an effort to reduce the hypercalciuria, she was given Poly Citra® (molar sodium and potassium citrate),* 45 ml/day. After 6 weeks, the dosage was reduced to 20 ml/day because of alkalosis. With this therapy the urinary excretion of calcium fell from 108 to 37.5 mg/24 hours. Balance studies revealed a negative calcium balance of −94 mg/day just before citrate therapy and −23 mg/day after 6 weeks of citrate therapy. The concentration of calcium in the serum was 11.4 mg/100 ml at the onset of citrate therapy and 11.1 mg/100 ml after 6 weeks. During citrate therapy the concentration of citrate in the serum was high, ranging from 6.2 to 8.3 mg/100 ml.

In February 1956 the concentration of calcium in the serum was 12.0 mg/100 ml and an additional 100 mg of calcium was given in the diet in the form of calcium lactate, 700 mg/day. After February 1956, the concentration of calcium in the serum remained between 10.9 and 12.0 mg/100 ml and the intake of calcium

* See recipe in footnote on page 261.

* Supplied by the Willen Drug Co., Baltimore, Maryland. This preparation supplies 1 meq each of sodium and potassium/ml.

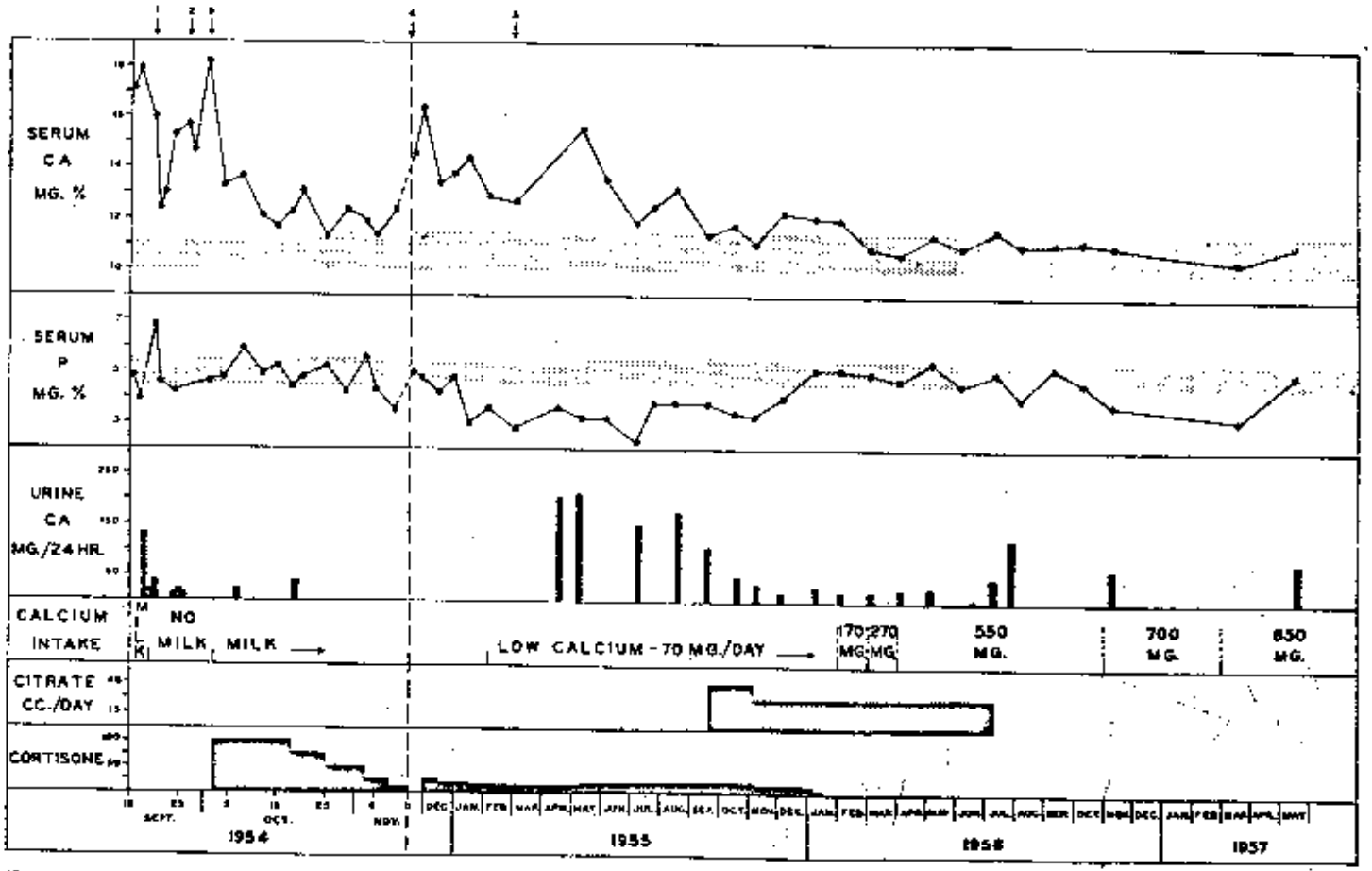

FIG. 3. *Course from September 1954 through June 1957.* The numbers at the top of the diagram indicate the following events: 1) Hypercalcemic "crisis" treated with 1 gram of sodium Versenate® intravenously over a 16-hour period. 2) Surgical exploration of the neck and anterior mediastinum revealed no parathyroid adenoma. 3) Second hypercalcemic "crisis" treated with sodium Versenate® intravenously. 4) Discharge from hospital. 5) Beginning of clinical improvement. Note: The dotted areas indicate the normal range for calcium and phosphorus. The citrate therapy [illegible] Company, Baltimore, Md.

was gradually increased to 550 mg of calcium/day in April 1956. The urinary output of calcium remained low. A balance study done in June 1956 disclosed a positive calcium balance of 152 mg/24 hours. Roentgenograms of the abdomen and skull made in July 1956 revealed no evident change in soft tissue calcification.

In July 1956, the citrate therapy was discontinued with no rise in concentration of calcium in the serum, but a rise in the urinary excretion of calcium did occur from 47 mg 24 hours to 121 mg/24 hours 1 month later. The concentration of citrate in the serum fell from 8.0 to 2.8 mg/100 ml.

Between July 1956 and December 1957 the concentration of calcium in the serum varied between 10.5 and 11.5 mg 100 ml. Urinary excretion of calcium in November 1956 was 65 mg/24 hours and in May 1957, 80 mg 24 hours. During this period the concentration of phosphorus in the serum gradually fell from 5.2 mg/100 ml in September 1956 to 3.2 mg/100 ml in January 1957, and in May 1957 was 5.0 mg/100 ml. The alkaline phosphatase activity remained constant between 15 and 20 Bodansky units. The dietary intake of calcium was gradually increased until in May 1957 she was taking 480 ml of milk daily, and either cheese or ice cream daily.

Roentgenograms of Long Bones: In March 1955, while the patient was receiving a diet low in calcium and 12.5 mg day of cortisone orally, roentgenograms of the long bones showed a further progression of the pathologic findings. The bands of subepiphyseal rarefaction had become wider with minute trabecular fractures noted at the ends of several of the long bones (Fig. 2C). This picture was practically the same in July 1955. In September 1955, when the patient was in negative calcium balance, roentgenograms disclosed remineralization of the subepiphyseal areas of radiolucency at the ends of the long bones with healing of the minute trabecular fractures. Further evidence of improvement was noted in roentgenograms made in February 1956 (Fig. 2D). Osteoporosis of the shaft had become less in degree while the relative density of the epiphyses had become less marked. Roentgenograms made in July 1956 disclosed normal bone structure.

Hypertension and Renal Function: The blood pressure had been persistently elevated to 160/100 mm Hg or higher until January 1956 when it began to fall and in March 1956 the blood pressure was 106/70 mm Hg.

Study of renal function disclosed that the concentration of nonprotein nitrogen in the serum remained elevated between 40 and 48 mg 100 ml. In January 1956, the urea clearance was 28% of normal, excretion of phenolsulfonphthalein was 37.5% in 2 hours, and the maximal specific gravity was 1.014. Urinalysis was normal. Urea clearance in January 1957 was 43% and excretion of phenolsulfonphthalein was [illegible]% in 2 hours, suggesting some improvement of renal function. Specific gravity, however, remained fixed at 1.010.

Growth: Until December 1955 growth in height had been relatively slow; however, during the next 3 months she grew 4.5 cm and during the next year, 11 cm (Fig. 4).

Neurologic Changes: During the study the patient displayed evidence of nervous system damage. The partial right facial palsy which was present at the time of admission, resolved in 2 weeks time. On October 25, 1954, a generalized convulsion was followed by intermit-

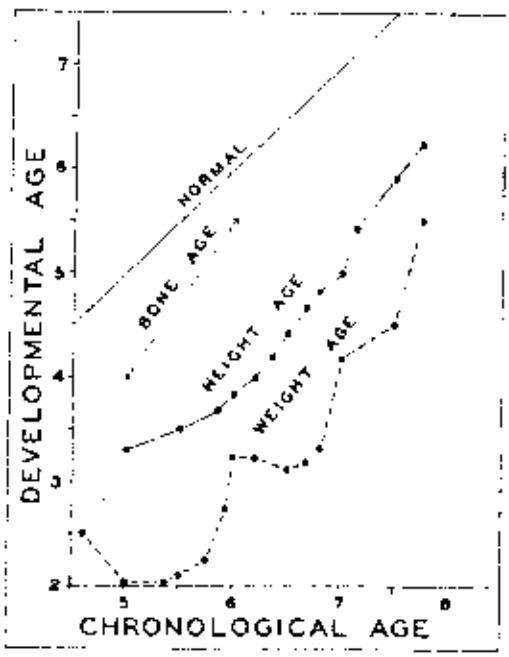

Fig. 4. Developmental curve illustrating advancement in height, weight and bone age during the period of treatment. (Height and weight standards from Wilkins, L.: The Diagnosis and Treatment of Endocrine Disorders in Childhood and Adolescence, 1st Ed. Springfield, Thomas, 1950.)

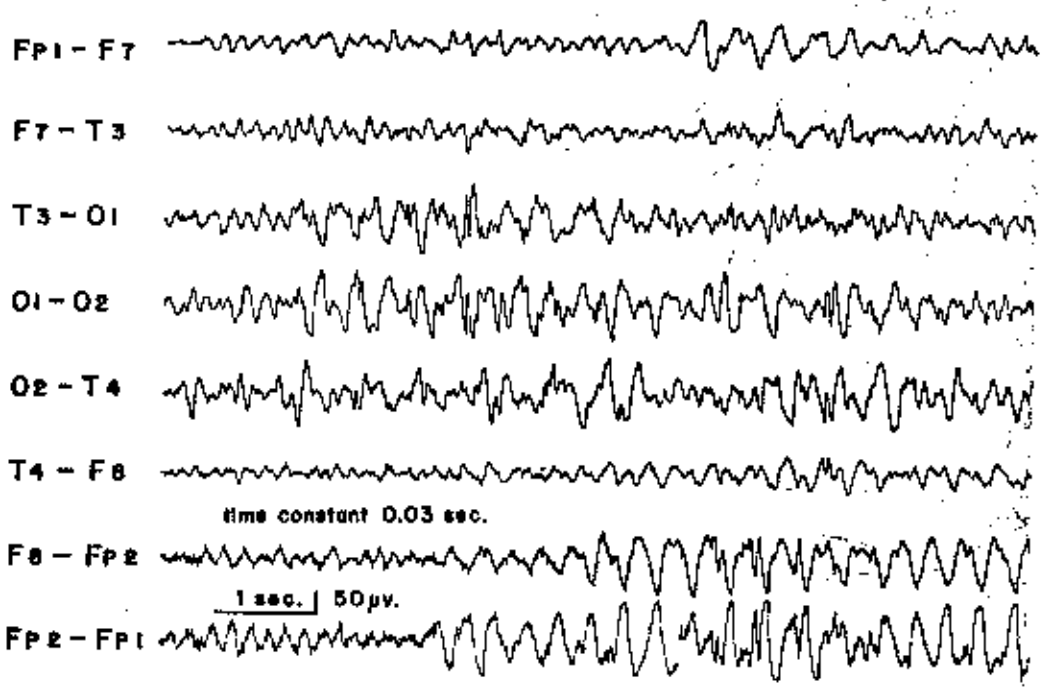

FIG. 5. Electroencephalogram made in June 1956 showing three independent foci or spikes associated with slow waves in left occiput (01), right occiput (02), and right pre-frontal (Fp2). Electrode labeling is International Standard. J.H.H. EEC #10705.

tent twitching of the right eyebrow and right hand for 3 days. She continued to have seizures characterized by aphonia and gradual loss of contact to the point of coma. These occurred at intervals of from 3 days to 1 month until December 1955. Five such episodes were observed during which the concentration of calcium in the serum was not elevated above the nonseizure levels. In August 1956, administration of diphenylhydantoin (Dilantin®) was begun and since then no seizures have occurred. An electroencephalogram in June 1956 was grossly abnormal with independent foci of spikes with slow waves (Fig. 5).

At times there was apparent hearing impairment. Evaluation disclosed normal reception but inability to perceive sound accurately, indicating a central inability to interpret sound.

In April 1956, a Wechsler Intelligence Test disclosed a verbal intelligence quotient (I.Q.) of 67, and a nonverbal I.Q. of 74; full I.Q., 67. It was again noted that she had difficulty deciphering the spoken word.

DISCUSSION

The patient manifested a clinical picture of unexplained mild idiopathic hypercalcemia of infancy at 10 months of age. No therapeutic measures were taken at this time to correct the hypercalcemia, and when hospitalized at 5 years of age symptoms of severe idiopathic hypercalcemia were evident. However, this patient differs in certain respects from other patients with severe idiopathic hypercalcemia. She does not show either the elfin facies or the osteosclerosis described by Schlesinger *et al.*[13] in a review of 10 cases.

The daily supplementation of vitamin D which the patient received would not normally be considered a toxic dosage, but the initial concentration of vitamin D in the serum was elevated (23 units/ml of serum) and only gradually fell to within normal limits 21 months after administration of vitamin D was discontinued, at the time when the concentration of calcium in the serum became normal.

The osseous findings in this case are of considerable interest since evidence of demineralization of bone was seen. Roentgenograms showed subepiphyseal rarefac-

tion, periosteal elevations, and relative rarefaction of the shafts of the long bones as compared to the epiphyses. These changes are similar to those described by Ross[1] and Debre[11] in cases of vitamin D intoxication in children. However, roentgenograms in this case did not show the marked increased density at the epiphyseal plate which is usually noted in both vitamin D intoxication and idiopathic hypercalcemia. Certain experimental findings are of interest in an attempt to understand the bone picture displayed by this patient. Harris and Innes[3] found that high doses of irradiated ergosterol given to rats initially resulted in increased deposition of calcium in the bone, particularly at the epiphyseal line. With prolonged therapy, demineralization occurred with resorption of the subepiphyseal spongiosa. With time they noted that the renal excretion of calcium increased, resulting in negative calcium balance. The demineralization is probably a direct effect of vitamin D on the bone tissue and it is possible that the demineralization of the bone in the patient studied by us was due to a prolonged cumulative effect of vitamin D. As hypercalcemia improved, remineralization took place, even in the face of negative calcium balance. The source of calcium for remineralization was therefore endogenous, either from soft tissue deposits or from redistribution in the bone itself. The latter possibility seems more likely since there was no evident decrease in roentgenographic evidence of soft tissue calcification at the time of remineralization. It is felt that the unusual duration of the disease fostered the changes of demineralization. Hypercalciuria may have acted as a safety valve to allow the disease to persist so long before severe symptoms of hypercalcemia were present.

In addition it should be emphasized that the patient has now been followed for 2 years since the concentration of calcium in the serum returned to normal (3¾ years in all). During this period she has received a full diet with 480 ml of milk and no added vitamin D, and on this regimen has been normocalcemic with a normal rate of growth. Though not conclusive, this follow-up does not implicate any other factor as a cause for hypercalcemia in the absence of supplemental vitamin D.

The fall in the concentration of phosphorus in the serum noted in this patient during convalescence is of interest. Howard and Meyer[14] reported a low concentration of phosphorus in the serum during the convalescent period in adults with vitamin D intoxication.

The concentration of citrate in the serum was initially elevated, a finding which Harrison[15] observed in hypervitaminosis D. He noted that elevated citrate levels have been found in a variety of conditions in which demineralization of the skeleton and hypercalcemia occur. Winberg and Zetterström[16] reported a low concentration of citrate in the serum in hypervitaminosis D, and Forfar and associates[12] noted a low concentration in idiopathic hypercalcemia, but too few determinations have been made in such patients to permit any conclusions concerning the significance of citrate levels in hypercalcemic states. The subsequent citrate levels disclosed low to normal values during cortisone therapy, high values during citrate therapy, and normal values after administration of citrate was discontinued and osseous healing had occurred.

The fundamental reason why idiopathic hypercalcemia should occur in infants receiving moderate dosage of vitamin D is not settled. There could be a physiologic difference in the response to vitamin D. Follis *et al.*[17] have stated that there is a wide variability in the vitamin D requirements in infancy. A second possibility is a metabolic defect in the degradation mechanism of vitamin D such that excessive accumulation of the vitamin could occur. In this regard Fyfe[18] demonstrated high concentrations of vitamin A in the serum with a greater than normal rise after oral administration of vitamine A to patients with idiopathic hypercalcemia of infancy. He suggested that there might be a concomitant defect in the metabolic handling of vitamin D. Fellers and

Schwartz[9] have reported studies of concentrations of vitamin D in the plasma of two infants with hypercalcemia, osteosclerosis, and mental and physical retardation. Both of these infants had high concentration of vitamin D in the serum. The levels were comparable to those found in two children who were given 100,000 units/day of the vitamin. This suggests some abnormality in the vitamin D metabolism of hypercalcemic infants. The assays of vitamin D in the serum of the patient studied by us also suggest failure of normal metabolism of vitamin D. On the other hand, Morgan and co-workers[10] have recently obtained vitamin D assays in the serum of two patients with idiopathic hypercalcemia in infancy. The results, performed by the same laboratory as the latter two determinations in the patient studied by us, were within normal limits. The paucity of determinations of concentrations of vitamin D in the serum of infants with hypercalcemia and the discrepant results reported prevent any conclusions from being made concerning the fundamental metabolic disturbance.

Whether the role of vitamin D is primary or secondary, it appears to have been contributory as a cause of the hypercalcemia in this particular case. Since a daily intake of 400 units of vitamin D has been shown to be adequate for normal growth and prevention of rickets in infancy,[11] it would seem advisable to limit the prophylactic vitamin D doses to this level rather than incur the danger of hypercalcemia on higher dosage, rare though it may be.

Clinically, several points are worthy of note. The retardation in growth rate persisted throughout the period of hypercalcemia. When the concentration of calcium in the serum returned to normal and the osseous abnormalities improved, an accelerated growth spurt occurred, followed by a normal rate of growth (Fig. 4). The hypertension gradually resolved, with normal blood pressure being obtained when the concentration of calcium in the serum returned to normal. Other effects of hypercalcemia left permanent residua. Renal function showed only slight improvement. Personality function and seizure frequency improved with time but the intelligence defect and perceptive hearing defect showed no appreciable improvement.

SUMMARY

A case of idiopathic hypercalcemia present from early infancy and diagnosed at 5 years of age is reported in which the serum assay of vitamin D indicated elevated levels. After discontinuation of supplemental vitamin D and a diet low in calcium the concentrations of calcium and vitamin D in the serum gradually returned to normal over a period of 18 months. Roentgenograms of the bones showed evidence of demineralization rather than increased density as reported in other cases of "idiopathic" hypercalcemia. During a subsequent 2-year follow-up the patient has maintained a normal serum calcium. The etiology is discussed with particular reference to the role of vitamin D in this case.

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Stanbury, J. B. (Ed.)

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Am. Pract. & Digest Treat. 2: 976-81 (1951)

CASE 190

Vitamin D Intoxication: A Metabolic Psychosis

Dr. Joseph C. Aub: When I saw today's patient on rounds when she was admitted, I looked at her and got discouraged and wandered on. Then by a lucky hunch (I suppose that's what it was) Dr. Gottschalk did a blood calcium, and from then on this problem unraveled and became a very interesting one.

I feel very strongly about this; I'm rather enthusiastic about this case. When I first examined her I found that she had something in her abdomen that was very interesting by physical examination; then I talked to her and walked away discouraged, as any doctor does when he doesn't know what to do for a patient. Actually these older people are very interesting. If you are going to study old people and really help them with their problems you mustn't get discouraged because they don't know what day it is.

Dr. Carl Gottschalk: Mrs. C., No. 737205, is a 59-year-old lady who came to the hospital because of anorexia and a loss of weight for a period of some years. The history is poor and very unreliable. The story apparently goes back for a period of 30 years. She has never had much appetite. She has never weighed a great deal. She did weigh as much as 110 pounds at one time, and over a period of about 30 years she gradually lost weight until now she weighs about 70 pounds. The weight loss has not been particularly striking in recent years, although she has

had less appetite in the past six months than she has had in years previously.

The history also noted the presence of many aches and pains. She has had generalized headaches for a long time; she has pains in her abdomen not particularly localized; her joints and muscles have been sore and painful in much the same vague way. The patient was studied in 1948 at an outside hospital; they found diverticula in the colon, but little else of significance. Her condition now is not terribly different from what it was in 1948 with perhaps one exception: her mental status is more vague. She is not quite as rational as she was. The history of the mental condition is rather poor. The family dates it back only to eight days prior to admission when they say she was beginning to have some trouble speaking and was a bit disoriented as to place and time. However, her physician dates it back for many months or years. As a matter of fact about six months ago she was sent to a psychiatric hospital's out-patient department, so I think we can be safe in assuming that her mental condition hasn't been what we might call normal for some time. It probably has been worse recently. In retrospect, we obtained an interesting history of the medication she has taken. The patient has been taking a proprietary drug which contains many vitamins, including vitamin D, 50,000 units per capsule, for about six weeks or two months. She has been taking these capsules after each meal, so we suspect that her vitamin D intake has been 150,000 units a day for about six to eight weeks. We also found she has been taking one grain of a bromide preparation three times a day for an indefinite period of time. She has also taken a sodium bicarbonate preparation. The patient has never vomited and has had no diarrhea.

The physical examination showed a small woman who on admission was quite disoriented and had a very poor memory, with recent and past memory loss. Without any treatment her mental status had improved somewhat. There was a mass in the abdomen right below the diaphragm just about in the mid-line. This mass was palpable, and as pointed out by Dr. Aub, had a systolic thrill and a localized murmur suggesting it might be an aneurysm of the aorta. The neurologic examination has been negative except for her mental state. There were no localizing neurologic signs.

The laboratory work shows normal urine, without albumin; the sediments are negative. The specific gravity is rather low, around 1.005 to 1.005 with a maximum specific gravity on the urine concentration test of 1.018. The hemoglobin is 12.5 Gm., with a normal white count and a normal smear. We have also done a bone marrow aspiration which we think is normal. The first laboratory test done was the NPN which turned out to be 38 mg. per 100 cc. When repeated several days ago it was 48. The patient's BMR is a little bit low—minus 5 and minus 9. Her eosinophiles have been counted several times; when fasting they are always quite low. They were 13 before an epinephrine test; four hours after 0.5 cc. of 1:1000 epinephrine they fell to 5. She has a serum sodium concentration of 138 and 132 mEq./L., with a low chloride of 83 and 87. This chloride may actually be lower still, as we have found that she has 5 mEq./l. of bromides in her blood; the sample for bromides was drawn after the patient had been here for about 12 days. The CO_2 has been high—35 mEq./l. of arterial blood; her arterial pH was 7.52. The venous pH is a bit lower, 7.45. The urea clearance test has shown 18 per cent of normal urea clearance. The PSP test was diminished, with 10 per cent excretion in each of the four periods. The PSP excretion in 1940 at an outside hospital was 20 per cent in 15 minutes; it is now 10 per cent. Her serum calcium values, done on two separate days, have been high; both have averaged 14.3 mg. per 100 cc., normal being 8.5 to 10.2. Phosphorus has been 4.8 and 5.3 mg., and alkaline phosphatase 4.2 and 2.9 units per 100 cc. The 24-hour calcium excretion, while the patient is on a low calcium diet, is 122 mg. (normal). The total protein initially was 8.3 Gm., and that was drawn just to get some idea of the patient's nutritional status; a repeated total protein has been 6.6 Gm. We haven't as yet got the results of the A/G ratio.

Dr. Allan Butler: May I ask a question? Has there been vomiting?

Dr. Gottschalk: No vomiting, either by history or in the hospital.

Dr. Walter Bauer: No diarrhea?

Dr. Gottschalk: No diarrhea.

Dr. Aub: Of course the 8.3 Gm. of serum protein was something I just couldn't explain; we looked very hard for multiple myeloma there and just now the total protein comes back 6.6 Gm.

Dr. Frederic Bartter: Was the 8.3 Gm. per 100 cc. measured by the falling drop method?

Dr. Aub: Yes. This young lady has lost an enormous amount of weight. She has an interesting finding in her abdomen. She has a pulsating mass with a thrill. There was a nice thrill there yesterday. How differently that sounds today than yesterday! When the total protein came in as 8.3 Gm. we wondered whether by any chance she had multiple myeloma around the aorta. Dr. Bauer is horrified at that; why not?

TABLE I.—*The Action of Various Substances on Calcium Metabolism in Outline*

	Ca Absorption	P Excretion in Urine
Vit. D.	+++	+
A. T. 10	+	+++
Parathyroid extract	0	++++

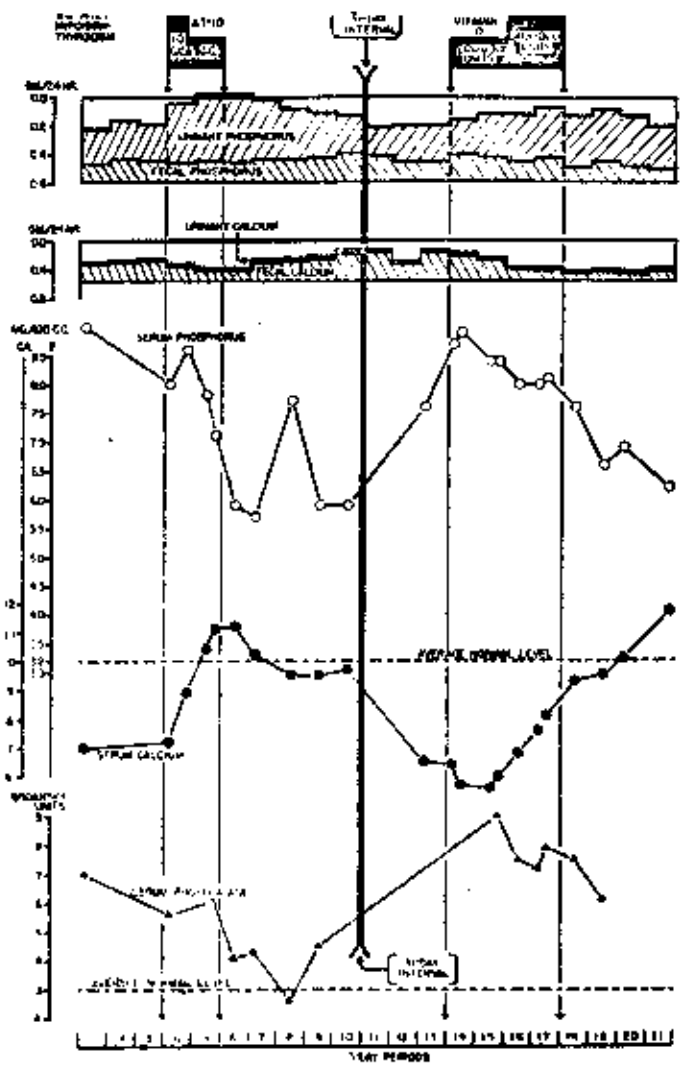

FIG. 1. Phosphorus and calcium balances, serum phosphorus, calcium and alkaline phosphatase in a patient with idiopathic hypoparathyroidism during administration of AT-10 and vitamin D. (Courtesy of Williams & Wilkins Company, Baltimore. Reprinted from Albright et al.[5]) Metabolic data in this and the succeeding figure arranged according to the following scheme: There is a horizontal base line; intake is charted downwards from this base line; the fecal and urinary excretions are then measured from the intake line towards towards the base line. If the output (fecal and urinary) exceeds the intake, the final level will be above the base line, if it does not the final level will be below the base line. Thus a positive balance is indicated by a clear area below the base line; a negative balance by a shaded area above the base line. The scales for nitrogen (not shown) phosphorus and calcium are so chosen that fluctuations in any one balance should be accompanied by equal fluctuations in the sum of the other two balances, provided that the changes in these balances represent changes in protoplasm or bone.

DR. BAUER: Around the aorta?

DR. BUTLER: In a recent article on vitamin D poisoning, the patient had a serum protein of 8 Gm. per 100 cc., and at the time it was taken he had an elevated serum calcium.

DR. AUB: I don't ever recall seeing that.

DR. BARTTER: Dr. John Dawson of the University of Leeds Medical School who was here last year studied a series of patients in whom vitamin D intoxication had been produced. He found that there was a significant elevation in serum protein in a great many (mean protein of 8.0 Gm. per 100 cc. compared to normal of 6.5 Gm. per 100 cc.).

DR. AUB: The total protein worried us very much. The other thing is that the patient has taken bromides, and probably has for a long time.

DR. PAUL D. WHITE: May I say a word about the abdominal mass? I don't think it is very important. It's probably an arteriosclerotic dilatation of the aorta, which many old people have.

DR. BARTTER: It seems to me that the finding of a high serum calcium level is reasonably well explainable. I have a lot more difficulty with the finding of alkalosis and high CO_2. As far as the calcium is concerned, it must be due to vitamin D intoxication, this having led originally to increased calcium absorption, with elevated serum calcium, which in some fashion caused renal damage, resulting in a rising NPN and serum phosphorus (Table I).

I have brought two slides to illustrate the metabolic effects of vitamin D_2. These studies[5] were carried out in patients with hypoparathyroidism, so that compensatory changes in parathyroid function would not obscure the results. In both studies the effects of vitamin D were compared with those of AT-10.

It is apparent (Fig. 1) that vitamin D resulted in (1) a marked fall of fecal calcium; (2) a rise of serum calcium, (3) a moderate rise of urinary phosphorus and (4) a fall of serum phosphorus. (AT-10, in contrast, produced a lesser decrease in fecal calcium, and a greater rise in urinary phosphorus.)

Figure 2 shows essentially the same changes. In this instance, however, the rise of urinary phosphorus with vitamin D was more marked, so that negative phosphorus and calcium balances were produced.

alkalosis. The rest of the picture seems to me to be very typical of vitamin D intoxication, as regards the serum calcium, inorganic phosphorus, serum protein, corneal opacification and the diminished renal function. The renal dysfunction unfortunately will be chronic and won't clear up promptly.

I wonder what her blood pressure was, because not infrequently you have an elevated blood pressure in such patients.

DR. AUB: 150/90 on admission.

DR. BUTLER: 150/90. In a case of vitamin D intoxication reported by Hyde and Richmond,[2] the blood pressure during the acute phase was normal, but then as the patient ran into chronic renal difficulty the blood pressure rose progressively. I was interested in the roentgen rays because not uncommonly you find calcification in the larger arteries and aorta. Could the mass one feels in the abdomen be related to the calcification of the aorta?

DR. AUB: There is no calcification there.

DR. BUTLER: There is a great deal of difference in the literature about the dose, both in terms of amount and duration, that causes vitamin D intoxication. If this patient had been bed-ridden during the time she was taking the vitamin D, then the demineralization of bone due to inactivity would enhance the effects of vitamin D in causing toxicity. I wonder if she had been rather inactive during the period of such therapy.

DR. AUB: She was like an anorexia nervosa; she was all over the bed when we first saw her. I think she was pretty active.

DR. BUTLER: The roentgen rays don't show demineralization?

DR. AUB: Not very much.

DR. JACOB LERMAN: I would like to ask Dr. Bauer and Dr. Bartter if vitamin D could act as a cortisone.

DR. BAUER: We don't know.

DR. BARTTER: May I say just one word in answer to your question? We think that vitamin D intoxication is a function of the amount of vitamin D and the amount of calcium in the intake, and that a lot of vitamin D will be less bad with no calcium, than less vitamin D with more calcium.

DR. AUB: At any rate, this patient is a more companionable person than when she came in. I think we forget that a chronic bromide intake makes people extraordinarily sluggish. We used to see quite a lot of it at the Huntington Hospital. Bromide isn't used much anymore, but people would come in with the mental deterioration of prolonged bromide intake, and it would make you think they had a metastasis in their central nervous system. This lack of companionability when this lady first came in, I think probably was due to bromide.

Dr. Cogan looked at her eyes and found something very interesting there. We have asked him to come over and talk about the effect of hypervitaminosis D on the eyes.

DR. DAVID G. COGAN: A few years ago Dr. Albright and Dr. Bartter were good enough to let me examine patients with hypercalcemia and about half of them had changes in their eyes which are probably pathognomonic of hypercalcemia.[4] The changes are subtle but they are none the less definite with slit-lamp examination. Grossly one sees a carpet-like gray opacification in the palpebral fissure on either side of the cornea (Fig. 3). With a slit lamp there are equally

FIG. 3. Corneal deposit of calcium (band keratopathy) in a patient with vitamin D intoxication. Note the hazy ring near the edge of the cornea, resembling arcus senilis but differing in being more prominent in the palpebral fissure. Several denuded areas may be seen, from which the calcium-laden epithelium has flaked off. (Courtesy of Dr. David G. Cogan.)

characteristic glass-like crystals in the bulbar conjunctiva. The corneal opacity is superficially not unlike arcus senilis and other changes in the limbus, but actually by slit lamp it is quite distinct, characterized by gray very superficial opacity (calcific) immediately beneath the epithelium in an otherwise normal cornea. As some of these calcific plaques slough off through the epithelium they leave a clear cornea in the corresponding area. The calcification always predominates in the periphery and in none of the cases has it extended far enough to give the patient any difficulty with his vision. We have observed these changes in patients with hypercalcemia from various causes such as hyperparathyroidism, uremia, vitamin D poisoning and sarcoid.

The mechanism is of some interest. Extravascular fluid diffuses into the cornea from the periphery and water is selectively abstracted from this fluid through the semipermeable membrane at the surface of the cornea. This results locally in a concentration of electrolytes beneath the surface membranes and those electrolytes which are near saturation level to begin with will precipitate out. Thus the formation of the calcific deposit beneath the epithelium is a supersaturation, and not a necrobiotic process. No doubt the loss of carbon dioxide through the epithelium is also of significance in localizing the deposition of calcium, as in the case of the lungs or in other acid forming organs such as the kidney and stomach where there is a similar subepithelial calcification.

In reference to the questions of the total blood protein in these patients with vitamin D intoxication, I looked through the records of the other patients and in only one was it above 8 Gm. per 100 cc.

The present patient is noteworthy in showing how rapidly the corneal calcification may occur. Apparently it can develop within six to eight weeks of vitamin D intoxication. The opacity is reversible; with restoration of normal blood calcium, the corneal opacity disappears.

Dr. Helen S. Pittman: Dr. Aub, this woman has been worse since she has been on vitamin D, but she was put on it because she was miserable. It's an overlay on something else.

Dr. Aub: Oh yes; she has been seedy for 20 years. She has been a feeding problem for 20 years, getting much worse in the last six months. Mentally she has been seedy too, getting much worse in the last six weeks.

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Editor's Follow-up Note

When she was seen in the clinic a month later, Mrs. C. had gained eight pounds, was clear mentally and was generally improved. She had given up all her medications, except for an occasional barbiturate capsule for sleep at night.

Cases From

The MEDICAL GRAND ROUNDS

Massachusetts General Hospital

Am. Pract. 3: 568–569, (1971)

Edited by JOHN B. STANBURY, M.D., BOSTON, MASSACHUSETTS

CASE 95

VITAMIN D POISONING *

DR. JACOB LERMAN: Our patient today presents an interesting problem of poisoning. Dr. Stall will present the case.

DR. BERNARD G. STALL, III: Mrs. B., No. 631593, is a 36-year-old woman who entered this hospital two weeks ago because of rheumatoid arthritis. This began three years ago with pain and stiffness in the neck, and subsequently there was involvement of all the joints and the development of moderately severe constitutional symptoms, consisting of a weight loss of 40 pounds, malaise, and easy fatigue. During this period she remained semi-ambulatory and has received a wide variety of medications and therapy, including salicylates, physiotherapy, immobilization of joints, a series of gold injections, and Ertron in doses of about 100,000 units of vitamin D every day for three weeks, seven months before admission. She has also been hospitalized on two occasions, once for 14 weeks. None of this treatment seemed to have any effect on the course of the disease, which had been gradually progressive, although there were partial remissions and exacerbations occurring apparently spontaneously. For the six months before admission she had noticed increasing nocturia, up to three or four times per night, frequency and urgency and occasionally incontinence of urination. For the three weeks before admission she was bothered with nausea and vomiting, especially after meals, and by alternating constipation and diarrhea.

She appeared chronically ill. There was widespread rheumatoid involvement of all the joints, with both acute inflammatory and chronic articular changes. There was a rheumatoid nodule 1 cm. in size on the left forearm below the olecranon. Except for a rather large pyramidal lobe of the thyroid, the remainder of the examination was not remarkable.

Laboratory data: White blood cell count was 10,100. The hemoglobin was normal. Urine specific gravity was fixed at 1.010. There was persistent albuminuria, 2 and 3 +, and the sediment contained a few red and white cells and granular casts. The sedimentation rate was 0.9 mm. per minute. The uric acid and NPN were normal. The congo red test showed 52 per cent of the dye retained in the serum.

The first two weeks of the hospital course were characterized by a recurrence of her nausea and vomiting and headache. She had been placed on the usual regimen for rheumatoid arthritis, namely, bed rest and joint exercises, salicylates and physiotherapy.

Studies of her spinal fluid and x-rays of the skull failed to demonstrate any cause for these symptoms. The blood calcium was determined and was found to be considerably elevated, 13.7 mg. per 100 cc. The phosphorus was 3.9 per cent and the alkaline phosphatase 3 units per cent.

At that point, medication was gone into more thoroughly and on checking with the family physician, it was found that she had been taking a vitamin preparation which contained 25,000 units of vitamin D in a dosage of four such tablets daily for a period of a month.

Her treatment was then altered so that she was partially mobilized and placed on a low calcium diet. Fluids were forced, and three days later the calcium was 9.7 mg. per 100 cc. and the phosphorus was 2.7 mg. per 100 cc. Symptomatically she is much improved.

The x-rays are of some interest in that there is considerably more calcification of the cartilaginous structures than one would expect for a person of her age. There is a good deal of calcification in the costal cartilage and also the thyroid cartilages. She is only 36 years of age.

DR. LERMAN: I should like to point out that Dr. Stall is entirely responsible for this fine detective work. As soon as he had a calcium report of 13, he got busy and chased down the nature of the drug she had taken. He obtained some sort of a name, which he investigated. It was a multi-vitamin preparation of which four tablets contained a total of 100,000 units of vitamin D. I think that we have the cause for her hypercalcemia.

DR. MARIAN W. ROPES: We surely are seeing a great deal of this type of poisoning. Some companies

* Presented on 9/30/48.

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put out a multi-vitamin product containing 25,000 to 50,000 units of vitamin D. The early toxic symptoms are not specific, such as headache, nausea and vomiting. In this case apparently, there is very little renal damage. So far, there is very little calcification of tissues. To be sure, she has the calcified cartilages but this change occurs, occasionally, at this age in normal individuals and somewhat earlier in rheumatoid arthritis. She has no calcification around the joints. Most of the cases seen in the past have been first suspected because of calcification in the synovial tissues and soft tissues around the joints. Her response to the forcing of fluids has been somewhat better than I thought it would be, partly because I thought she had more renal damage than she apparently had. Actually, I think the blood calcium level is down to stay, and probably the excretion is at a safe level.

I am sure that I do not know how we are going to prevent patients from taking the multi-vitamin capsules containing large doses of vitamin D. With Ertron most of the patients knew they were taking vitamin D. In her case, she said she had had no vitamin D as such except in the spring.

A Physician: Has she had enough gold to account for the renal symptoms?

Dr. Stall: She did not seem to react either favorably or unfavorably.

Dr. Greene Fitzhugh: What is the highest dose you would consider safe per day?

Dr. Ropes: That is a very difficult question. In cases that need vitamin D, 50,000 units can be given for short periods of time. I think that we have no evidence as to what the limiting dose is. It is true that some people get poisoning from 50,000 units a day. Whether or not it is because of some underlying renal damage or because of hypersensitivity, we do not know.

This patient's congo red test is interesting and makes one wonder whether she already had some damage to the kidney which made her more sensitive to vitamin D. I doubt that. I am sure that a dose of 100,000 units and over is toxic to a fair percentage of people, but not anywhere near 100 per cent, and many people can take 300,000 or more units without trouble. On the other hand, since we can't pick those out, and since the symptoms are so insidious and rarely recognized until damage has been done, I think no person should take vitamin D in higher doses than 100,000 units.

Dr. Paul D. White: There are a good many preparations, now, with large amounts of vitamin D in them.

Dr. Ropes: Yes. I do not remember the names of the products, but at least two or three companies put out capsules that I have seen containing 50,000 units, along with other vitamins. They are sold as multi-vitamin capsules.

Dr. Lerman: This patient demonstrated certain interesting points that are common to all patients with vitamin D poisoning. The excretion of calcium is very high. She had several four-plus Sulkowitch tests of the urine. We do not know whether today's urine specimen still shows it.

It is also surprising how long the effect of the poisoning lasted in her case. She stopped taking the medication when she came to the hospital; yet 10 days to two weeks later she continued to have these severe headaches and high urinary and blood calcium. This shows that the effect is not transitory and that the damage can go on long after the drug has been stopped.

Dr. Stall: This patient's eyes were also examined by means of a slit lamp, and it was found that she also had a band keratitis.

Editor's Follow-up Note

The patient remains in the hospital for a therapy of her rheumatoid arthritis. Her course has been one of slow improvement, and at the present time she is under separate investigation for a slowly enlarging thyroid gland. Her blood calcium and phosphates are normal.

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American Jour. of Clinical Nutrition
5:533-541

The Pathogenesis of Idiopathic Hypercalcemia in Infancy

By Thomas Stapleton, D.M., M.R.C.P.,* William B. Macdonald, M.D., M.R.C.A.P.,† and Reginald Lightwood, M.D., F.R.C.P.‡
With the Technical Assistance of J. W. J. Evans

The purpose of this article is to review the chemical changes which have been found in cases of idiopathic hypercalcemia, and to discuss the etiology and treatment of the disease. The clinical features have been described elsewhere.[1] In summary, all cases have commenced in the first year of life, sometimes as early as a few weeks after birth, but usually at about five months, with anorexia, vomiting, constipation and failure to gain weight normally. Some cases have one or more of the following features: thirst, polyuria, occasional spikes of fever, a systolic murmur, hypertension, osteosclerosis, a peculiar facies and mental retardation.[2] In general it can be said that the earlier the onset and the more severe the symptoms, the more likely are complicating features to be present.

CHEMICAL DESCRIPTION

The characteristic chemical changes are a rise in the level of serum calcium and blood urea. Other changes may occur as well: hypercholesterolemia, rise in α_2 globulin, acidosis, and a low serum alkaline phosphatase.

Calcium

Because of the possibility of an inaccurate laboratory estimation and because a transient rise in the serum calcium level can occur in other diseases, we have only considered the diagnosis of idiopathic hypercalcemia when two estimations of the serum calcium have been at least 12 mg 100 ml. The highest recorded value is 24 mg 100 ml by Cordeiro,[3] but in the United Kingdom the highest is 19 mg 100 ml.[4] The distribution of the increase of serum calcium between the protein-bound and soluble fractions has not been determined. There is no constant relationship between the degree of hypercalcemia and plasma protein levels. The daily excretion of calcium in the urine is raised. Amounts as large as 118 mg in 24 hours have been found.[9] Other reports[1,6,7,8] all give figures well above the normal daily excretion which is of the order of 20 to 30 mg.[5]

In spite of the increased urinary calcium excretion, the calcium balance is strongly positive (Fig. 1). In reported balance studies the calcium retention expressed as a percentage of calcium intake has varied between 31 and 69 per cent in eleven cases, with an average of 49 per cent.[1,6,8,9]

Phosphorus

The serum phosphorus is usually in the normal range of 3.5 to 5.5 mg 100 ml sometimes slightly higher, sometimes lower. In the early stages of the illness, when the impairment of renal function is minimal, there appears to be no constant relationship to the serum calcium level. In the more advanced stage of the illness the serum phosphorus may be abnormally high.

When the serum calcium is high the phosphorus balance is strongly positive (Fig. 1). In seven reported balance studies,[4,5,8] the phosphorus retention expressed as a percentage

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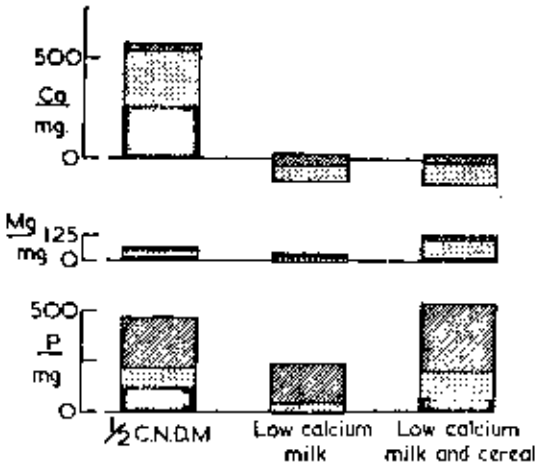

Fig. 1. Treatment related to daily calcium, magnesium and phosphorus balances, which are averages of four-day collections. (Urinary excretion hatched, stool excreted dotted, positive balance black). (From: *Lancet* i: 962, 1956.)

of phosphorus intake has varied between 26 and 84 per cent with an average of 51 per cent.

Magnesium

We have estimated the serum magnesium in seven children. Values ranged from 1.8 to 2.7 mg/100 ml. confirming the findings of other workers.[6] In only one case did there appear to be any relation with the rise and fall of serum calcium (Fig. 2) and in that patient the magnesium also paralleled the changes in serum phosphate and urea. It therefore seems likely that the changes were due to fluctuations in renal function. The magnesium balance seems to be unaffected throughout the course of the illness[8,9] (Fig. 2).

Bicarbonate

In most cases of idiopathic hypercalcemia the serum bicarbonate is within normal limits. Some infants may have an abnormally low serum bicarbonate even when renal function is not greatly impaired.[10] It is possible that some infants with hypercalcemia have an associated renal acidosis.[11] There appears to be no direct relationship between serum calcium and bicarbonate.

Sodium, Potassium Chloride

The serum concentration of these electrolytes are within normal limits in idiopathic hypercalcemia of infancy. There is no disturbance of the sodium, chloride or potassium balances.[9]

Proteins

In his original description of the biochemical changes in idiopathic hypercalcemia, Payne[12] stated that the plasma proteins showed an in-

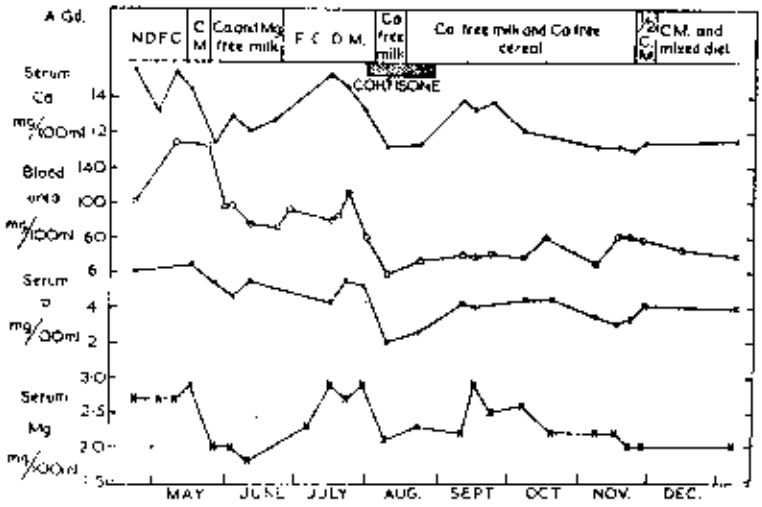

Fig. 2. Treatment related to blood urea, and serum calcium, phosphorus and magnesium. (Modified from *Acta Paediat.* 44: 559, 1955.)

crease in the α_2- and γ-globulin fractions. These findings have been confirmed by other investigators, but only in some cases of idiopathic hypercalcemia.[6,13] Only two out of seven infants studied by us have had total serum proteins higher than 7 g/100 ml, but in all of the seven the α_2-globulin fraction was significantly raised, although the γ-globulin was normal. The α_1- and β-fractions are occasionally slightly raised.

Cholesterol

In severe cases serum cholesterol levels may be of the order of 300 to 400 mg/100 ml. In infants with an illness of intermediate severity, the serum cholesterol is often increased to 250 to 300 mg/100 ml, and returns to normal levels when the serum calcium falls and clinical improvement commences.

TABLE I

Serum Calcium Levels Related to Serum Citrate Levels and Diet

Patient	Diet	Serum calcium mg/100 ml	Serum citrate mg/100 ml
C. Hm.	Vitamin D fortified dried milk	13.4	3.0
	Low-calcium diet	12.0	1.5
	Low-calcium diet with added solids	13.1	3.6
	Low-calcium diet with added solids	11.3	1.8
S. Ek.	Vitamin D fortified dried milk	13.1	2.7
	Low-calcium diet	11.1	2.2
J. Gh.	Vitamin D fortified dried milk	13.2	2.6
	Vitamin D fortified dried milk	12.1	2.0
	Low-calcium milk	11.1	1.0
	Low-calcium milk	11.0	1.3
	Equal parts whole milk and low-calcium milk	11.7	2.7
A. Pr.	Vitamin D fortified dried milk	15.1	3.1
	Vitamin D fortified dried milk	13.7	2.8
	Low-calcium diet	11.8	3.5
	Low-calcium diet	12.7	2.1
	Low-calcium diet	12.2	2.0
	Low-calcium diet	11.5	1.7
	Low-calcium diet with added solids	11.2	4.0
	Low-calcium diet with added solids	10.9	3.0

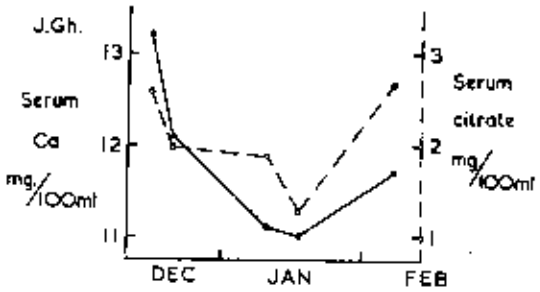

Fig. 3. The variable relationship between the serum citrate and serum calcium levels.

Alkaline Phosphatase

Low concentrations of this enzyme are often found in the active phase of the disorder, particularly in severely affected cases when osteosclerosis is a complicating feature. Levels of 4 to 8 King-Armstrong units are not uncommon. The concentrations return to normal during recovery and sometimes become higher than normal when affected children are treated with calcium-free diets, and radiologic evidence of a decrease in osteosclerosis develops.

Citrates

It has been stated that the serum citrate level is inversely related to the serum calcium.[7] In four cases studied by us no constant relationship has been found. (Table I and Fig. 3, 4).

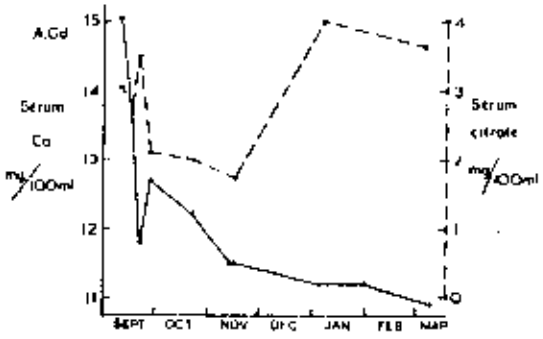

Fig. 4. The variable relationship between the serum citrate and serum calcium levels.

Urea

The blood urea is raised, although not necessarily throughout the whole active course of the disease. In some infants it is of the order of 160 to 180 mg/100 ml even in the absence of dehydration.

Vitamin A

Plasma vitamin A levels were estimated in eight infants with idiopathic hypercalcemia and were significantly higher than those found in normal infants, or in marasmic infants, both in the fasting state and four hours after a standard oral test dose of vitamin A.[14]

PATHOGENESIS

The clinical, chemical and pathologic findings closely resemble those which are found in persons who have received excessive intakes of vitamin D. (Fig. 5) The following evidence suggests that a large intake of vitamin D is a main causative factor:

(1) All infants with the disease have received vitamin D either as a supplement or in a fortified infant food.

(2) The vitamin D intake of infants in the United Kingdom, where the disease has been commonest, has increased during the past fifteen years largely as a result of the increased fortification of infant foods: the extent of this increase was not fully appreciated until recently. This is shown in the tables reproduced from the report by a sub-committee of the British Paediatric Association[15] (Tables II, III). These figures permit an approximate estimate to be made of the likely intake of vitamin D of an infant fed on the full-cream milk, referred to in Table II fortified with crystalline calciferol 400 I.U. per dry ounce, the calciferol-fortified cereal referred to in Table III, together with the generally recommended quantity of cod-liver oil compound:

For example, the vitamin D intake of an infant who receives each day

	Approximately
(a) 1½ pints of a full-cream dried milk containing 400 I.U. vitamin D per dry oz	1,725 I.U.
(b) 1 oz of a cereal containing 1,500 I.U. vitamin D per dry oz	1,500 I.U.
(c) 1 teaspoonful of national cod-liver oil compound (Welfare Food Service)	700 to 800 I.U.
would be about 4,000 I.U.	

(3) The disorder has occurred in Finland, especially among premature babies who have had interstitial plasma cell pneumonia. These infants had all received several hundred thousand units of vitamin D as stoss therapy.[16,17]

An excessive vitamin D intake, however, cannot be the sole factor leading to the development of the disorder, because thousands of infants in the United Kingdom and elsewhere must have received as large doses of vitamin D as those few hundreds who have developed the disease: and some infants with the disease have not received excessive doses of vitamin D. Some infants have been affected in the early weeks of life. These three facts [illegible] that idiopathic hypercalcemia may be due [illegible] par-

TABLE II

The Amounts of Crystalline Calciferol Added to a Proprietary Brand of Full-Cream Dried Milk, and Reports of Assays of Vitamin D in the Milk

Date	Crystalline Calciferol added I.U./dry oz
Up to August 1942	70
From August 1942	128
From October 1943	403
From November 1944	460
From October 1951	400

Age of milk months	Year of manufacture	Assays
7 to 12	1950	392
13 to 18	1952	463
		405
3 to 6	1952	560

TABLE III

The Amount of Calciferol Added to a Dried Cereal and Reports of Assays

Date	Crystalline Calciferol added I.U./dry oz	Assays: Age of food months	Assays: I.U./dry oz
Up to March 1946	1,000		
From March 1946	2,000	0 to 2	1,110 to 1,870
		13 to 28	758 to 1,200

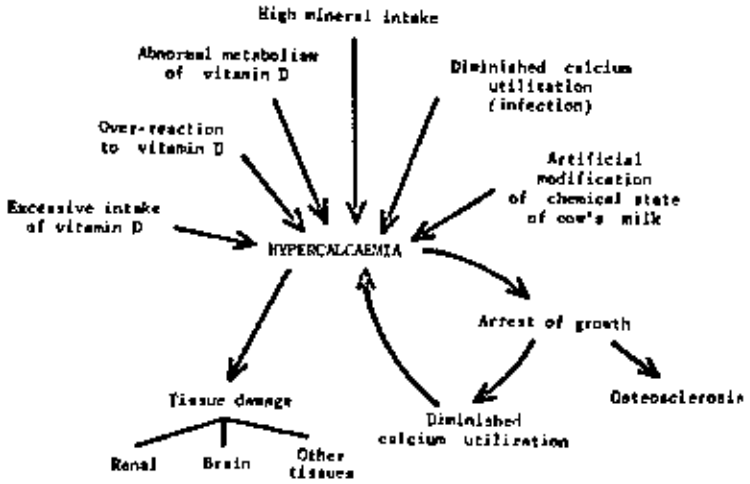

Fig. 5. The pathogenesis of idiopathic infantile hypercalcemia (Modified[30] from Helvet. Paediat. Acta 10: 140, 1955.)

ticular response of some infants to vitamin D, either an extreme of the physiologic variation, or an inborn metabolic defect which causes them to react in an abnormal manner.[18,19]

The fall in the high serum calcium when cortisone is given resembles that observed in sarcoidosis with hypercalcemia.[20] In both instances, it may be caused by an anti-vitamin D effect.

Conditioning Factors

Alternatively, or in addition, there may be conditioning factors which make some infants more susceptible to a given dose of vitamin D.

(1) All the infants reported as suffering from idiopathic hypercalcemia have been artificially fed. The high calcium and phosphorus content of cow's milk (approximately 120 mg/100 ml and 95 mg/100 ml, respectively), compared to those in human milk (approximately 30 mg/100 ml and 15 mg/100 ml, respectively) must at least predispose to the development of the disease.

(2) Impaired rates of growth lower the net requirement of calcium, phosphorus and vitamin D. This was probably a factor in the infants who had had interstitial plasma cell-pneumonia reported from Finland.[18] Infections have preceded the onset of the disease in cases reported from the United Kingdom.[5,21]

(3) Idiopathic hypercalcemia has been recognized in infants in many countries (Finland, Norway, Portugal, Sweden, Switzerland, United Kingdom and United States of America). Although the vitamin D fortification of milk is much less in America than it has been in the United Kingdom, many American mothers must have given their infants supplementary vitamin preparations; the disease has not been overlooked, yet the number of cases apparently has been small. A possible explanation may be in the use of evaporated milks in the North American Continent, whereas in the United Kingdom dried milks are in general use. It has been suggested that the essential fatty acid content of dried milks may be lower than that of evaporated milks,[22] (both, of course being cow's milk have a lower essential fatty acid content than human milk). This is an attractive hypothesis which would explain the geographic distribution of the illness, and might help to account for the high plasma cholesterol levels found in affected infants, as there is some evidence that a deficiency of essential fatty acids interferes with sterol metabolism and can be followed by a raised plasma cholesterol.[23-25] Sinclair's claim is not supported by analyses of different preparations of cow's milk for their essentially fatty acid contents.[26]

While investigating this problem we have made an interesting observation. An infant with idiopathic hypercalcemia and an infant

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Fig. 6. Section of kidney stained for calcium from a female infant with idiopathic hypercalcemia who died at age of 11 months (no treatment having been given for comparison with Figure 7).

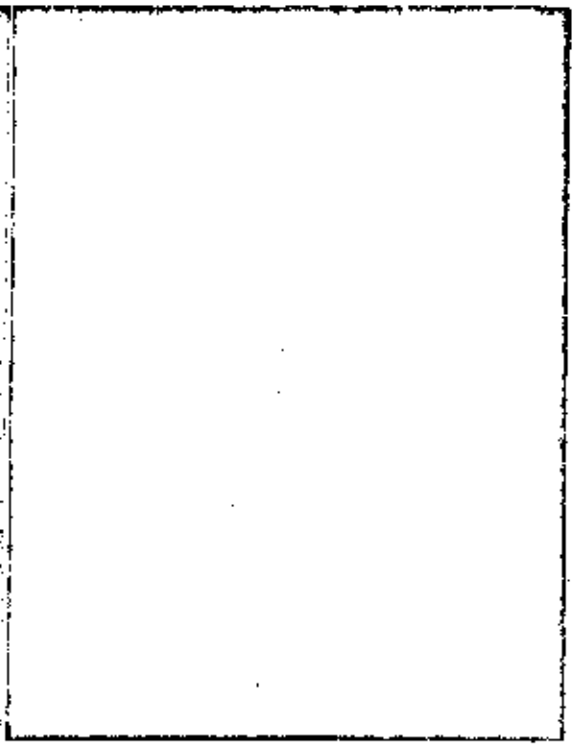

Fig. 7. Section of kidney from a rat six weeks old which had been given 500,000 units calciferol 12 days previously. (Alizarin × 16.)

with a normal serum calcium level were given by mouth cotton seed oil, which is rich in essential fatty acids. In both instances there was a fall in the serum calcium level, with a rebound on discontinuing the oil.

The raised vitamin A levels in idiopathic hypercalcemia[11] support the possibility of abnormal fat metabolism in this disease.

COMPLICATIONS

The complications of idiopathic hypercalcemia appear to be due to the high serum calcium, its duration of action, and the age of the infant when it develops. The type of complication is determined by the location of metastatic calcification in the body and the overall effect of calcium on tissue metabolism.

Kidneys

Renal involvement is evidenced by azotemia. Calcification and fibrosis of the kidneys have been reported by several investigators,[2,7,13,27–29] and it seems that this organ is the most constant site of calcium deposition. Calcium deposits are concentrated at the cortico-medullary junctions (Figures 6, 7), and are seen as concretions within the lumina of the tubules and as amorphous deposits in the interstitial tissue. Similar changes at this site occur in rats given toxic doses of vitamin D, and also in rats given smaller doses of vitamin D together with acetazolamide.[31]

Renal Function

The high blood urea may fall to normal limits after two to three weeks of treatment independently of hydration changes. This is in keeping with the improvement in renal function (Table IV), and resembles that which occurs in sarcoidosis with hypercalcemia following treatment.[32] It seems unlikely that kidneys with the severe damage and fibrosis seen in some

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fatal cases of idiopathic hypercalcemia could improve to this extent in such a short time, and it is probable that earlier and reversible changes occur. There might be a clearing of kidney tubules obstructed by calcium casts as has been reported in vitamin D poisoning studied by renal biopsy.[18] The impaired inulin and urea clearances which improve during treatment would support this theory, unless altered permeability of tubular epithelium in this disease nullifies the clearance results. An alternative or additional explanation is that a high level of ionized calcium may inhibit enzyme activity in renal tubular cells without causing irreversible changes.

Our experiments done on young rats poisoned with vitamin D and receiving a high-calcium diet show no reduction in the oxygen uptake of kidney slices. The oxygen uptake of kidney slices of healthy young rats is not diminished by the addition of high concentrations of calcium or vitamin D to the Krebs-Ringer medium. These experiments, however, do not deny the possibility of a lesser degree of inhibition of metabolic processes not directly concerned with aerobic oxidation, but with more specialized organ function.

Vitamin D in its soluble phosphorylated form is an activator of aerobic oxidation in tissues other than bone, as well as being an activator of other enzymes such as alkaline phosphatase.[34] Thus it is reasonable to assume that if a depression of tissue metabolism occurs in hypercalcemia it is more likely to be due to the hypercalcemia than to a primary vitamin D effect.

Other Sites of Calcification

Calcification of blood vessels[27] of the myocardium and heart valves[14,29] and in subcutaneous tissues[36] have been described. Osteosclerosis is a common feature in the more severe grades of the illness. Calcification has also been found in the lungs and the fundal glands of the stomach.[13]

The systolic murmur which is frequently found in affected infants may be due to calcification in cardiac valves, the hypertension to thickening or calcification of arterial walls.

TABLE IV

Renal Function Tests*

C. Hm. (Female)

Age in Months	Serum calcium mg/100 ml	Blood urea mg/100 ml	Urine specific gravity	Inulin clearance: U mg/100	V ml	P mg/100 ml	Time mins	Uncorrected ml/min	Corrected To surface area 1.73M² ml/min	Urea clearance: U mg/100	V ml	P mg/100 ml	Time mins	Uncorrected ml/min	Corrected To surface area 1.73 M² ml/min	Urea clearance-inulin clearance ratio
10	13.4	70	1.014 (19 hr)	391	57.5	58	60	6.5	34	465	57.5	71	60	6.3	33	1.03
10½	12.0	68	1.010 (14 hr)	540	49	63	60	7.1	37	565	49.0	69	60	6.7	35	1.05
13	11.2	58	1.022 (18 hr)	300	63	33	82	7.0	37	565	63	68	82	6.4	33	0.90
20	19.8	42	1.022 (15 hr)	398	62	29	70	12.1	56	550	62	58	70	8.4	39	0.69
				413	64	30	60	11.9	79	471	64	58	60	8.6	40	0.58
				222	112	18	60	23.0	83	119	112	46	60	16.6	60	0.72
				616	54	16	70	39.1	108	860	54	46	70	14.1	52	0.47

* The clearances were done when the infant was sedated with a barbiturate. The urine volumes include the 30 ml. water used for bladder washings.

Note the low inulin and urea clearances in the severe stage of the disease, with an improvement and fall in the urea/inulin clearance ratio when the serum calcium fell.

TREATMENT

The efficacy of treatment depends on the stage of the illness at which it is given. Some infants with hypercalcemia recover without special treatment. On the other hand, some of the severely affected infants have changes which are largely irreversible. In most cases, recovery can be accelerated by treatment. The chief methods of lowering the serum calcium are (1) a low-calcium diet, and (2) cortisone.[4-6,8,9,30-38]

A low-calcium diet is the mainstay of treatment.[9,36] This can be in the form of a low-calcium milk, produced either by treating cows' milk with a calcium-free ion exchange resin,[39,40] or as a commercial dried milk preparation, Locasol (Trufood) containing 7 mg calcium per 100 ml of reconstituted whole milk. In addition, older infants can be given a low calcium cereal (Glaxo) containing approximately 0.1 mg of calcium per gram of cereal. This cereal with its low-calcium content and its high inorganic phosphorus and phytate content results in an appreciable negative calcium balance due to a high content of calcium in feces.[5,7,37]

Low-calcium cereal has the added advantage of a high caloric value so that weight gain is better (and this may be an important factor in accelerating cure if calcium utilization improves). For infants who are entirely bottle fed it can be added to the milk in small amounts (up to a dracham per ounce has been found satisfactory).

The disadvantage of the low-calcium diet is that the preparation of the low-calcium milk and cereal, to be fully effective, requires the use of water of low-calcium content. In certain areas, this means distilled water.

The response of the serum calcium level to diet is not always quick and it may be desirable to give cortisone. Given orally in doses of 25 to 50 mg per day, it results in a fall in serum calcium within few days. The effect is not permanent, as [illegible] continuing the cortisone, the serum calc[illegible] may rebound to a high level.[5] The main value of cortisone is its use as a short term form of therapy combined with the low-calcium diet. After two to four weeks it can be discontinued, by which time the calcium-lowering effect of the diet should be well established.[9] Used over long periods there is the risk of undesirable side effects such as hyperadrenocorticism, and masking of intercurrent infections.

The mode of action of cortisone is not understood, although its main effect appears to be in blocking absorption of calcium from the bowel. The administration of cortisone to infants with idiopathic hypercalcemia[4,5] and adults with sarcoidosis[30] results in negative calcium balances from fecal losses. Its structural similarity to vitamin D has prompted the suggestion of competition for substrate.[35]

It is important that infection should be avoided as much as possible, and if contracted, it should be promptly treated, especially if the infant is on cortisone therapy.

Other measures have been used to limit calcium absorption from the bowel. There have been no reports of phytates being used alone for the treatment of hypercalcemia in infants, but they have been used in the treatment of hypercalcemia in sarcoidosis in adults.[41] There seems to be no contraindication to their use in infants and they should increase the fecal loss of calcium if added to the low calcium diets. The disodium salt of ethylene diamine tetra acetic acid has been used as a calcium-extracting agent.[4] We believe that this treatment carries a serious risk[42] and consider it unnecessary in view of the effectiveness of other forms of treatment.

It is possible that vegetable oils containing high percentages of essential fatty acids may have some use in idiopathic hypercalcemia.

ACKNOWLEDGMENT

We wish to thank Dr. J. D. Pickup and Dr. E. L. Simons for sending us the infant's kidney, and Dr. K. H. Porter for preparing the section used for Figure 7.

We also wish to thank all those who have written to us about cases in their own countries.

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FAT-SOLUBLE VITAMINS.

XXIII. THE INDUCTION OF GROWTH-PROMOTING AND CALCIFYING PROPERTIES IN FATS AND THEIR UNSAPONIFIABLE CONSTITUENTS BY EXPOSURE TO LIGHT.*

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(Received for publication, April 24, 1925.)

In our report of the discovery of induction of growth-promoting and calcifying properties in various materials by exposure to light (1) we inserted brief mention of the fact that growth-promoting properties had also been imparted to fats. At that time, the rats used in these experiments had been on the fat diet for only a few weeks and it was not considered advisable to kill them immediately for data on the deposition of calcium salts. In spite of the absence of this information, we were nevertheless convinced, in the light of other experiments (2, 3, 4, 5), that in all the instances where a growth-promoting property had been imparted, we were dealing with the production of an antirachitic activation. We have previously expressed our conviction that the antirachitic factor functions, not only in the retention of calcium in rickets, but also in the adult, in which rickets is never observed (6). In the sense in which we use the term, the antirachitic factor represents specifically the organic agent which promotes normal calcium anabolism. It may cure rickets, it may promote growth, or it may simply prevent excessive loss of lime from the body as revealed by metabolism experiments on animals which show no gross abnormality. The specific capacity in which it functions in an animal depends upon the condition of the animal, both with respect to age and nutrition, and upon the composition of the ration furnished. These relations must of necessity be rather intricate and can hardly

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be discussed in this paper from the different angles in which we have come to appreciate them. For the present we must confine our treatment to the antirachitic activation of various fats and their constituents. We have studied this matter in some detail, using growth, increased calcium content of bone, and "line tests" as criteria. We have paid particular attention to the mechanism of the reaction with respect to time, nature of light, permanency of the activation, kind of fat, constituents of fat which can take the activation, and the rôle of sterols. The subject is so immense in its potentialities that we consider our treatment far from satisfactory. We are adding to our information as rapidly as facilities permit.

EXPERIMENTAL.

Part I. Experiments with Irradiated Fat on Growing Rats.

We have already stated that at the time of our discovery of the activation of fats by exposure to light we used olive oil and lard for the experiments (1), but we did not mention the fact that we compared them with cod liver oil in the effect produced. This comparison was made on the assumption, that if cod liver oil owed its activity to light, it should not undergo any deterioration on further exposure. Obviously this disregarded many factors possibly involved, but at any rate it furnished us a tangible means of approach to the problem.

As before, we used a quartz mercury vapor lamp of the Cooper-Hewitt BY type run at 40 volts and 4.5 amperes as our source of light. The fats were exposed to its radiations at a distance of 22 inches in 100 cc. quantities contained in a glass dish measuring 8 by 10 inches. They were stirred every 5 to 10 minutes. The lard and olive oil were irradiated 30 minutes, but the cod liver oil was irradiated 17 hours.

The rats used were our usual stock animals reared under our standard conditions designed to produce animals low in their reserve of the antirachitic factor. They were confined in our standard cages provided with false, screen bottoms and were fed a basal diet of purified casein, 18; salt 40, 4 (4); yeast, 8; agar, 2; and dextrinized corn-starch, 68. When the fats were fed, they were mixed in with the above ration, 2 parts for each 98 gm. of the mixture.

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It has already been reported that at the end of 3 weeks the rats on the irradiated olive oil weighed from 92 to 98 gm. in contrast with the controls at 67 to 73 gm., and that those on the irradiated lard weighed 92 to 125 gm. compared with their controls at 74 to 94 gm.

Chart I in presenting the completed records of these experiments shows that the difference in weight of the different groups

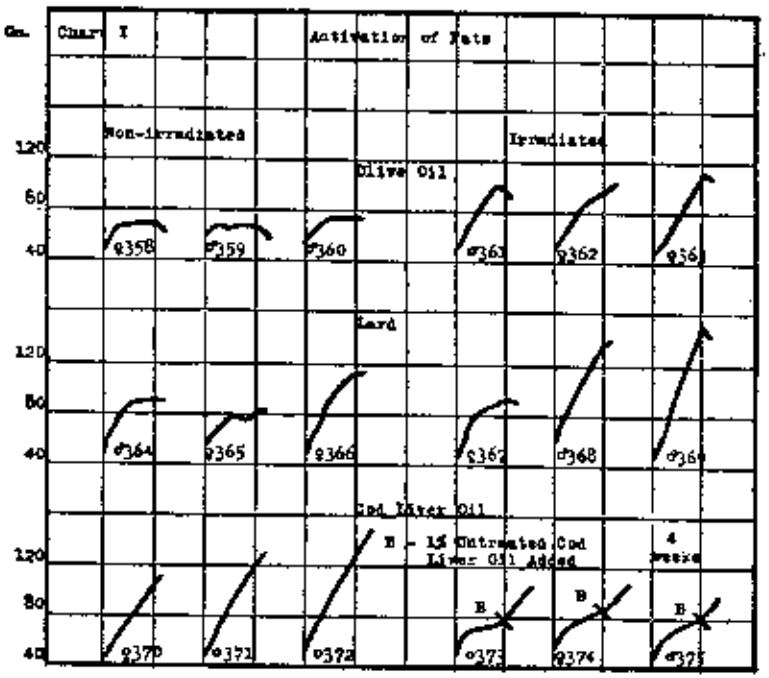

Chart I.

was somewhat increased with prolongation of the experiment. In general both the lard and olive oil rats benefited by irradiation of the fat. One exception, *viz.* Rat 367, was undoubtedly due to a catarrhal condition and therefore merits little consideration.

Contrary to expectations, cod liver oil lost its activity by prolonged irradiation. This apparently was not due to an induced toxicity because the further addition of 1 per cent of untreated oil gave an almost immediate response in growth.

With the termination of the above experiments the animals were killed and the bones removed for ash analyses. These were made on the alcohol-extracted humeri and femora as described in another publication (7). The results are presented only in part (Table I) to conserve space. They show, what we have previously shown to be true for other constituents of the ration, that hand in hand with the stimulation of growth, calcification of the bones is also increased.

That deactivation of the cod liver oil was probably caused by destruction of activated constituents of the oil is supported by the results presented in the first five sets of curves shown in Chart II. In these experiments olive oil was exposed to the ultra-violet rays for respectively 30 minutes, 5 hours, 10 hours, and 17 hours. One

TABLE I.

Increase in Ash Content of Bones with the Feeding of Irradiated Olive Oil.

2 per cent non-irradiated olive oil.				2 per cent irradiated olive oil.			
Rat No.	On ration.	Ash.		Rat No.	On ration.	Ash.	
		Humerus	Femur.			Humerus	Femur.
	days	*per cent*	*per cent*		*days*	*per cent*	*per cent*
358	35	48.8	50.9	361	35	53.9	52.3
359	35	46.4	47.1	362	35	56.1	56.2
360	35	50.0	49.2	363	35	58.3	56.9
Average.....		48.4	49.1	Average.....		56.1	54.8

group served as a control without exposure. The data show that at 30 minutes and even 5 hours exposure, apparently maximum effects of activation comparable to those of our previous trials were secured, but with longer exposure, 10 and 17 hours respectively, such activation had been entirely destroyed.

The last set of curves in Chart II gave us our first data on the permanency of the activation. The test was run on one of our older samples of olive oil, this one in particular having stood in a cork-stoppered Erlenmeyer in a dark cabinet for 5 to 6 weeks before it was used. As seen in the chart it was decidedly growth-promoting. Since these results were obtained we have carried out other tests at intervals of months which will be discussed later in this paper.

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Chart III presents further data on the activation of olive oil with rats taken from two different litters. One-half of each litter

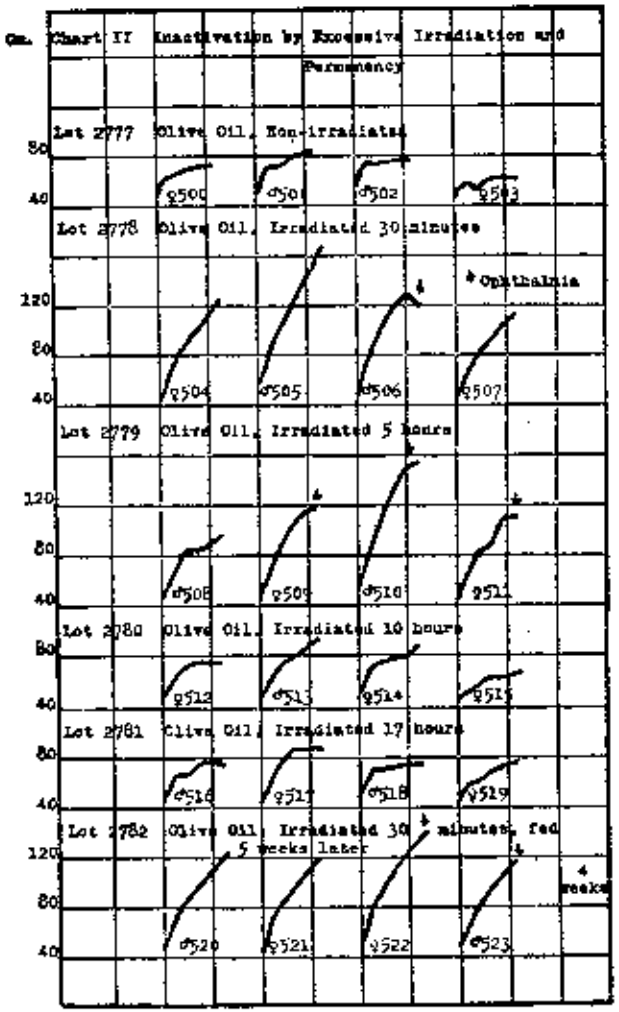

Chart II.

was irradiated and the others were kept as controls. If there remained any doubt in our minds as to the effect of light on the fats or as to the reliability of our technique this dispelled it.

Zucker, Pappenheimer, and Barnett (8), and Steenbock, Jones and Hart (9) showed some time ago that the calcifying properties of cod liver oil were not destroyed by saponification, definitely associating in this way the antirachitic property with the unsaponifiable constituents of the fat. To see if this was also the case with artificially activated oils we irradiated olive oil as before for 30 minutes and then saponified it by boiling it in 50 gm. quantities with 120 cc. of a 20 per cent solution of KOH in alcohol for

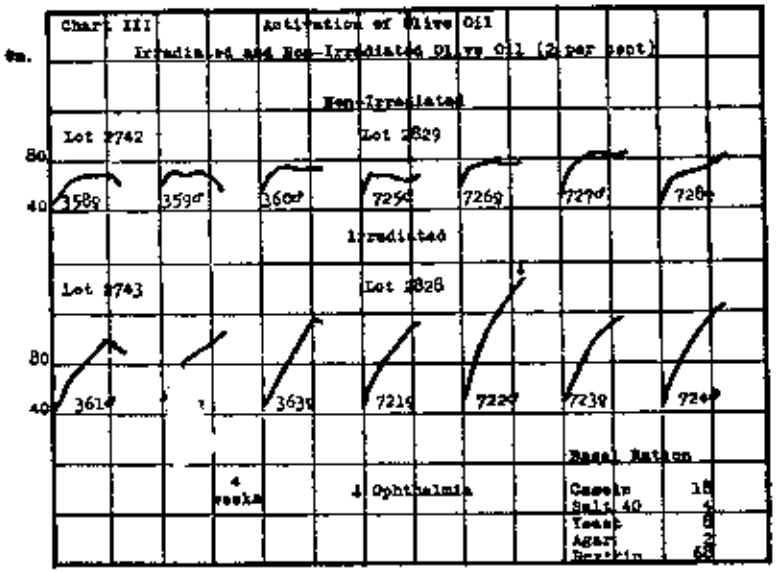

Chart III.

30 minutes. After cooling, the product was diluted with distilled water and shaken out three times with ether. The ether extract was evaporated on the basal ration in a quantity equivalent to 2 per cent of the oil. This was fed to the first group. For comparison there was also prepared a similar product from non-irradiated oil. This was fed to a second group at the same level of intake. A third group received 2 per cent of irradiated oil without any treatment.

Chart IV in the first three sets of curves shows the growth made by the animals on these materials. Irradiated oil and its saponi-

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fiable constituents are seen to be fully equivalent to each other, while the unsaponifiable fraction from non-irradiated oil—pre-

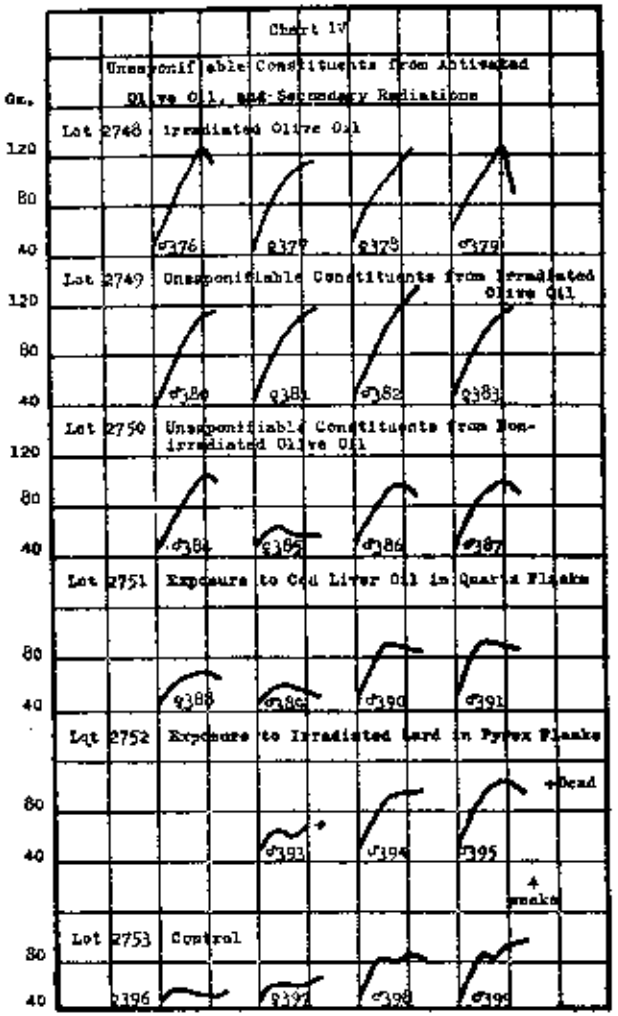

Chart IV.

sented at the bottom of the chart—showed itself to induce little more growth than the basal ration itself.

In Chart IV are also shown two sets of curves, those of Lots 2751 and 2752, which present the results of our first attempts to

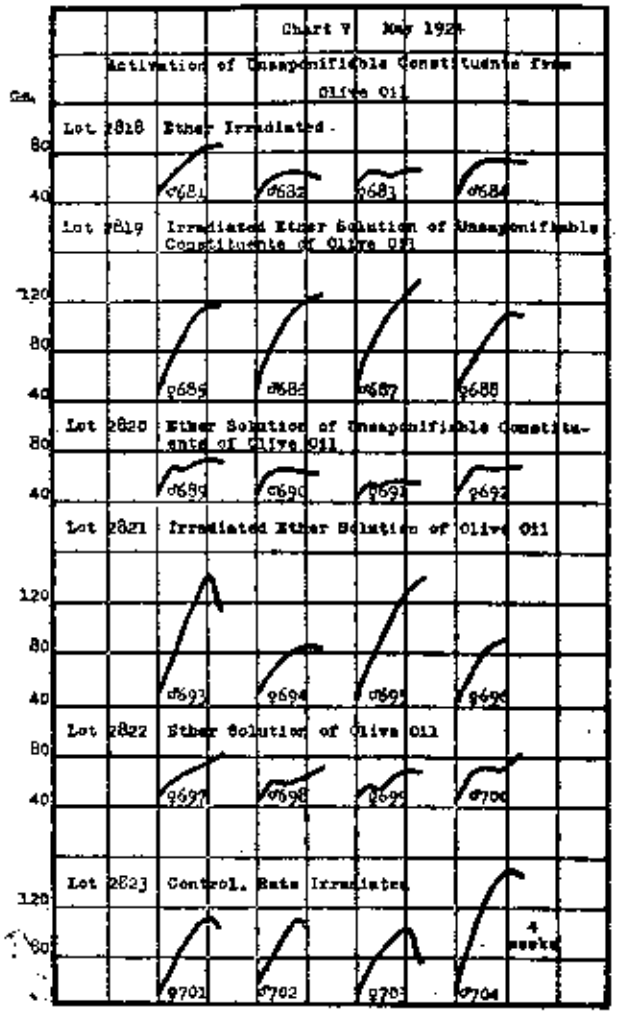

Chart V.

determine the nature of the calcifying and growth-promoting activity of our artificially activated fats and cod liver oil. We

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tried to ascertain if these fats give off secondary radiations; in a general way possibly to be compared to a phosphorescence of which we were reminded. We placed two 250 cc. quartz flasks filled with cod liver oil in our rat cages in such a manner that the animals were confined in close proximity to them. As a matter of fact we reduced the size of our cages to one-fourth of their usual dimensions so that the animals were usually in contact with the flasks.

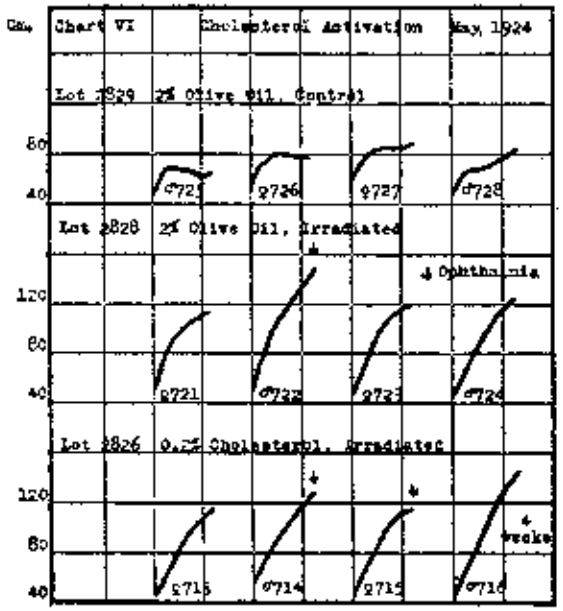

Chart VI.

In another case we took irradiated lard contained in two 200 cc. Pyrex flasks. Needless to say, in all these experiments light other than diffuse daylight and incandescent Mazda lamp light was excluded from the room, for, although these experiments were carried out in March (1924) when solar radiation is not intense no reliance was placed on window glass to filter out the active rays, all windows except those of northern exposure were kept shaded with window curtains. As seen from the chart our results were entirely negative.

Chart V presents still further data on the activation of the unsaponifiable constituents of fats. Here we took the unsaponifiable constituents from olive oil (obtained as before) and irradiated them in ether solution contained in a quartz flask. After irradiation, the solution was evaporated on the ration in such quantity that each 100 gm. received an amount equivalent to 2 gm. of oil. There were also fed portions of the basal ration on which had been evaporated an irradiated ether solution of the unsaponifiable constituents, an irradiated ether solution of olive oil, as well as non-irradiated olive oil and non-irradiated unsaponifiable constituents. In all cases where olive oil or its preparations were fed they were given in such a quantity that 100 gm. of ration received the unsaponifiable constituents from 2 gm. of oil. To further control our experiments, one group of rats on the basal ration was directly irradiated 10 minutes daily 6 days out of the week. As Chart V shows, positive results were obtained only when irradiated unsaponifiable constituents were fed as such or as contained in olive oil, and where the rats were directly irradiated.

With the demonstration that the unsaponifiable constituents of a crude fat could be activated, we next attempted to ascertain if any of the known constituents of this fraction were concerned in this reaction. We had in mind specifically the sterols. In a preliminary way, we dissolved cholesterol prepared from pigs' brain in ether and irradiated it in a quartz flask. After 30 minutes of exposure under the same conditions as the fats, the solution was evaporated on the ration so that 0.2 gm. of cholesterol was added to each 100 gm. of ration. Chart VI shows that this cholesterol was as active as 2 per cent of irradiated olive oil. Previous trials had shown us that non-irradiated cholesterol was entirely inactive, thus furnishing us with the suggestion that at least one sterol was capable of being activated. We speak of it here as a suggestion only because sterols are well known to be purified with difficulty and we had no guarantee that slight amounts of unrecognizable impurities might not have been responsible. Certainly the results of experiments carried out with cholesterol of the degree of purity as purchased on the market cannot be considered final.

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Part II. Experiments with Irradiated and Non-Irradiated Fats on Rachitic Rats.

During the spring of 1924 when practically all of our experiments were of short duration—6 to 8 weeks or less—we were obliged to breed our rats continuously in order to secure a sufficient number of young. This proved to be a heavy drain on the vitality of our females and made it necessary to secure new breeding stock at an early date. To provide such in excellent condition, we departed from our usual procedure and irradiated all our stock with ultra-violet light and also supplemented their ration with 5 per cent of butter fat and fresh green grass, *ad libitum*. These additions may have been unnecessary; but at all events we were soon well supplied with young. However, we found that our troubles had but just begun, for when we put the young on the basal synthetic ration used in our growth experiments they all grew normally without the addition of any of the antirachitic factor. This compelled us to use, either the line test as developed at the Johns Hopkins Laboratory (10), or else the prevention of rickets, as criteria of the induction of calcifying properties.

Before this time we had had but limited experience with rickets and with the line test merely enough to demonstrate that these methods gave the same results as our growth method; but we had also experienced that while the McCollum Ration 3143 (11) was more satisfactory than the Sherman and Pappenheimer Ration 84 (12), it left much to be desired. In the first place, it did not contain a sufficiency of vitamin A for animals as raised in our stock. Ophthalmia often became incident in our animals before the symptoms of rickets had developed to a sufficient extent to make changing of the ration for an experimental trial permissible. This was true even when we made it a point to use yellow corn which McCollum does not specifically recommend. In the second place, the progress of rickets in our animals was far more variable than could be countenanced with indifference, necessitating most careful control. The variation in the incidence of rickets suggested to us that after rickets had been produced a corresponding variation might also be shown in the response of the animals to the addition of the antirachitic factor. We began to suspect that McCollum's

Diet 3143 carried far more protein than necessary—approximately 33 per cent—and especially too much gelatin. Sometimes our animals developed anorexia—a reaction not unusual with high gelatin diets—and at other times, they had diarrhea.

In the course of these experiments we have accordingly cast about for other more suitable rations. After a large number of trials we found that our Ration 2965 composed of yellow corn 76, wheat gluten 20, calcium carbonate 3, and sodium chloride 1, gave far more consistent results than any other tested. This also had the advantage of greater ease of preparation and reduced cost. Before its general use we also used our Ration 2966 which is the same as Ration 2965 with 5 parts of gelatin substituted for 5 of yellow corn. This was found to possess no superior qualities over Ration 2965. At other times we have used our Ration 3075 carrying 1 part of alfalfa in place of 1 of yellow corn in Ration 2965. This serves to increase the vitamin A content which is sometimes desirable when the experiment is run over a long period of time and severe symptoms of rickets are desired. In this case this amount of alfalfa did not add materially to the amount of the antirachitic factor in the ration, but with 5 parts of alfalfa we found the rickets-producing properties of the ration considerably decreased. It is, however, impossible to make any definite statement in regard to the amount of alfalfa which can be used with safety because it varies in its rickets-producing properties according to the extent it has been previously exposed to light. This we have already reported on for Ration 3143 exposed to ultra-violet light (5), and has also been found by us to be true for clover. 5 per cent of clover cured in the dark has no antirachitic properties, but the same amount cured in the sun may prevent rickets entirely. When completely activated it takes but a few per cent of most naturally occurring foods in a rickets-producing ration to change it completely to one physiologically satisfactory.[1]

In studying the activation of oils by light as determined by the line test, we found it desirable first of all to find out in what time a response to the antirachitic addition was to be expected. For this purpose we used a sample of commercial cod liver oil as the antirachitic agent. Six rats, all from one litter, were kept for

[1] Unpublished data.

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35 days on Ration 3143 to develop marked symptoms of rickets. 2 per cent of cod liver oil were then added to the ration and after the 2nd day one animal was killed daily and examined for calcium deposition by silver staining in the metaphyses of the distal ends of the radii and ulnæ. As shown in Table II it was not until the end of the 5th day that a positive line test was obtained. In another trial, with as large an amount of cod liver oil as 10 per cent of the ration, the response was not definitely positive in 2 days. These results, in a general way, correspond to those obtained by McCollum and coworkers (13) who obtained a posi-

TABLE II.

Time Necessary for Production of Positive Line Test.

Lot No.	Rat No.	Basal ration No.	Dietary addition.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
				days	gm.	gm.	gm.	
2837	775	3143	2 per cent cod liver oil.	2	80		5	Negative.
	776		2 " " " " "	3	79		7	"
	777		2 " " " " "	4	97		6	"
	778		2 " " " " "	5	92		5–6	Positive.
	779		2 " " " " "	6	74		5–6	"
	780		2 " " " " "	7	75		5	"
2904	1080	3143	10 " " " " "	2	44	44	4.5	Incipient(?).
	1081		10 " " " " "	2	47	45	4	"
	1082		10 " " " " "	2	79	67	7	"

tive test in 5 days. Evidently, a comparatively low level of antirachitic, over a long period of time, is to be preferred to a high level, over a short period. We have accordingly seen fit to keep our rats at least 7 days and usually 10 to 11 days on the test ration.

It was also necessary for us to know at what level irradiated oils could be fed to best advantage. Table III shows what results are to be expected when irradiated olive oil is fed at lower levels than 2 per cent. It is seen that in a period of 9 days 0.1 per cent was entirely inactive while 0.2 per cent gave fairly positive results. This corresponds with the activity of many samples of cod liver oil as obtained on the market. Such activity is not, however, to be considered a maximum attainable with artificially

TABLE III.

Amounts of Irradiated Olive Oil Necessary for Positive Line Test.

Lot No.	Rat No.	Basal ration No.	Dietary addition.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
				days	gm.	gm.	gm.	
2917	1146	3143	None.	9	85	87	7–8	Negative.
	1147		0.1 per cent irradiated olive oil BI.	9	82	84	8	"
	1148		0.1 " " " " "	9	85	87	8	"
2918	1149		None.	9	84	81	78	"
	1150		0.2 per cent irradiated olive oil BI.	9	77	87	7–8	Questionable.
	1151		0.2 " " " " "	9	64	64	6	Positive.
	1152		0.2 " " " " "	9	70	73	7	"
2919	1153		None.	9	72	78	6–7	Negative.
	1154		0.5 per cent irradiated olive oil BI.	9	72	78	6	Positive.
	1155		0.5 " " " " "	9	65	70	7	"
	1156		0.5 " " " " "	9	62	67	6	"
2920	1157		None.	9	77	80	8	Negative.
	1158		1.0 per cent irradiated olive oil BI.	9	67	70	8	Positive, broad line.
	1159		1.0 " " " " "	9	62	65	6	" "
2921	1160		None.	9	67	74	8	Negative.
	1161		2.0 per cent irradiated olive oil BI.	9	70	70	6	Practically complete healing.
	1162		2.0 " " " " "	9	75	76	6	"

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TABLE IV.

Permanency of Activity Imparted to Olive Oil by Irradiation.

Lot No.	Rat No.	Basal ration No.	Dietary addition.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
				days	gm.	gm.	gm.	
3060	1838	2905	0.5 per cent irradiated olive oil BI kept in bottle in dark 8 mos.	11	84	86	7	Positive.
	1839		1.0 per cent irradiated olive oil BI kept in bottle in dark 8 mos.	11	92	95	8–9	"
	1840		2.0 per cent irradiated olive oil BI kept in bottle in dark 8 mos.	11	84	80	7	"
3161	2326		0.1 per cent irradiated olive oil BI kept in bottle in dark 10 mos.	11	73	77	7	Traces.
	2327		0.2 per cent irradiated olive oil BI kept in bottle in dark 10 mos.	11	85	92	7	"
	2328		0.5 per cent irradiated olive oil BI kept in bottle in dark 10 mos.	11	77	82	6–7	Positive.
	2329		1.0 per cent irradiated olive oil BI kept in bottle in dark 10 mos.	11	79	81	7	Very broad.
	2330		2.0 per cent irradiated olive oil BI kept in bottle in dark 10 mos.	11	79	74	6	Almost healed.
3160	2320		0.1 per cent irradiated olive oil D II kept exposed to air and light 9 mos.	11	104	120	8	Negative.
	2321		0.2 per cent irradiated olive oil D II kept exposed to air and light 9 mos.	11	119	138	8	"
	2322		0.5 per cent irradiated olive oil D II kept exposed to air and light 9 mos.	11	95	105	7–8	"
	2323		1.0 per cent irradiated olive oil D II kept exposed to air and light 9 mos.	11	114	119	7	"
	2324		2.0 per cent irradiated olive oil D II kept exposed to air and light 9 mos.	11	85	94	6–7	"

activated preparations. It is also well known that cod liver oils differ widely in their potency, depending upon their source and method of preparation.

In December we (14) reported that an olive oil activated in April, 1924, had not depreciated in antirachitic potency after 8 months standing in the dark in a stoppered bottle. Hess and

TABLE V.

Optimum Time of Exposure for Antirachitic Activation of Olive Oil Fed at a 2 Per Cent Level.

Lot No.	Rat No.	Basal ration No.	Time of exposure.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
			min.	days	gm.	gm.	gm.	
2901	1061	3143	0	7	65	67	6	Negative.
	1062		5	7	65	64	5	Deposit in center.
	1063		10	7	65	65	5	Narrow line.
	1064		15	7	60	62	5	Heavy line.
	1065		20	7	62	58	5	Very heavy line.
	1066		30	7	62	60	5	" " "
2902	1067		0	7	68	77	6	Negative.
	1068		5	7	67	67	7	Fairly heavy line.
	1069		5	7	67	67	5	" " "
	1070		10	7	75	74	7	Very heavy line.
	1071		10	7	77	72	5	" " "
	1072		15	7	67	64	6	Heavy line.
2903	1073		15	7	74	70	5	" "
	1074		0	7	75	77	5	Negative.
	1075		20	7	72	72	7	Very heavy line.
	1076		20	7	69	67	5	Heavy line.
	1077		30	7	57	52	5	Very heavy line.
	1078		30	7	54	54	6	Heavy line.

Weinstock (15) at the same time reported a persistence of activity for a 6 month period. We are now able to report another test after a 10 month interval which showed results substantially the same as before (Table IV). These results are not to be taken as indicating that the artificially induced activity cannot be destroyed readily under certain conditions. We have found one sample of olive oil, activated in May, 1924, and kept in our office till February, 1925, in a beaker covered with a watch-glass, entirely

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inactive when fed at a level of 2 per cent. It had also lost its yellow color completely even though exposed to only diffuse daylight.

As to time of exposure with our equipment, we have selected 30 minutes, though at times we have used 15 minutes. This apparently gave optimum results, as shown in Table V, with

TABLE VI

Antirachitic Activation of Olive Oil with the Carbon Arc.

Lot No.	Rat No.	Ration No.	Time of exposure of dietary addition of 2 per cent olive oil.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
			min.	*days*	*gm.*	*gm.*	*gm.*	
2983	1442	2966	0	10	94	102	9	Negative.
	1443		5	10	125	134	11	"
	1444		5	10	112	120	11	"
	1445		15	10	90	91	8	"
	1446		15	10	100	97	9	"
	1447		30	10	87	84	6–7	Not continuous.
2984	1448		30	10	105	94	7–8	" "
			hrs.					
	1449		1	10	84	82	5–6	Broad.
	1450		0	10	87	86	5	Negative.
	1451		2	10	102	100	6	Broad.
	1452		2	10	90	88	6	"
	1453		1	10	90	89	6–7	"
2985	1454		5	10	84	74	5	Narrow but definite.
	1455		5	10	89	84	6	" " "
	1456		10	10	100	98	7	" " "
	1457		10	10	85	77	7	" " "
	1458		0	10	97	97	7	Negative.
2986	1459		17	10	109	117	9	Positive(?).
	1460		17	10	97	94	7	"
	1461		0	10	99	100	7–8	Negative.

olive oil exposed in a thin layer. In the experiments there reported 25 cc. of olive oil were measured out in a Petri dish 9.7 cm. in diameter, giving a depth of oil from 3 to 4 mm. This was exposed at a distance of 2 feet from our quartz Cooper-Hewitt mercury arc run at 50 volts and 4 amperes. This strength of

current with our apparatus gave a light of low intensity and as the burner had been in daily use for some months its original efficiency was no doubt much reduced.

Other sources of ultra-violet can also be used as shown by the results of Table VI. Here we used an open carbon arc run at 50 to 60 volts at a distance of 20 inches from the oil. The oil was contained in a Petri dish of the same size as before—25 cc. to a dish. To prevent excessive heating and contamination of oil with flakes of carbon, we used an air current from an electric fan. 5 and 15 minute exposures gave entirely negative results and even 30 minutes gave only a faint deposit of calcium salts. Though the data of carbon arc and quartz mercury vapor lamp illuminations are not absolutely comparable they suggest that the carbon arc is not as efficient in effecting the antirachitic activation as the mercury arc. More data are required before a final statement can be made, but it can be accepted that varied sources of ultra-violet light can be used. We have also obtained activation with the iron arc and by exposure to sunlight.

Hess and Weinstock (16) have reported very interesting results, using Corning glass filters in attempting to determine to what particular rays the antirachitic action of light on rats might be due. They found that "in order to be of value in rickets, ultra violet waves must have a wave length not longer than 303 or possibly 313 millimicrons."

We have also used Corning filters,[3] in part the same as those used by Hess and Weinstock, to determine in a preliminary way what rays are responsible for the antirachitic activation of foods. For this purpose we measured 2 cc. of olive oil in inverted porcelain crucible covers about 4 cm. in diameter; covered them with 2 inch squares of the various filters and exposed them simultaneously at a distance of about 20 inches from our mercury arc. The period of exposure was 1 hour. Table VII presents the data obtained. In the first place it shows that Pyrex if sufficiently thin—approximately 1.5 mm.—will transmit the activating rays of the intensity used; and that window glass and plate glass and the Corning glass

[3] The transmission spectra of these filters for ultra-violet light have been published in Technologic Papers 119 and 148 of the U. S. Bureau of Standards.

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TABLE VII.

Antirachitic Effect of Mercury Arc with 8 Per Cent Olive Oil Irradiated 1 Hour Through Filters.

Lot No.	Rat No.	Ration No.	Filter.	Filter thickness.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
				mm.	days	gm.	gm.	gm.	
2998	1521	2960	Pyrex.	6.00	10	93	100	8	Negative.
	1522		"	6.00	10	84	85	7–8	"
	1523		"	5.02	10	90	102	7	"
	1524		"	5.02	10	95	107	7	"
	1525		"	4.00	10	85	92	7	"
3001	1538		"	4.00	10	80	87	7–8	"
	1539		"	3.00	10	88	91	6–8	"
	1540		"	3.00	10	94	104	7–8	"
	1541		"	2.50	10	95	107	7	"
	1542		"	2.50	10	77	81	6–8	Broken line.
3002	1543		"	2.00	10	98	103	6–8	Negative.
	1544		"	2.00	10	100	102	7	"
	1545		"	1.49	10	88	88	6	Broken line.
	1546		"	1.49	10	80	82	6–7	" "
	1547		"	0.99	10	90	96	6–7	" "
3003	1548		"	0.99	10	110	115	8	Continuous line.
	1549		"	0.75	10	110	109	6–8	Broad line.
	1550		"	0.75	10	100	98	8	" "
	1551		Window.	2.92	10	90	88	6–7	Negative.
	1552		"	2.92	10	112	108	6	"
3005	1559		"	1.01	10	107	119	9–10	"
	1560		"	1.01	10	105	107	7	"
	1561		Plate.	4.15	10	120	140	9–10	"
	1562		"	4.15	10	114	127	10	"
	1563		"	2.78	10	107	112	8	"
3006	1564		"	2.78	10	114	125	10	"
	1565		G-586-A	3.55	10	97	104	6	"
	1566		"	3.55	10	95	98	6	"
	1567		G-38-H	3.43	10	107	112	7	"
	1568		"	3.43	10	95	96	7	"
3007	1569		G-385-DP	3.42	10	90	95	7–8	"
	1570		"	3.42	10	97	104	7–8	"
	1571		None.		10	90	89	6–7	Almost complete healing.
	1572		"		10	92	92	8	" "

TABLE VII—*Concluded.*

Lot No.	Rat No.	Ration No.	Filter.	Filter thickness.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
				mm.	days	gm.	gm.	gm.	
3107	2027	2965	G-586-A	3.55	10	103	107	7–8	Negative.
	2028		"	3.55	10	92	90	5–6	" *
	2029		"	3.55	10	113	118	8	" †
	2030		"	3.55	10	95	95	5–6	" †
3108	2033		Water.	140	10	103	101	7–8	Continuous line.‡
	2034		"	140	10	97	93	7	" " ‡
	2035		"	25	10	98	86	6	" " ‡
	2036		"	25	10	130	125	8–9	" "

* Irradiated 4 hours.
† Irradiated 8 hours.
‡ Irradiated 5 hours.

filters G-385-DP, a colorless glass, G-38-H no viol C, a yellow glass, and G-586-A, a purple glass, are impermeable. Pyrex, as is well known, is more transparent to the violet end of the spectrum; and in harmony with this we find that while 1.5 mm. of Pyrex allowed activation to take place, 1.01 mm. of window glass did not.

Inspection of spectrograms taken by Dr. H. P. Gage of the Corning Glass Works showed that the window glass filter transmitted light of even shorter wave-lengths than 313 millimicrons while Pyrex of 1.5 mm. transmitted down to 297 and less. This locates the activating light approximately in the region of the spectrum of no greater wave-length than that spoken of by Hess when using light on the animals directly, but it is to be emphasized that quantitative as well as qualitative factors are involved and absolute conclusions are impossible. In the long time exposures of our olive oil considerable energy in the form of light of shorter wave-lengths than that recorded in the spectrograms may have penetrated the filter and affected the activation. Results obtained with filters in this manner are therefore far from definite. Experiments are now in progress in cooperation with Dr. Farrington Daniels in which we are using monochromatic light on a quantitative basis.

We were surprised at the result obtained with the purple glass

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filter, G-586-A—which was negative—not because of its transmission spectrum but because of results published by Hess and Weinstock (16). They made a special point of the fact that, while filter G-586-A transmitted rays which possessed antirachitic properties, another filter, G-86-B, a neutral glass, did not. These filters both transmitted approximately the same rays of the short end of the spectrum, but differed essentially beyond 405 millimicrons. They were led to suggest a neutralizing action of light of one wave-length upon another. We have not been able to confirm their results in this particular, because, not only have we found that an 8 hour exposure under G-586-A is without effect in activating olive oil but a 2 hour daily exposure of rats for 10 days also failed to cure rickets as Hess and Weinstock reported. These results on rats were obtained in duplicate with two different filters, using a new lamp in one case at a distance of 12 inches. Since we obtained these results, Hess and Weinstock (17) also found that filter G-586-A did not allow the passage of light to activate oils, but as far as we know they have not yet reported further results on rats directly.

Knowledge of the transmissibility of various filters for the antirachitic rays is of great importance because of the limited occurrence of antirachitic foods in nature. No doubt, solar radiations of the required wave-lengths are in general able to produce only a superficial effect due to their limited ability to penetrate most materials occurring in nature. Among these, water (18) is a well known exception. That the specific rays of the ultra-violet concerned in antirachitic activation are able to penetrate water, we determined in the following manner. We filled a 250 cc. quartz Florence flask half full of distilled water and, after adding 25 cc. of olive oil and stoppering, inverted it and weighted it down in a glass battery jar almost filled with water. The whole was then exposed to the radiations from a quartz mercury vapor lamp from above for 5 hours. In one case the exposure was made under 25 mm. of water and in another case under 140 mm.

Table VII shows that the olive oil was activated in both cases. Our experiments have not yet determined through what depth of water the rays of the intensity used by us will penetrate, but they suggest how it is possible for cod and other fish to have activated oils stored in their body. Undoubtedly they obtain the active

TABLE VIII.

Antirachitic Activation of Fresh Fats as Compared with Old Fats.

Lot No.	Rat No.	Ration No.	Dietary addition.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
				days	gm.	gm.	gm.	
2774	482	3143	2 per cent untreated cocoanut oil A — 7 yrs. old.	6	130	125	8	Negative.
	483		2 " " " " " " — 7 " "	6	94	97	6–7	"
	484		2 " " " " " " — 7 " "	6	135	129	7	"
	485		2 " " irradiated " " " — 7 " "	6	100	100	6	"
	486		2 " " " " " " — 7 " "	6	116	117	6	"
	487		2 " " " " " " — 7 " "	6	84	82	4–5	"
2840	781		2 " " untreated " " — 2 yrs. old.	8	77	77	5	"
	782		2 " " " " " — 2 " "	6	75	75	6	"
	783		2 " " irradiated " " — 2 " "	7	72	65	5	Positive.
	784		2 " " " " " — 2 " "	8	70	65	5	"
	785		2 " " " " " — 2 " "	8	72	62	4–5	"
2863	885		2 " " untreated fresh cocoanut oil.	7	82	87	6	Negative.
	886		2 " " " " " "	10	87	82	6	"
	887		2 " " irradiated " " "	7	77	78	4	Positive.
	888		2 " " " " " "	10	88	87	5	"
3162	2331		5 " " untreated " " "	11	89	90	6	Negative.
	2332		5 " " " " " "	11	104	108	6	"
	2333		10 " " " " " "	11	118	112	5	"
	2334		10 " " " " " "	11	112	115	6	"
	2335		20 " " " " " "	11	125	128	6	"
	2336		20 " " " " " "	11	104	102	6	Few specks.
3002	1791	2965	2 " " fresh corn oil — Mazola.	10	82	85	7	Negative.
	1792		2 " " " " " — "	10	80	88	7	"
	1793		2 " " irradiated fresh corn oil — Mazola.	10	95	95	8	Positive.
	1794		2 " " " " " " — "	10	105	98	7–8	"

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3003	1799	2 per cent untreated corn oil — 10 yrs. old.	10	87	87	7	Negative.
	1800	" " " " " — 10 " " "	10	78	80	6–7	"
	1801	" " irradiated " " — 10 " " "	10	68	73	6	"
	1802	" " " " " — 10 " " "	10	98	102	8–9	"
2838	769	3143 2 " " untreated cottonseed oil — 10 yrs. old.	11	77	73	5	"
	770	2 " " " " " — 10 " " "	7	70	69	5	"
	771	2 " " " " " — 10 " " "	17	90	82	4–5	"
	772	2 " " irradiated " " — 10 " " "	7	99	100	7–8	"
	773	2 " " " " " — 10 " " "	11	87	90	6	"
	774	2 " " " " " — 10 " " "	17	79	74	4–5	"
2997	1517	2963 2 " " fresh cottonseed oil — Wesson.	10	73	78	6	Positive.
	1518	2 " " " " " " " "	10	85	85	7	"
	1519	2 " " untreated " " — " " "	10	110	119	8	Negative.
	1520	2 " " " " " — " " "	10	93	100	8	"
2775	489	3143 2 " " peanut oil — 6 yrs. old.	6	93	92	3–4	"
	489	2 " " " " " — 6 " " "	6	79	72	5	"
	490	2 " " " " " — 6 " " "	8	102	93	4–5	"
	491	2 " " irradiated " " — 6 " " "	6	100	100	5	"
	492	2 " " " " " — 6 " " "	6	124	119	8	Faint line.
	493	2 " " " " " — 6 " " "	6	101	99	6	Negative.
3149	2255	2965 2 " " untreated oleo oil — 6 yrs. old.	11	112	117	5	"
	2256	2 " " " " " — 6 " " "	11	99	107	6–7	"
	2257	2 " " irradiated " " — 6 " " "	11	102	112	7	"
	2258	2 " " " " " — 6 " " "	11	80	92	6	"
3148	2252	10 " " butter fat.	10	82	86	6–7	"
3130	2143	2 " " " "	11	81	90	4	"
	2144	2 " " " " irradiated.	11	84	83	6	Positive
	2145	2 " " " " "	11	102	106	6	"
3106	2023	6 " " " " "	11	107	112	6	Almost healed.
	2024	10 " " " " "	11	92	98	6	"

compounds from small fish living in rather shallow waters which in turn consume smaller organisms thoroughly exposed to ultraviolet radiations. This suggestion is not new. Saleeby (19) when advocating greater hygienic utilization of sunlight expressed himself as follows: "Not one word against cod liver oil do I suggest. . . . . The vitamin in it was probably made by sunlight falling on the green plankton in the far waters of the North Atlantic, and thence, via other creatures, reached the cod, and the cod's liver, whence at length it gets into the blood of Sheffield children." Drummond and Watson have also pointed out how growth-promoting properties are synthesized by diatoms grown in the sunlight in synthetic media and how these diatoms ultimately and indirectly provide food for the cod (20).

When attempting to activate other fats than lard and olive oil we have obtained variable results. One of the first oils that we used was a sample of cocoanut oil which had been kept in a mason jar in the laboratory for 7 years. It had been exposed to the diffuse light of the laboratory and was decidedly acid. Table VIII shows that when added to Ration 3143 at a 2 per cent level after irradiation, it gave negative results. Another sample, an unrefined oil kept in the laboratory for 2 years in a tin container, while inactive before irradiation, was readily made antirachitic. A sample of fresh cocoanut oil extracted by pressure from cocoanut meats in the laboratory was found inactive, but could be readily activated. In general with laboratory-prepared cocoanut oil we have not as yet been able to demonstrate antirachitic properties by using the line test when as much as 20 per cent of the ration was fed over a period of 10 days or when as much as 60 per cent of oil equivalent was fed as the isolated unsaponifiable constituents. We are inclined to believe that the reported antirachitic potency of commercial cocoanut oil (13, 21) may have been acquired by the usual practice of drying the copra of commerce in the sun. This remains to be tested out experimentally.

A fresh sample of corn oil gave negative results before irradiation and positive results afterwards as shown in Table VIII. But again with a sample of corn oil approximately 10 years old, negative results were obtained even after 30 minutes irradiation. The same was true of cottonseed oil of approximately the same age. The corn oil had been kept in a tightly stoppered tin container

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about one-third full and the cottonseed oil in a loosely stoppered glass bottle. Peanut oil and oleo oils, both 6 years old, kept in tin cans, could not be activated. The fresh oils have not yet been tested.

As the result of the widespread recognition of the nutritive value of milk and its products the idea that milk is a panacea for human and animal ills has gained widespread credence. It is, however, common knowledge in medical circles that neither the consumption of cow's milk nor human milk even in large quantity is necessarily a guarantee against the incidence of rickets. Our data on butter fat (Lots 3148, 3130, and 3106, Table VIII) support other evidence (13) of its inefficiency in this capacity. This obviously is due not so much to a poverty in compounds which can be antirachitically activated as to a lack of radiation. Like whole milk and egg yolk (20) the potency of which we have increased from 15- to 20-fold, it too can have its potency increased.

Part III. Experiments with Irradiated and Non-Irradiated Unsaponifiable Constituents on Rachitic Rats.

In Part I we described experiments bearing on the antirachitic activation of the unsaponifiable constituents of olive oil as determined by our growth method. We now wish to present data using the "line test" for confirmation of these results, as well as for a more definite characterization of the nature of the compounds which can be activated.

First of all, we again prepared an ether solution of the unsaponifiable constituents of olive oil. This we administered before and after irradiation to rachitic rats. The ether solution was evaporated on the ration in amounts equivalent to 2 per cent of olive oil and when irradiated was always exposed to our mercury arc for 30 minutes at a distance of approximately 2 feet. Table IX confirms our other work in showing that the irradiated material induced calcium deposition in the rachitic rat while the untreated did not. From these results, it is not, however, to be inferred that the ability to become antirachitically active is a property common to unsaponifiable compounds in general. We shall not treat of this matter in detail at this time, but in a preliminary way we show in Table X how mineral oil (Stanolax), for example, can-

not be affected in this manner. This corresponds with data obtained by Hess and Weinstock (17).

TABLE IX.

Activation of Unsaponifiable Constituents of Fats.

Lot No.	Rat No.	Ration No.	Dietary addition.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
				days	gm.	gm.	gm.	
2854	839	3143	Unsaponifiable matter equivalent to 2 per cent olive oil.	11	84	92	8	Negative.
	840		" "	11	77	87	8	"
	841		" "	9	85	82	6	"
	842		Unsaponifiable matter equivalent to 2 per cent olive oil irradiated.	9	82	80	6	Positive.
	843		" "	11		72	6	"
	844		" "	11	102	98	6–7	"

TABLE X.

Irradiation of Mineral Oil.

Lot No.	Rat No.	Ration No.	Dietary addition.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
				days	gm.	gm.	gm.	
2830	731	3143	0.5 per cent untreated mineral oil (Stanolax).	26	114	110	6	Negative.
	733		0.5 per cent irradiated mineral oil (Stanolax).	26	87	85	6	"
2817	677		2.0 per cent untreated mineral oil (Stanolax).	26	89	89	5–6	"
	680		2.0 per cent irradiated mineral oil (Stanolax).	26	97	96	5–6	"

Our earlier experiments of May, 1924, showing that ordinary cholesterol represented one of a group of compounds taking the activation, led us to concentrate our attention on cholesterol. We used cholesterol prepared from pig's brain by cold acetone

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extraction after drying with plaster of Paris. Purification of it was effected by saponifying it with potassium hydroxide dissolved in alcohol, then extracting it with ether, and repeatedly crystallizing from alcohol. Samples 1 and 2 were recrystallized eight times from 95 per cent alcohol which gave us a product melting at 147°C. (uncorrected). Irradiation of them was carried out in ether solution in a quartz flask with an exposure of 30 minutes at a distance of 2 feet.

Lots 2967 and 2981 in Table XI show that cholesterol prepared as above was activated to a very high degree. One-hundredth of 1 per cent of irradiated cholesterol in a ration, with the total consumption by the rat of 6 mg., gave a very broad calcium line in the distal ends of its radii and ulnæ in 10 days. These results, however, did not assure us definitely that cholesterol represented the acceptor for the radiant energy because sterols are well known to be purified with difficulty. We accordingly took measures to purify our cholesterol further.

4.93 gm. of cholesterol (already recrystallized eight times and activated by exposure to ultra-violet light) were benzoylated by the method of Dorée and Gardner (22), using redistilled benzoyl chloride and pyridine. After pouring into water, the product was filtered off; boiled up with alcohol; cooled; and filtered. This latter was repeated twice and gave us a preparation of cholesterol benzoate melting at 147°C. (uncorrected), with the characteristic changes which this compound undergoes when exposed to higher temperatures.

When fed to rats it was found inactive (Lot 3004). After saponification by boiling with potassium hydroxide, dissolved in alcohol, then extraction with ether, and recovery of the cholesterol by crystallization from alcohol, a cholesterol was obtained which was also found inactive. Its melting point remained unchanged at 147°C. (uncorrected). Irradiation in ether solution, however, was able to restore its activity.

At present we are unable to explain definitely the negative results obtained with the benzoate as prepared from the active cholesterol, because we do not know whether the benzoate as such or the cholesterol in combination with it is absorbed. It appears, however, that the cholesterol is totally inactivated because mother liquors from the recrystallizations of the benzoate when fed to two rats were also found inactive.

TABLE XI

Antirachitic Activation of Cholesterol.

Lot No.	Rat No.	Ration No.	Dietary addition.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
				days	gm.	gm.	gm.	
2967	1338	3143	2 per cent olive oil irradiated.	10	97	98	6	Positive.
	1339		0.2 per cent cholesterol.	10	104	109	7–8	Negative.
	1340		0.2 " " "	10	118	119	7–8	"
	1341		0.2 " " " I irradiated.	10	115	107	6–7	Positive.
	1342		0.2 " " " I "	10	120	114	6	"
	1343		0.2 " " " I "	10	106	96	4	"
2981	1429	2966	0.2 " " " II.	10	84	89	8	Negative.
	1430		0.2 " " " II.	10	80	87	8	"
	1431		0.2 " " " II irradiated.	10	97	88	5	Very broad.
	1432		0.1 " " " II "	10	84	76	5	Almost complete healing.
	1433		0.05 " " " II "	10	85	77	6	" "
	1434		0.01 " " " II "	10	77	77	6	Very broad.
3004	1653		0.2 per cent benzoate of irradiated cholesterol I.	10	92	105	6–8	Negative.
	1654		0.2 " " " "	10	87	93	6–8	"
	1655		0.2 per cent cholesterol III from benzoate of irradiated cholesterol I.	10	90	102	5–6	"
	1656		0.2 " " " " " "	10	90	96	7–8	"
	1657		0.2 per cent irradiated cholesterol III from benzoate of irradiated cholesterol I.	10	97	105	7–8	Almost complete healing.
	1658		Mother liquors from benzoate. " " " "	10	102	114	8	" " "
3064	1809	2965	" " " "	10	112	107	10–11	Negative.
	1810		" " " "	10	102	115	9	"
3069	1775		0.1 per cent irradiated cholesterol III-A prepared through acetate from cholesterol III.	10	99	107	8	Broad.
	1776		" " " "	10	87	90	7	Almost complete healing.

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The cholesterol obtained from the benzoate was purified still more. We took 1 gm. of it after recrystallization from alcohol and acetylated it by heating with acetic anhydride over a Bunsen burner for 10 minutes. On pouring into water the acetate separated out. It was recrystallized from alcohol, giving 0.925 gm. of product, melting at 113.8°C. (uncorrected). 0.5 gm. of this was saponified, yielding after crystallization from alcohol 0.77 gm. of cholesterol. This cholesterol again gave the same melting point; namely, 147°C. (uncorrected) as before. When irradiated and fed at a level of 0.1 per cent, it gave a very broad line test with Rats 1775 and 1776.

Up to this time we had always irradiated our cholesterol in ether solution to good effect. Table XII shows that it can also be activated in crystal form when exposed under our usual conditions. Apparently the time of exposure can be varied tremendously because after an exposure of 17 hours in place of the usual 30 minutes, it is seen that the activity of the cholesterol had been decreased to only a barely detectable degree. It should be emphasized that we have presented only a part of the data. Actually we have irradiated cholesterol for 1 hour, 2 hours, 5 hours, and 10 hours, as well as at 30 minutes and 17 hours, and each preparation was fed at the various levels ranging from 0.01 to 0.2 per cent. Our results showed no discrepancies in the entire series. In view of the fact that the cholesterol was exposed to the air in an open dish during these irradiations, it indicates a remarkable stability on the part of the cholesterol which stands in rather sharp contrast to what we have observed for olive oil.

Table XIII presents some data that we obtained with phytosterol from corn oils. Here we used the same corn oils on which we have already reported in Part II. From these we prepared phytosterols by saponifying them with sodium hydroxide dissolved in alcohol and then extracting the dried soaps with ether. The resultant ether extract was evaporated to dryness, taken up in hot alcohol, and the phytosterols were crystallized out on cooling. Purification was effected by repeated crystallization from hot alcohol in like manner. Phytosterol I was recrystallized four times; phytosterol II, nine times; and phytosterol III, five times. All were beautifully white crystalline compounds, melting respectively at 137°, 138°, and 138.5°.

TABLE XII.

Excessive Irradiation of Cholesterol.

Lot No.	Rat No.	Ration No.	Dietary addition.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
				days	gm.	gm.	gm.	
3000	1934	2066	0.01 per cent cholesterol V irradiated 30 min. in ether solution.	10	139	145	9	Broad.
	1935		0.05 per cent cholesterol V irradiated 30 min. in ether solution.	10	102	105	7-8	Almost completely healed.
	1936		0.1 per cent cholesterol V irradiated 30 min. in ether solution.	10	97	102	7-8	" "
	1937		0.2 per cent cholesterol V irradiated 30 min. in ether solution.	10	95	100	9	" "
3003	1954		0.01 per cent cholesterol V irradiated 17 hrs. in ether solution.	11	102	107	8-9	Not definitely positive.
	1955		0.05 per cent cholesterol V irradiated 17 hrs. in ether solution.	11	104	113	7-8	Positive.
	1956		0.1 per cent cholesterol V irradiated 17 hrs. in ether solution.	11	102	100	7	Quite broad.
	1957		0.2 per cent cholesterol V irradiated 17 hrs. in ether solution.	11	107	107	8	Very broad.
	1958		0.01 per cent cholesterol V irradiated 30 min. as crystals.	11	82	85	6-7	Quite broad.
	1959		0.05 per cent cholesterol V irradiated 30 min. as crystals.	11	77	75	5-6	Almost completely healed.

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3094	1960		0.1 per cent cholesterol V irradiated 30 min. as crystals.	11	95	99	6	Very broad.
	1961		0.2 per cent cholesterol V irradiated 30 min. as crystals.	11	103	100	6-7	Almost completely healed.
3096	1978		0.01 per cent cholesterol V irradiated 17 hrs. as crystals.	11	85	85	6-7	Negative.
3104	2007		0.05 per cent cholesterol V irradiated 17 hrs. as crystals.	10	93	100	9	Positive.
	2008		0.1 per cent cholesterol V irradiated 17 hrs. as crystals.	10	89	90	7	"
3108	2031		0.2 per cent cholesterol V irradiated 17 hrs. as crystals.	10	109	97	5	"

TABLE XIII.

Activation of Phytosterol.

Lot No.	Rat No.	Ration No.	Dietary addition.	On modified ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
				days	*gm.*	*gm.*	*gm.*	
3062	1795	2965	0.175 per cent phytosterol III from fresh corn oil (Mazola).	10	90	92	7	Negative.
	1796		" "	10	94	94	7	"
	1797		0.175 per cent irradiated phytosterol III from fresh corn oil (Mazola).	10	88	82	6-7	Positive.
	1798		" "	10	79	76	6-7	"
3063	1803		0.2 per cent phytosterol II from old corn oil.	10	90	94	7	Negative.
	1804		0.2 " " " II " " " "	10	135	116	0-1	(Positive but caused by fasting.)
	1805		0.2 " " irradiated phytosterol II from old corn oil.	10	82	87	8-9	Negative.
	1806		" "	10	95	102	8	Traces.
3026	1645	3143	0.2 per cent phytosterol I from old corn oil.	10	75	88	6	Negative.
	1646		0.2 " " " I " " " "	10	82	95	7	"
	1647		0.2 " " irradiated phytosterol I from old corn oil.	10	102	116	9	"
	1648		" "	10	90	96	7	"
3078	1883	2965	0.5 per cent irradiated phytosterol II from old corn oil.	10	80	80	7	Narrow line.
	1884		" "	10	59	65	6	Broad line.
	1885		1.0 per cent irradiated phytosterol II from old corn oil.	10	77	75	7	" "
	1886		" "	10	87	85	7	" "

The data shown in the table corroborate in a general way with the conclusions that we arrived at from our experiments carried out with the oils themselves with the exception that when one of the phytosterols was fed at a higher level than 0.2 per cent of the ration it gave positive results. Apparently there was still a trace of material in the oil which was capable of being activated. It is not clear from these results whether this inactivation represents an isomerization of the sterol or presence of a contamination. We shall report on that later.

When we first observed that old cocoanut oil could not be activated, we suspected that probably the acidity might be responsible. To determine this, we added 5.44 gm. of oleic acid to 100 cc. of fresh olive oil, which gave it an acidity equal to that of the cocoanut oil, and then irradiated the mixture. We found as shown in Table XIV that this did not interfere with the activating processes. We also dissolved the unsaponifiable constituents of olive oil in old cocoanut oil which could not be activated in such amounts that 2 gm. of cocoanut oil carried the unsaponifiable constituents from 2 gm. of olive oil. As the table shows, when this mixture was irradiated it became antirachitically active. We believe as a result of these experiments that we are justified in assuming that the failure of the activation of the old oils is not due to an interference with the process of activation itself but to some molecular change in the unsaponifiable constituents which renders them incapable of accepting the activation.

In this connection it is interesting to note the results obtained by Fridericia (23) when determining what he believed to be vitamin A. He found that old fats, or oxidized butter fat, when melted with fresh untreated butter fat, destroyed the ability of the latter to maintain growth in rats. As Fridericia makes no mention of the incidence of ophthalmia in his animals it is very probable that his animals ceased to grow because of the destruction of the antirachitic factor rather than of vitamin A. This is all the more probable because 10 per cent of butter fat as used by him in compounding his ration would not supply a great excess of the antirachitic agent. His results from this standpoint can be brought into harmony with our own. Quite recently we had occasion to observe that olive oil activated in the presence of oleic acid had lost its activity after standing in the dark for 4 months. This is in sharp

TABLE XIV.

Analysis of Failure of Activation of Old Fats.

Lot No.	Rat No.	Ration No.	Dietary addition.	On ration.	Initial weight.	Final weight.	Daily consumption.	Line test.
				days	gm.	gm.	gm.	
2963	1344	3143	2 per cent irradiated olive oil.	9	115	110	7-8	Positive.
	1345		2 " " olive oil acidified with oleic acid.	9	98	93	6	Negative.
	1346		" "	9	85	75	3-4	Positive (animal sick with respiratory trouble).
	1347		2 per cent oil acidified with oleic acid, then irradiated.	9	105	108	6-7	Positive.
2962	1348		" "	9	75	72	5-6	"
	1349		" "	9	110	112	8	"
	1351		Irradiated non-saponifiable extract of cocoanut oil A equivalent to 2 per cent of oil	9	97	96	7	Negative.
2964	1352		"	9	87	87	7	"
	1356		2 per cent cocoanut oil A plus non-saponifiable extract of olive oil equivalent to 2 per cent olive oil.	9	117	118	5-6	"
	1357		"	9	130	131	7-8	"
	1358		2 per cent cocoanut oil A irradiated with non-saponifiable extract of olive oil equivalent to 2 per cent olive oil.	9	105	102	5-6	Positive.
1359			"	9	102	96	6	"
1360			"	9	130	126	9	"

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contrast to our observations with unacidified olive oil. Furthermore, activated cholesterol has been observed to lose its activity by evaporation with hydrochloric acid.

SUMMARY.

Evidence of the antirachitic activation of fats is presented in considerable detail using the line test, growth, and increased calcium content of bone, as criteria.

Excessive irradiation of olive oil as well as cod liver oil was found to result in their inactivation. Evidently the compounds activated are rather labile in the presence of other constituents of these fats.

Activated olive oil, kept in a stoppered bottle in the dark, was found unimpaired in activity after 10 months.

As in cod liver oil, the antirachitic potency of irradiated fats lies in their unsaponifiable constituents.

Mineral oil cannot be activated.

A ration composed of yellow corn 76, wheat gluten 20, calcium carbonate 3, and sodium chloride 1, was found more satisfactory for producing rickets than any hitherto reported.

With the various rickets-producing rations used, a test period of 7 to 11 days was found sufficient to produce a positive reaction.

Activation was induced by light produced by the quartz mercury vapor lamp, the open carbon arc, the iron arc, and by sunlight.

Activating rays of the quartz mercury vapor lamp were not able to penetrate the Corning glass filters G-385-DP, G-38-H, G-586-A, window glass, nor plate glass. Thin Pyrex was somewhat permeable.

Water was found to transmit the activating rays, thus demonstrating how cod liver oil may possibly acquire its activity.

Old oils such as cocoanut oil, corn oil, oleo oil, peanut oil, and cottonseed oil, all acid in reaction, could not be activated.

The same was true of the unsaponifiable constituents of old oils, including phytosterols with the exception of one case. In this, the irradiated phytosterol showed some activity when fed at high levels.

Cholesterol, purified by repeated crystallizations from alcohol, and then as the benzoate and acetate, could be activated.

The activity of irradiated cholesterol could not be destroyed by long continued irradiation either in crystal form or in ether solution.

The failure of activation of old oils does not lie in an inhibition of the reaction by compounds formed on standing because mixtures of unsaponifiable constituents of fresh oils with old oils can be activated; furthermore, acidification of fresh fats does not prevent activation.

Further data will be presented later.

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VITAMIN D AND TISSUE CITRATE*

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The increased elimination of urinary citrate effected by the administration of vitamin D (1) raises the question whether this is the result of a lowering of the renal threshold or a response to an increased accumulation in body tissues, or both. The reports in the literature on tissue citrate in this connection are limited and conflicting. While Freeman and Chang (2) and Harrison and Harrison (3) found an increase in serum citrate in the dog and the infant, respectively, when vitamin D was administered, Oelsé (4) was unable to find any "significant" difference in the level of serum citrate in rachitic as compared with normal infants. Shock treatment with vitamin D actually reduced the citrate level.

The wide distribution of citrate in the body (5, 6) and its high concentration in bone (7), as well as the large number of metabolic reactions in which citrate or its precursors participate (8), made it desirable to determine whether vitamin D could increase tissue citrate. In an ultimate analysis, such an effect could be the result of either its increased synthesis or its decreased destruction.

EXPERIMENTAL

Our experiments were carried out with female Sprague-Dawley rats, generally 5 weeks old. They were maintained in hanging wire baskets and were fed *ad libitum*. The rations were cereal-free and semisynthetic in nature. They were identical in their organic composition, as previously described (1). In brief, they represented respectively a low phosphorus (0.017 per cent) rachitogenic ration No. 23A, a non rachitogenic ration, lower in phosphorus, half normal in calcium content (0.30 per cent P, 0.22 per cent Ca) No. 41A, a low calcium ration (0.01 per cent) No. 11D, a ration low in calcium, phosphorus, and magnesium (0.02 per cent) No. 23—, and a ration in which all inorganic additions had been limited to 0.40 per cent sodium chloride (No. S-6). In all of these rations the required inorganic ingredients were incorporated at the expense of an equal weight of glucose. However, when sodium bicarbonate was fed (Tables II, IV),

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the glucose ingredient was not reduced. Vitamin D, when required by our experimental plan, was given as a cottonseed oil (Wesson) solution of calciferol by dropper. The amount ranged from 75 to 150 I.u. given every 3 days unless otherwise indicated. Control rats were usually equal in number to those given vitamin D.

After the rats had been kept on their respective rations from 8 to 36 days, they were anesthetized with ether, blood samples were withdrawn into an oxalated syringe by heart puncture, and the desired soft tissues were removed rapidly and dropped into liquid air for storage. Blood, as obtained, was ejected at once into 5 ml. of 10 per cent trichloroacetic acid (TCA) and centrifuged after 30 minutes. The precipitate was suspended in 10 per cent TCA and citrate was determined on the combined supernatant fluids within 24 hours after collection. For the analysis of citrate in bone, the right humerus was extracted with ether, dried to constant weight at 105°, then weighed and ground to a powder in a mortar. Weighed samples were extracted four times with 5 ml. portions of 1 N sulfuric acid. Analyses were made on aliquots of the combined extracts. Control analyses showed that none of the citrate was lost by the previous ether extraction. For the analysis of tissues other than blood or bone, the frozen samples were weighed, then ground in a mortar with quartz sand and 10 to 20 ml. of 20 per cent TCA, centrifuged, and reextracted with an equal volume of TCA. Liver tissue presented certain difficulties because of its high content of reducing substances. To remove these, the frozen tissue was homogenized in ethanol and centrifuged, and the residue dried at 105°. Citric acid was determined on an extract of this residue obtained with four successive 5 ml. portions of 1 N H_2SO_4.

All citrate determinations were made essentially according to the pentabromoacetone technique of Speck, Moulder, and Evans (9) with the chromogenic reagent of Dickman and Clontier (10). Recovery determinations for added citrate were made with each series of analyses.

For a check on the rachitic state of our animals and the effect of vitamin D, bone ash was occasionally determined on ether-alcohol-extracted femora, and inorganic phosphorus and calcium determinations were made on blood sera. The calcium was precipitated directly as the oxalate and titrated by the method of Wang (11). Inorganic phosphorus was determined by the method of Fiske and Subbarow (12). As vitamin D produced its maximal antirachitic effect after the rats had been on their rations for 15 days, most of the soft tissues were taken for analysis at the termination of this period.

Results

Table I shows that vitamin D increased the citrate content of blood, kidney, bone, and possibly that of heart and small intestine, leaving only the liver citrate unaffected. The citrate values for both blood and bone were remarkably consistent within groups. The blood values were precisely reproducible for comparable groups. The increases for heart and small intestine are open to some question because of the extensive overlapping of values, but there is no question about the relative position of the others.

As was found to be true for urinary citrate, increases in citrate were produced with the rats on either normal or rachitogenic rations. Furthermore, the highest citrate values were again obtained with a low phosphorus intake, but the greatest increase, induced by vitamin D, was found with rations adequately supplied with phosphorus. Although alkalosis, induced by feeding sodium bicarbonate or otherwise, is known to increase urinary citrate (5, 13–17), our results substantiate those of Leonards and Free (18) that it has little or no effect on the level of blood citrate (Table II). Vitamin D was equally effective in elevating blood citrate, whether or not sodium bicarbonate was fed. This effect was demonstrable with rats kept on a normal ration, a rachitogenic ration, or on a ration from which all inorganic salts except sodium chloride had been omitted. It is, therefore, evident that the effect of vitamin D on blood citrate was not mediated

TABLE I

Effect of Vitamin D on Citrate Content of Various Tissues

Ration No.	Blood	Heart	Kidney	Small intestine	Liver	Bone
	γ per ml.	γ per gm.	γ per gm.	γ per gm.	γ per gm.	mg. per gm.
11A + vitamin D	21 (13–32)	53 (17–97)	38 (17–83)	76 (18–112)	12 (8–21)	5.23 (4.28–6.07)
11A	13 (8–20)	43 (25–65)	18 (9–31)	61 (26–84)	13 (6–20)	3.39 (2.52–4.01)
Increase due to vitamin D, %	61.5*	23.2	111*	24.6		54*
23A + vitamin D	29 (20–36)	61 (21–89)	60 (26–120)	79 (46–120)	11 (6–21)	6.05 (5.63–6.77)
23A	20 (10–31)	55 (16–143)	52 (10–113)	62 (23–83)	11 (5–10)	4.40 (3.03–6.03)
Increase due to vitamin D, %	45*	11	15	27.4		38†

Each value is the average of from eleven to twenty-three rats after 15 days on the ration. The values in parentheses represent the range.

* $P < 0.01$.

† $P < 0.02$.

through an increased systemic alkalinity, although vitamin D has been shown to increase the alkalinity of the urine of rachitic rats (19). Furthermore, although vitamin D is well known to be a determinant of the level of serum calcium and phosphorus, the increased level of serum citrate could

TABLE II

Effect of Vitamin D on Blood Citrate

Ration No.	Days on ration	With vitamin D	Without vitamin D	Increase due to vitamin D
		γ per ml.	*γ per ml.*	*per cent*
11A	15	21 (17)	13 (14)	61.5
11A + 10% $NaHCO_3$	15	25 (5)	15 (6)	66.7
23A	15	29 (18)	20 (23)	45.0
23A + 10% $NaHCO_3$	15	31 (7)	20 (8)	55.0
S-4	21	28 (6)	17 (6)	65.0
S-4 + 10% $NaHCO_3$	21	26 (2)	17 (2)	53.0

The figures in parentheses give the number of animals.

TABLE III

Effect of Vitamin D on Serum Calcium and Phosphorus

Ration No.	Calcium	Phosphorus
	mg. per cent	*mg. per cent*
11A + vitamin D	10.36 (9) (9.96–10.86)	8.80 (8) (7.60–9.60)
11A	6.72 (7) (5.90–7.56)	9.30 (10) (7.90–10.80)
23A + vitamin D	11.25 (3) (10.00–11.80)	4.99 (4) (3.78–6.30)
23A	10.21 (5) (9.61–10.80)	1.80 (5) (1.54–2.26)

The top figures in parentheses give the number of animals, the lower figures the range in values.

not be correlated with a change in the level of either of these constituents (Table III). These findings are in complete agreement with those of Harrison and Harrison (3).

Our studies of the citric acid content of bone (Table IV) show that it also was increased by vitamin D. This was true even in the limited number of cases in which only a single dose of 72 i.u. of vitamin D had been given 15 days before the end of the experiment. In harmony with urinary

excretion, the bones contained more citrate when the rats were on the low phosphorus ration than when they received adequate supplies of phosphorus.

Our data do not support the very limited data of others (7, 20) that the amount of citrate in bone is reduced in rickets. With rachitogenic rations we obtained values which represented an increase of from 16 to 30 per cent above the values obtained with normal rations. Dickens' conclusions are based on only one kitten and Nicolaysen's findings are complicated by the

TABLE IV

Effect of Vitamin D on Bone Citrate

Ration No.	Days on ration	No. of rats	Fetter ash	Citrate in bones		Increase in citrate
			per cent	*mg.*	*mg. per gm.*	*per cent*
11A + vitamin D	15	6	51.8	0.405	5.50	56.0
11A	15	6	49.8	0.267	3.57	
11A + vitamin D	15	6	55.1	0.348	4.90	52.6
11A	15	6	52.5	0.211	3.21	
11A + 10% $NaHCO_3$ + vitamin D	15	6	55.6	0.338	5.08	67.6
11A + 10% $NaHCO_3$	15	6	51.8	0.196	3.03	
23A + vitamin D	15	6	45.1	0.115	5.01	17.0
23A	15	6	44.2	0.380	5.46	
23A + vitamin D	15	6	48.9	0.458	6.20	65.3
23A	15	6	42.1	0.221	3.75	
11D + vitamin D*	22	4	43.4	0.186	2.79	59.0
11D	22	4	42.0	0.104	1.75	
11D + vitamin D	21	4	45.8	0.311	3.99	61.0
11D	21	4	48.2	0.170	2.43	
23− + vitamin D	15	4	27.8	0.157	2.66	32.0
23−	15	4	25.3	0.125	2.01	

* 72 to 525 i.u. of vitamin D on the 1st day only.

fact that his rations contained considerable quantities of calcium and phosphorus. It is, however, possible that this discrepancy can be resolved by further analysis of the rachitic state in these instances. Dickens, for example, found less citrate in cartilage than in true bone tissue, and a decreased citrate content in the entire bone when rickets was produced on a low calcium diet.

No correlation could be found between citrate levels and bone ash, but it was evident that the citrate values were the lowest in bones which had been produced by low calcium rations. It would thus seem that the amount of citric acid is more directly related to the calcium nutriture of the animal than to the rachitic state *per se*. Our rats on the low phosphorus ration

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were severely rachitic after 15 days as evidenced by the wide metaphyses, routinely observed, and the lowered serum inorganic phosphorus, although the percentage of ash was not greatly lowered.

In agreement with the work of Leonards and Free (18), bicarbonate feeding did not increase bone citrate. On the other hand, vitamin D increased bone citrate regardless of the bicarbonate content of the ration.

It is of interest that the increase in tissue citrate effected by vitamin D is duplicated by the action of fluoroacetate (21), though to a considerably lesser degree; both substances increase citrate in all the tissues examined except liver. According to Busch and Potter (22), the inability of fluoroacetate to build up liver citrate is due to the fact that the liver has, in addition to the tricarboxylic acid cycle, an alternate pathway for citrate metabolism. Apparently the liver, and other organs as well, can catabolize large amounts of citrate. Hepatectomy has been found to increase urinary citrate (5). The feeding of large quantities of citrate to the intact animal apparently produces very little or no increase in its excretion (13–15, 17, 23, 24). In rabbits and cats, citric acid has been found to be higher in portal than in arterial blood (25).

SUMMARY

The citrate content of blood, bone, kidney, heart, and the small intestine of rats on normal or low phosphorus rachitogenic rations was increased by physiological doses of vitamin D. It did not affect liver citrate. Comparable to changes in urinary citrate, tissue citrate was highest in rats kept on a low phosphorus ration, but vitamin D produced the biggest increase with phosphorus-containing rations. Vitamin D was as effective in increasing blood and bone citrate in the presence of dietary bicarbonate as in its absence, although bicarbonate itself had little or no effect. It appears that the increase in urinary citrate is a reflection of its increased accumulation in certain tissues.

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Biological [illegible]ivity of 25-Hydroxyergocalciferol in Rats[1]

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ABSTRACT 25-Hydroxyergocalciferol is 1.5 times more effective than either vitamin D_2 or D_3 in curing rickets in rats (60 units antirachitic activity per microgram). It is effective in inducing intestinal calcium transport and mobilization of bone mineral in rats and acts much more rapidly in these two systems than does vitamin D_2.

In 1966, Lund and DeLuca (1) described a highly biologically active metabolite of vitamin D_3 and with the accumulation of evidence DeLuca (2) suggested that it might well represent the metabolically active form of the vitamin. Ponchon and DeLuca (3) developed a chromatographic system which resolved this fraction into seven radioactive metabolites, only one of which retained the ability to cure rickets in rats. Blunt et al. (4) isolated the biologically active metabolite in pure form from hog blood and identified it as 25-hydroxycholecalciferol (25-HCC). It was synthesized (5) and its biological activity was shown to be greater than and more rapidly acting than that of vitamin D_3 (6). Subsequent work demonstrated that vitamin D_3 must be hydroxylated in the 25 position before it can exert its biological activity, establishing the 25-HCC as the circulating active form of the vitamin (7). Drescher et al. (8) showed clearly that a similar metabolite was formed from vitamin D_2 in rats and chicks. It was isolated and identified as 25-hydroxyergocalciferol (25-HEC) by Suda et al. (9, 10). This metabolite, like 25-HCC, is more antirachitic in rats than vitamin D_2 and acts more rapidly to initiate intestinal calcium transport and bone mineral mobilization. It is the purpose of this paper to report these results.

MATERIALS AND METHODS

Vitamin D_2 and 25-Hydroxyergocalciferol (25-HEC). The crystalline vitamin D_2 was obtained from Phillips Duphar, Weesp, The Netherlands. 25-HEC was isolated in pure form from the plasma of hogs, as previously described by Suda et al. (9, 10). The exact concentrations of these substances were determined by ultraviolet absorption at 265 mμ using a molar extinction coefficient of 19,400.

Preparation of Rats. Male weanling rats of the Holtzman strain[2] were housed individually in hanging wire cages and given food and water ad libitum.

For the rickets cure test (line test assay), rats were fed the rachitogenic diet of Steenbock and Black (11) for 21 days. The rachitogenic diet consisted of the following: (in %) ground corn, 76; wheat gluten,[3] 20; $CaCO_3$, 3; NaCl, 1 and water-soluble vitamin mixture, 0.2. The water-soluble vitamin mixture consisted of: (in %) thiamin, 0.5; riboflavin, 0.5; pyridoxine, 0.5; calcium pantothenate, 2.8; nicotinamide, 2.0; inositol, 20; folic acid, 0.02; vitamin B_{12}, 0.002; biotin, 0.01 and glucose monohydrate,[4] 73.7. After a 21-day depletion period, a single dose of 0.1 μg of either vitamin D_2 or 25-HEC was administered orally in 0.1 ml cotton seed oil[5] by stomach tube. Controls received the oil vehicle alone. Seven days later the rats were killed, their radii removed and stained with $AgNO_3$ solution. The degree of new calcification was scored visually as described in the U. S. Pharmacopoeia (12).

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[2] Holtzman Co., Madison, Wis.

[3] Wheat gluten, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Cerelose, Corn Products Refining Co., New York.

[5] Wesson oil, the Wesson Oil Co., New Orleans, La.

In intestinal transport experiments, rats were fed for 5 to 6 weeks a purified diet which contains 0.47% Ca and 0.3% P (13). The diet used consisted of the following: (in %) glucose monohydrate,[4] 64.2; casein,[7] 18.0; cystine, 0.2; cotton seed oil,[5] 10.0; $CaCO_3$, 1.09; equimolar mixture of KH_2PO_4 and K_2HPO_4, 0.9; roughage,[8] 3.0; choline chloride, 0.2; Ca- and P-free salt mixture, 2.0 and water-soluble vitamin mixture which was described above, 0.1. The Ca- and P-free salt mixture contained: (in %) KCl, 57.7; NaCl, 20.9; $MgSO_4$, 17.9; $FeSO_4 \cdot 7H_2O$, 3.22; $CuSO_4 \cdot 5H_2O$, 0.078; NaF, 0.113; $CoCl_2 \cdot 6H_2O$, 0.004, KI, 0.01; $MnSO_4 \cdot H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$, 0.44 and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.005. In addition, the rats received three times a week a supplement of fat-soluble vitamins in cottonseed oil which supplied 70 μg of β-carotene, 105 μg of 2-methyl-1,4-naphthoquinone and 875 μg of α-tocopherol weekly. After 5 to 6 weeks, the rats exhibited a serum calcium concentration of 4.5 to 5.0 mg/100 ml. These rats were given a single dose of 0.25 μg of either 25-HEC or vitamin D_2 intravenously in 0.02 ml of 95% ethanol. Controls received the ethanol vehicle alone. Three, 4, 6, 8, and 12 hours after administration, the rats were killed and calcium transport measurements in everted duodenal sacs as described earlier by Martin and DeLuca (14) were carried out. The data are expressed as ratios of ^{45}Ca concentration on the serosal side to that on the mucosal side.

In experiments on the mobilization of bone mineral, rats were fed the purified diet described earlier, except that calcium was omitted. After 3 weeks on this low calcium diet serum calcium concentrations fell to 3.5 to 4.0 mg/100 ml. At this time the rats were given a single dose of 2.5 μg of either 25-HEC or vitamin D_2 intravenously in 0.02 ml of 95% ethanol. Controls received ethanol alone. Four, 8, 12, 16 and 24 hours after administration, the rats were killed and the serum calcium concentration was determined by Webster's method (15). The rise in serum calcium in rats on this diet reflects increased mobilization of bone material.

RESULTS

The 25-hydroxyergocalciferol was extremely antirachitic in rats as shown in table 1. Routine line test assays in our own laboratory and independently at the Wisconsin Alumni Research Foundation Testing Laboratories[10] showed this metabolite to have 60 IU of antirachitic activity per microgram. This activity was 1.5 times that of vitamin D_2 or vitamin D_3 and was comparable to that exhibited by 25-hydroxycholecalciferol (4, 6).

When the abilities of vitamin D_2 and 25-HEC to promote intestinal calcium transport were compared (table 2), it was found that, whereas vitamin D_2 required 6 hours to cause a significant increase in calcium transport ratio, there was a significant increase only 3 hours after administration of 25-HEC. In addition, the responses to 25-HEC at 6 and 8 hours were much greater than those to vitamin D_2 at the same times ($P < 0.02$ and $P < 0.001$, respectively). Its speed of action was

TABLE 1

Antirachitic activity of 25-hydroxyergocalciferol

Compound	IU/μg
Ergocalciferol	41±1[1]
25-Hydroxyergocalciferol	60±5

[1] Standard deviation. Each value represents the average of two separate assays of eight determinations each.

TABLE 2

Response of intestinal calcium transport to intravenous 25-hydroxyergocalciferol

Hours after injection	Ratio serosal ^{45}Ca/mucosal ^{45}Ca			
	Vit D_2		25-HEC	
0	1.50±0.14[1]			
3	1.40	±0.39	1.94*	±0.41
4	1.54	±0.36	2.09	±0.65
6	1.98*	±0.30	2.74**	±0.49
8	2.06***	±0.12	3.51***	±0.23
12	3.35**	±0.70	3.58***	±0.59

[1] The values represent averages from five rats ± standard deviation. Each animal received 0.25 μg of either vitamin D_2 or 25-HEC. Control animals received vehicle alone. Significance of difference between the response at each time interval for a given treatment and that at 0 time: * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

[6] See footnote 4.

[7] "Vitamin-free" test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[8] See footnote 5.

[9] Celu flour, Chicago Dietetic Supply House, Chicago, Ill.

[10] Wisconsin Alumni Research Foundation Testing Laboratories, 426 Walnut Street, Madison, Wis.

roughly comparab that of 25-HCC (6) with which a significant response was obtained already at 3 hours, whereas about 6 hours were required for a comparable response from vitamin D_2.

The 25-HEC was also an effective bone mineral mobilizing agent (fig. 1). It clearly acted more rapidly than does vitamin D_2 but is not likely to achieve the activity of 25-HCC or of 25-hydroxydihydrotachysterol₃ in this regard (6, 16).

DISCUSSION

The present results support the belief that the circulating active form of vitamin D_2 is 25-hydroxyergocalciferol (9, 10) which was isolated and identified in this laboratory. They also lend considerable support to the belief that all of the D vitamins and dihydrotachysterols must be hydroxylated in the 25 position before they are physiologically active.

There is little doubt that the 25-hydroxycholecalciferol represents the circulating active form of vitamin D_3 (7, 17). This evidence is based on the much more rapid action of 25-HCC on both intestine and bone in vivo than of vitamin D_3 and on its complete effectiveness on isolated bone and intestine as compared to the complete lack of effectiveness of D_3 in these systems (18, 19). Thus far, the present report provides a clear demonstration that 25-HEC acts more rapidly than D_2 in vivo on intestine and bone. In fact, its speed of action compares very favorably with that of 25-HCC (6). Thus it appears likely that 25-HEC represents the circulating active form of vitamin D_2.

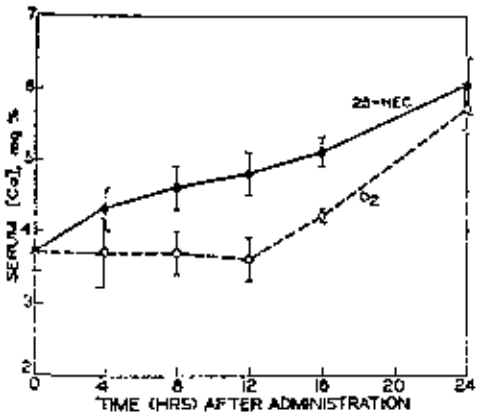

Fig. 1 Plasma calcium response of rats on a low calcium, vitamin D-deficient diet to either 2.5 μg vitamin D_2 or 25-hydroxyergocalciferol. The vertical bars represent standard deviation of values from five animals.

Up to the present time 25-hydroxycholecalciferol represented the most potent antirachitic substance known (4, 6). Clearly 25-hydroxyergocalciferol is at least equally effective as 25-HCC in the cure of rickets in rats. Because man responds equally well to vitamin D_2 and D_3 as do rats, it is likely that 25-HEC will be as effective as 25-HCC in man as an antirachitic agent. Crude 25-HEC has little effect on calcium absorption in chicks (8) as might be expected from the fact that D_2 is one-tenth as active as D_3 in chicks.

25-HEC is an effective bone mineral mobilization agent, but it does not appear quite as effective as 25-HCC (6). However, an exact comparison must await additional investigation.

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25,26-Dihydroxycholecalciferol, a Metabolite of Vitamin D_3 with Intestinal Calcium Transport Activity*

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ABSTRACT: A metabolite of vitamin D_3 (40 μg) has been isolated in pure form from the plasma of eight pigs given 250,000 IU of vitamin D_3/day for 28 days. It has been unequivocally identified as 25,26-dihydroxycholecalciferol by means of mass spectrometry and ultraviolet absorption spectra. This metabolite has some activity in intestinal calcium transport, but is virtually inactive in the cure of rickets and in the mobilization of bone mineral in rats.

Since the successful identification of 25-HCC[1] (Blunt *et al.*, 1968a) and 25-HEC (Suda *et al.*, 1969), it has been strongly suggested that all vitamin D compounds must be hydroxylated in the 25 position before they are active. Both 25-HCC and 25-HEC were 1.4–1.5 times more active than vitamin D_3 or D_2 in curing rickets in rats (Blunt *et al.*, 1968b; Suda *et al.*, 1969). Not only were they more active than their parent vitamins in the stimulation of intestinal calcium transport and in the mobilization of bone mineral, but they acted more rapidly (Blunt *et al.*, 1968b; Suda *et al.*, 1970a). In addition, 25-HCC was effective in isolated target organs while vitamin D_3 itself was without effect (Olson and DeLuca, 1969; Trummel *et al.*, 1969). Therefore, 25-hydroxy D vitamins seemed to be at least the circulating or hormonal active forms of the vitamins (DeLuca, 1969).

Recent studies on the metabolism of [^3H]vitamin D_3 have demonstrated that other metabolites, more polar chromatographically than either vitamin D_3 or 25-HCC, are present in certain tissues and blood following administration of physiological doses of the radioactive vitamin D or 25-HCC (Ponchon and DeLuca, 1969; Haussler *et al.*, 1968; Lawson

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[1] Abbreviations used are: 25-HCC, 25-hydroxycholecalciferol; 25-HEC, 25-hydroxyergocalciferol; 21,25-DHCC, 21,25-dihydroxycholecalciferol; 25,26-DHCC, 25,26-dihydroxycholecalciferol.

et al., 1969; Cousins *et al.*, 1970a,b). Although the biological activity of these polar metabolites is still unclear, their identification appeared important to a thorough understanding of the mechanism of vitamin D action.

More recently one of these polar metabolites has been isolated in pure form from hog plasma and identified as 21,25-DHCC (Suda *et al.*, 1970b). It was found to have a marked action on mobilization of bone mineral while having a small effect on intestinal calcium transport and in the cure of rickets in rats (Suda *et al.*, 1970b).

Another polar metabolite has now been isolated in pure form from hog plasma and unequivocally identified as 25,26-DHCC. This metabolite was shown to have activity in intestinal calcium transport while having no effect on bone mineral mobilization and in the cure of rickets in rats. It is the purpose of this communication to report these results.

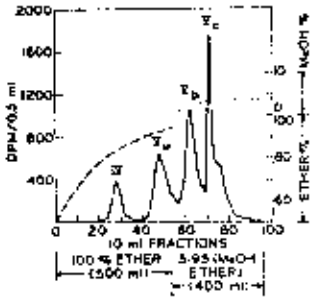

FIGURE 1: Silicic acid column chromatographic profile of plasma peak V. The column was eluted with an ether Skelly B methanol gradient as described in the text. (——) Radioactivity; (-----) gradient; 10-ml fractions were collected and tubes 70–90 were used in further purification.

Methods and Results

General Procedures. Ultraviolet spectra were recorded with a Beckman DB-G spectrophotometer. Samples were dissolved in ethanol. A molar extinction coefficient of 18,000 at 265 mμ was used for vitamin D_3 and 25-HCC (Blunt *et al.*, 1968a). A molar extinction of 18,000 at 265 mμ was assumed for 25,26-DHCC as well. Mass spectra were determined with an MS-902 mass spectrometer (electron energy, 70 eV; ionizing current, 450 μA; source temperature, 135–150° above ambient) using a direct introduction probe.

All radioactive determinations were carried out with a Packard Tri-Carb liquid scintillation spectrometer, Model 3003, equipped with an external standardization system. Samples for tritium determination were evaporated to dryness with a stream of air, dissolved in toluene-counting solution (2 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene per l. of toluene), and counted.

Acetylation Reactions. About 1 μg of the metabolite was allowed to stand at room temperature for approximately 5 hr in a solution of 5 μl of acetic anhydride and 5 μl of pyridine. The mass spectrum of the derivative was obtained by transferring the entire mixture to the probe tip and allowing the solvent to evaporate.

Silyl Ether Formation. Complete silylation was accomplished by treating the metabolite with about 10 μl of silylating reagent (pyridine–hexamethyldisilazane–trimethylchlorosilane, 7:2:1, v/v) for approximately 2 hr.

Periodate Cleavage. To about 1.5 μg of the metabolite in 20 μl of methanol a drop of 5% aqueous $NaIO_4$ was added. After 20 min the solution was dilated with water and extracted with chloroform. The chloroform solution was concentrated to a few microliters which were transferred to the probe tip for mass spectral analysis.

Isolation of Metabolites. Plasma (14.1 l.) was obtained from eight hogs which were maintained on normal stock rations supplemented with 250,000 IU of vitamin D_3 daily for 28 days. The plasma was made 70% saturated with $(NH_4)_2SO_4$, and the precipitate was extracted with methanol chloroform and partitioned to remove less polar metabolites of vitamin D_3 as previously described (Suda *et al.*, 1970b).

Exactly 100 IU (2.5 μg) of [1,2-^{3}H]vitamin D_3 which was prepared in this laboratory, was dosed intravenously to each of 51 chickens, which were maintained on a vitamin D deficient diet (Jungle *et al.*, 1967) for 27 days. Twenty hours after administration blood was collected by decapitation giving 133 ml of plasma after centrifugation. This was extracted with methanol-chloroform, and ^{3}H-labeled polar metabolites were partitioned and applied to a silicic acid column (Ponchon and DeLuca, 1969) to obtain the ^{3}H-labeled peak V as previously described (Suda *et al.*, 1970b).

The radioactive peak V taken from chicken plasma was then mixed with the extract from the hog plasma. The combined extract was then applied in 20 ml of chloroform to a large 150-g, 60-cm, multibore silicic acid column measuring stepwise in diameter 1.0, 2.0, 3.0, and 4.0 cm. The column was eluted with the Skelly B (*n*-hexane, bp 67–68°) ether–methanol gradient as previously described (Suda *et al.*, 1970b). The peak V was eluted as a single peak in the 5% methanol region. The peak V was then rechromatographed on a regular straight silicic acid (20-g) column as described by Ponchon and DeLuca (1969) except that the constant-volume mixing chamber contained 250 ml of 50% diethyl ether in Skelly B and the holding chamber contained 500 ml of 100% diethyl ether. As soon as the holding chamber became empty, it was filled with 400 ml of 5% methanol in diethyl ether. Fractions of 10 ml were collected. The elution profile is shown in Figure 1. Clearly the original peak V has been resolved into at least three components, which were designated Va, Vb, and Vc. The peak Va has been identified as 21,25-DHCC (Suda *et al.*, 1970b). The peak Vc collected in tubes 70–90 was then rechromatographed on a Celite partition column, especially designed for peak V metabolites as previously described (Suda *et al.*, 1970b). The column was developed with the mobile phase (20% chloroform 80% Skelly B equilibrated with 90% methanol 10% water) with 5.5-ml fractions collected. The profile of radioactivity from that column is shown in Figure 2. The peak Vc has now been resolved into four peaks. The major peak Vc,3 (tubes 77–90) was then applied in 0.1 ml of methanol to a 60 × 1 cm Sephadex LH-20 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) which was developed in methanol. Fractions of 1 ml were collected. Figure 3 shows the profile of radioactivity and 265-mμ-absorbing material which was eluted from that column. The absorbancy at 265 mμ exactly coincided with the radioactivity plot. The ultraviolet spectrum of the material is shown in Figure 4, giving maximum absorption at 265 mμ and minimum absorption at 228 mμ. A total of 40 μg of the metabolite was isolated.

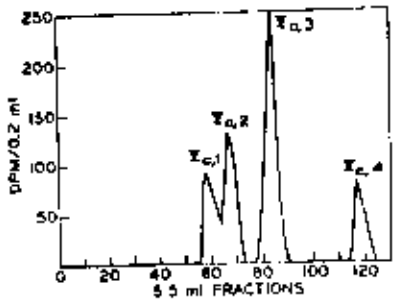

FIGURE 2: Partition chromatography of the peak Vc metabolite from Figure 1. Celite was used as the support. The mobile phase consisted of 20% chloroform 80% Skelly B equilibrated with the stationary phase (90% methanol-10% water). Fractions (5.5 ml) were collected and tubes 77–90 were used in further purification.

Identification of the Metabolite. The ultraviolet spectrum of the metabolite which exhibited absorption at λ_{max}^{EtOH} 265 mμ, indicated an unchanged *cis*-triene chromophore (vitamin D_2 shows λ_{max}^{EtOH} 265 mμ). Peaks at *m/e* 136 and 118 (136-H_2O) in the mass spectrum of the metabolite (Figure 5) confirmed this assignment, since these ions are characteristic for the vitamin D triene system (see, for example, Blunt *et al.*, 1968a, and Suda *et al.*, 1969). The molecular weight of 416 suggested the incorporation of two additional oxygen functions into the vitamin D_2 ring system; both of these had to be located in the sidechain, since the peaks at *m/e* 271 and 253 (271-H_2O) which correspond to loss of the entire sidechain (cleavage of C-17–C-20 bond) in the mass spectrum of vitamin D_2 itself, are also present in the spectrum of the metabolite (Figure 5). The metabolite formed a diacetate (mol wt 500) on treatment with acetic anhydride in pyridine, but a trimethylsilyl ether (mol wt 632, Figure 5) resulted upon silylation of the compound. These transformations establish that both additional oxygen functionalities are present as hydroxy groupings and that one of these should be located on a tertiary carbon center. The exact structure of the compound followed from an interpretation of the mass spectra of the metabolite and its derivatives. The metabolite spectrum (Figure 5) in addition to peaks already discussed shows a peak at M − 18 − 31 (367) suggesting the presence of a CH_2OH group. Since the acetylation results suggested one tertiary

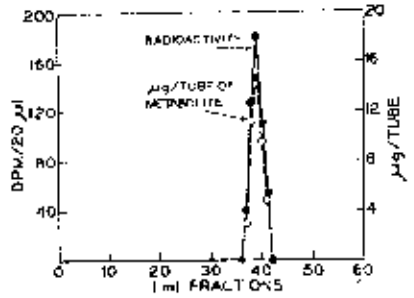

FIGURE 3: Chromatographic profile of the major peak Vc, 3 (tubes 77–90 of Figure 2) on a Sephadex LH20 column. The column was eluted with methanol. Fractions (1 ml) were collected. The micrograms per tube were calculated on the basis of a molar extinction coefficient of 18,000 at 265 mμ assumed for this metabolite.

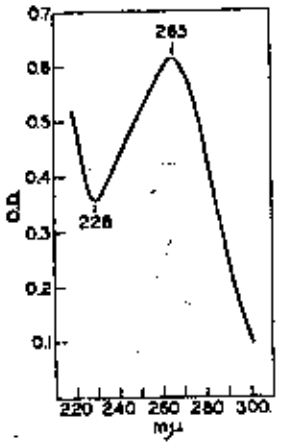

FIGURE 4: Ultraviolet absorption spectrum of the plasma peak Vc metabolite isolated from Sephadex LH20 columns.

hydroxyl function, the loss of 31 mass units pointed toward a 20,21- or 25,26-dihydroxylated side chain. The former possibility appeared unlikely since cleavage of the C-20–C-22 bond should be expected with elimination of the side chain fragment comprising carbons 22–27. (For example, 20-hydroxycholesterol shows a very intense peak at *m/e* 317 due to this cleavage.) The mass spectrum of the trisilylated metabolite confirmed a 25,26-substitution pattern. The relatively intense peak at *m/e* 529 corresponds to loss of 103 mass units [M − $CH_2OSi(CH_3)_3$] and the peak at *m/e* 219 can result by cleavage of the C-24–C-25 bond as shown in Figure 6. The peak at *m/e* 208 corresponds to *m/e* 136 in the spectrum of the metabolite shifted by one silyl grouping. The mass spectrometric evidence is summarized in Figure 6. Further proof for the presence of a vicinal glycol grouping was provided by periodate cleavage of the metabolite which gave a compound of mol wt 384 as expected for the transformation of a 25,26-dihydroxy compound to the corresponding 25-keto derivative. These data, therefore, establish the structure of this new metabolite as 25,26-DHCC (Figure 7).

Biological Activity of 25,26-Dihydroxycholecalciferol. The line test assay of 25,26-DHCC was carried out in rats as previously described (U. S. Pharmacopoeia, 1955) except that it was given intrajugularly in 20 μl of ethanol. 25,26-DHCC failed to show significant antirachitic activity, thus its antirachitic activity if any must be less than 4 IU/μg.

Bone mineral mobilization response to 25,26-DHCC intravenously was tested as previously described (Blunt *et al.*, 1968b) except that only 0.25 μg of 25,26-DHCC was administered. In addition, intestines were removed from these rats and intestinal calcium transport was measured by the everted sac technique as previously described (Blunt *et al.*, 1968b). Precise comparison of biological activity of 25,26-DHCC to that of 21,25-DHCC was not possible, because of the limited amount of the 25,26-DHCC isolated. As shown in Table I, 0.25 μg of 25,26-DHCC elicited no significant elevation of serum calcium concentration at 12 hr after administration. Under the same conditions, 0.25 μg of 25-HCC elevated serum calcium concentration from 4.7 to 6.6 mg %. On the other hand, 0.25 μg of 25,26-DHCC significantly

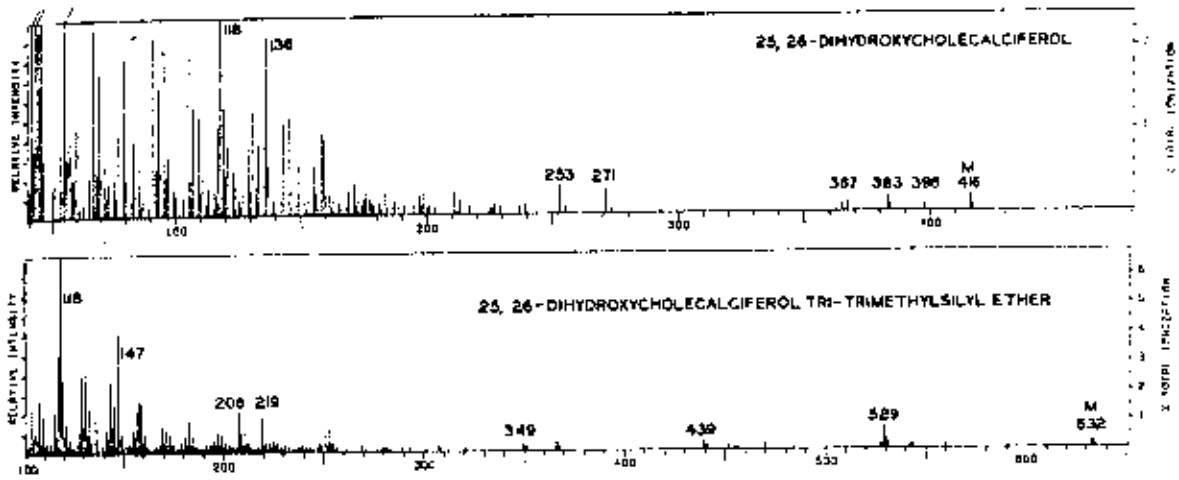

FIGURE 5: Mass spectra of the plasma peak Vc and its tritrimethylsilyl ether derivative.

TABLE I: Effect of Intrajugular Administration of 0.25 or 2.5 μg of 25,26-DHCC, 21,25-DHCC, or 25-HCC on Calcium Transport and Serum Calcium of Rats on a Low Calcium Diet.

	12 hr after Administration			
	Calcium Transport ^{45}Ca Serosal/^{45}Ca Mucosal		Bone Mobilization Serum Ca mg%	
Group	0.25 μg	2.5 μg	0.25 μg	2.5 μg
Control	1.6 ± 0.2[a] (4)	1.5 ± 0.3[a] (5)	4.7 ± 0.1[a] (4)	4.4 ± 0.4[a] (5)
25,26-DHCC	2.8 ± 0.6 (5)		5.1 ± 0.2 (5)	
21,25-DHCC		2.6 ± 0.8 (5)		6.5 ± 0.2 (5)
25-HCC	4.0 ± 0.5 (3)	5.9 ± 1.6 (5)	6.6 ± 0.6 (3)	7.3 ± 0.5 (5)

[a] Standard errors of the mean. Numbers in parentheses show the number of animals in each group.

increased intestinal calcium transport. 25,26-DHCC was about one-half as active as 25-HCC in the stimulation of intestinal calcium transport.

Discussion

In a series of experiments on the metabolism of vitamin D in this laboratory, it has been strongly suggested that all vitamin D compounds must be hydroxylated in the 25 position before they are effective (DeLuca, 1969). Further biological evidence established 25-HCC and 25-HEC at least as the circulating or hormonal form of vitamin D_2 and D_3, respectively (Blunt *et al.*, 1968b; Suda *et al.*, 1970a; DeLuca, 1969; Trummel *et al.*, 1969; Olson and DeLuca, 1969). In continuing investigation, Cousins *et al.* (1970a,b) demonstrated that 25-HCC is rapidly metabolized to a more polar metabolite (peak V) in intestine, bone, and kidney. This conversion in the case of intestine involves an even more polar metabolite (peak VI) as intermediate. Haussler *et al.* (1968) and Lawson *et al.* (1969) have also reported metabolites of vitamin D_3

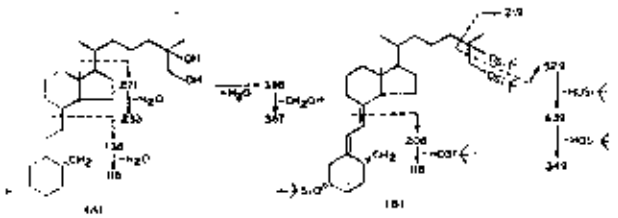

FIGURE 6: Schematic representation of bond ruptures leading to prominent ions in the mass spectra of 25,26-DHCC and its tritrimethylsilyl ether derivative.

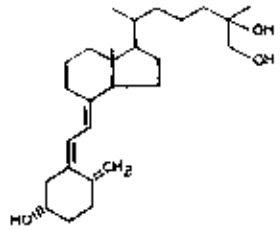

FIGURE 7: Structure of 25,26-DHCC.

more polar than 25-HCC. Myrtle *et al.* (1970) have claimed that their intestinal peak 4B, which corresponds to our peak V chromatographically, is biologically more active than 25-HCC in intestinal calcium transport *in vivo*. Ponchon and DeLuca (1969, 1970), however, failed to confirm this high degree of biological activity for peak V. The rapidity of appearance of these polar metabolites in the target tissues after ^{3}H-labeled 25-HCC administration indicates that they nevertheless are important to a complete understanding of the mechanism of vitamin D action. Continued experiments in this laboratory have shown that at least some of these polar metabolites appear in blood making their isolation from the plasma of pigs given large doses of vitamin D_3 feasible.

In previous work in this laboratory, the peak V fraction of blood has been resolved into at least three major components (Va, Vb, and Vc). The quantitatively major metabolite (Va) has already been identified as 21,25-DHCC (Suda *et al.*, 1970b). Another (major Vc; Vc,3) has now been isolated in pure form using isolation techniques similar to those used for the 21,25-DHCC.

The identification of Vc,3 as 25,26-DHCC was more straightforward than that of Va as 21,25-DHCC. A molecular ion of 416 demonstrated the addition of two oxygen atoms to the vitamin D_3 structure. The oxygen atoms were clearly on the side chain since loss of the side chains from both the parent vitamin and the metabolite gave identical *m/e* 271 fragments. One of these had to be a tertiary hydroxyl since it did not form an acetate but did form a trimethylsilyl ether. A loss of 18 and 31 from the molecular ion suggests a CH_2OH grouping. This must be adjacent to a tertiary hydroxyl. This limits the possibilities to the 20,21 and to the 25,26 positions. The 25,26 position of the added hydroxyls was clearly established by the mass spectra of the trifrimethylsilyl ether derivatives as described in the Results section, especially the *m/e* at 219 resulting from the cleavage of the C-24–C-25 bond.

The addition of a hydroxyl to either the 21 or the 26 position of 25-HCC has led to a decrease in the biological activity of this circulating active form of vitamin D (Suda *et al.*, 1970b, and present report). It is especially interesting that the 21 hydroxylation yields a compound preferentially active on bone while the 26 hydroxylation yields a compound which has only significant activity in the intestine. These observations may at least be important in designing chemical synthesis of vitamin D analogs with specific activities on bone or intestine. Biologically, it is difficult to assess the importance of either the 21,25-DHCC or the 25,26-DHCC especially since their origins are not clear. They may be functionally important in the tissue where they are generated, or they may be intermediates on the route to inactivation. These questions are currently under investigation.

Acknowledgment

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Vol. 35, No. 2, 1969 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

25-HYDROXYERGOCALCIFEROL: A BIOLOGICALLY ACTIVE METABOLITE OF VITAMIN D_2

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SUMMARY. A polar, biologically active metabolite of vitamin D_2 (ergocalciferol) has been isolated in pure form from the plasma of pigs fed 500,000 I.U. of vitamin D_2 per day for 26 days. By means of its behavior during gas-liquid chromatography, ultraviolet spectra, mass spectrometry and nuclear magnetic spectrometry, its structure has been positively identified as 25-hydroxyergocalciferol.

In this laboratory over the course of 5 to 6 years, evidence has accumulated that cholecalciferol is metabolized to a major biologically active metabolite (1, 2). Recently this metabolite was isolated in pure form from hog plasma and identified as 25-hydroxycholecalciferol (3, 4). Strong evidence that this substance represents the metabolically active form of vitamin D_3 has also been obtained (5, 6). Ergocalciferol (vitamin D_2) is also converted to a biologically active metabolite (7) and it has now been possible to isolate it in pure form and identify it as 25-hydroxyergocalciferol.

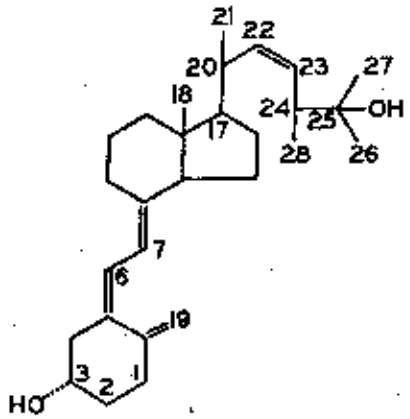
The blood plasma from 4 hogs fed for 26 days on a ration supplying 500,000 I.U. ergocalciferol/day to each animal was obtained. The α_2 globulin which binds the

vitamin and its metabolites was precipitated by 70% saturation with $(NH_4)_2SO_4$ and extracted with methanol and chloroform. Silicic acid column chromatography of the chloroform phase yielded a purified metabolite fraction (IV) which on rechromatography on silicic acid columns and finally on partition columns yielded 314 µg of pure metabolite. Purity was established by both tlc and glpc. The compound showed the expected absorption, λ_{max} ether = 264 mµ, in its spectrum and it gave characteristic isopyro and pyro forms on glpc. High resolution mass spectra* showed a molecular weight of 412.3341 establishing a formula of $C_{28}H_{44}O_2$ (calc. 412.3341). A characteristic fragment of mass 271 ($C_{19}H_{27}O$, M- side chain) for both the metabolite, ergocalciferol, 25-hydroxycholecalciferol, and cholecalciferol demonstrated the additional oxygen in the side chain. A peak at m/e 59 (C_3H_7O) and the loss of 58 m.u. (m/e 354, $C_{25}H_{38}O$) from M^+ in the mass spectrum of the metabolite but not in that of ergocalciferol strongly suggested a hydroxyl function at C-25. Confirmation was obtained when the trimethylsilyl ether of the metabolite was prepared, purified and subjected to mass spectrometry. The spectrum of this derivative (MW = 556, as expected for the disilyl ether) exhibited an intense peak of mass 131 ($C_6H_{15}SiO$), only possible for a C-25-O-silyl function.

The nmr spectrum of the metabolite, obtained with a Varian Associates HA-100 instrument and time averaging

*Determined on an MS-9 mass spectrometer (A.E.I.) coupled on-line to a Sigma-7 computer (Scientific Data Systems). For a description of the system, see A. L. Burlingame in "Advances in Mass Spectrometry," E. Kendrick, Ed., The Institute of Petroleum, London, 1968, p. 15; A. L. Burlingame, D. H. Smith, and R. W. Olson, Anal. Chem., 40, 13 (1968).

computer, showed a strong singlet at δ 1.24 ppm which was not present in the original ergocalciferol spectrum while two doublets at δ 0.87, (J = 7.0 cps) and at δ 0.98 ppm (J = 6.0 cps) originally present in the ergocalciferol spectrum were absent from that of the metabolite. Substitution of a hydroxyl group at only one position, namely the 25 position, could eliminate two doublets and give a strong singlet in this fashion. Two doublets remained at the δ 0.79 ppm (J = 6.5 cps) and at the δ 0.81 ppm (J = 7.0 cps) in the metabolite spectrum corresopnding to the 21 and 28 methyl groups. The singlet due to the 18 angular methyl group was present in both. Clearly then the structure of the metabolite is established as 25-hydroxyergocalciferol (Figure 1).



25 HYDROXYERGOCALCIFEROL

Figure 1. Structure of 25-hydroxyergocalciferol.

ACKNOWLEDGMENT. We would like to thank Miss Martha Petrie, Chemistry Department, University of Wisconsin for recording the nmr spectra, Dr. H. Whitlock

for help in nmr interpretation, and Drs. A. L. Burlingame and D. H. Smith of the University of California, Space Sciences Laboratory for the use of the MS-9 mass spectrometer and high resolution mass spectra. Supported by the Harry Steenbock fund of the Wisconsin Alumni Research Foundation

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The Isolation and Identification of 25-Hydroxyergocalciferol*

T. Suda,† H. F. DeLuca,‡ H. K. Schnoes, and J. W. Blunt§

Biochem. 8: 3515-3520, (1969)

ABSTRACT: A polar metabolite of vitamin D_2 (314 μg) has been isolated in pure form from the blood of four pigs given 100,000 IU of ergocalciferol/day for 26 days. It has been identified as 25-hydroxyergocalciferol by means of ultraviolet spectra, gas-liquid partition chromatography, nuclear magnetic resonance spectra, mass spectra, and mass spectra of its trimethylsilyl ether derivative. It is about 1.5 times more active than vitamin D_2 or D_3 in curing rickets in rats.

Lund and DeLuca (1966) described for the first time a polar metabolite of vitamin D_3 which appeared to be at least as biologically active as the parent vitamin in curing rickets in rats. This metabolite was described in man (DeLuca *et al.*, 1967; Avioli *et al.*, 1967), chicks (Drescher *et al.*, 1969), and hogs (Blunt *et al.*, 1968a), proved to possess all the biological activities of the parent vitamin, and acted more rapidly (Lund and DeLuca, 1966; Morii *et al.*, 1967). This metabolite was isolated in pure form and identified as 25-hydroxycholecalciferol (Blunt *et al.*, 1968a,b). Evidence has been obtained that it represents the metabolically active form of the vitamin (Blunt *et al.*, 1968c; Trummel *et al.*, 1969).

Recently it was shown that the peak IV metabolite is also formed from vitamin D_2 in both rats and chicks (Drescher *et al.*, 1969). This metabolite has now been demonstrated in hogs, isolated in pure form, and identified as 25-hydroxyergocalciferol. It is the purpose of this communication to report these findings.

Methods and Results

General Procedures. All radioactive determinations were carried out by means of a Packard Tri-Carb Model 3003 liquid scintillation counter equipped with an automatic external standardization system. Samples to be counted were evaporated to dryness with a stream of air, dissolved in toluene-counting solution (2 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)benzene] per l. of toluene), and counted.

Ultraviolet spectra were recorded with a Beckman DB-G spectrophotometer. Samples in this case were dissolved in diethyl ether.

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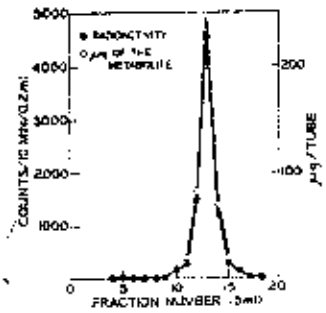

FIGURE 3: Partition chromatography of the peak IV metabolite from porcine plasma. Celite was used as the support. The stationary phase consisted of 80% methanol 20% water equilibrated with Skelly B while the mobile phase was the Skelly B which was equilibrated with the 80% methanol–20% water; 5-ml fractions were collected.

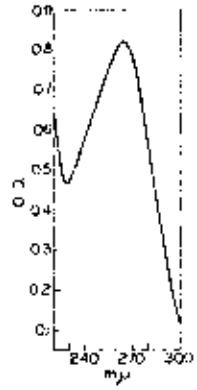

FIGURE 4: Ultraviolet spectrum of 25-hydroxyergocalciferol.

tition column. For this, 200 ml of Skelly B was equilibrated at 10° with an equal volume of 80% methanol–20% water; 15 ml of the methanolic phase was mixed with 20 g of Celite and dry packed into a 60 × 1 cm column in 2-cm portions. The upper phase was used as the mobile phase. The residue obtained from tubes 63–98 from the silicic acid column (after removal of solvent by a flash evaporator) was applied in a small volume of mobile phase (1–3 ml) to the partition column. The column was developed with mobile phase with 5-ml fractions collected. Tubes 11–17 contained the peak IV metabolite. These were recombined, evaporated to dryness as before, and rechromatographed on another identical partition column. The profile of radioactivity from that column is shown in Figure 3. Optical density at 265 mμ was also measured in each fraction and the calculated micrograms of peak IV metabolite are plotted assuming a molar extinction coefficient of 19,400. Clearly the absorbancy at 265 mμ exactly coincides with the radioactivity plot. The ultraviolet spectrum of the material is shown in Figure 4. A total of 0.314 mg of metabolite was isolated. The purity of the peak IV metabolite thus obtained from the Celite column was examined by gas–liquid partition chromatography, as shown in Figure 5. Note that a double peak of pyro and isopyro derivatives of the metabolite is evident having a retention time of 14.0 and 15.8 min. This together with the ultraviolet λ_{max} at 265 mμ demonstrates that the triene structure of the parent vitamin D_2 is retained by the metabolite. In any case both the ultraviolet and gas–liquid partition chromatographic data demonstrate a high degree of purity for the isolated metabolite. It may be of interest that the retention times for the pyro- and isopyroergocalciferol are 7.0 and 7.9 min under the same conditions.

For the purpose of identification it was necessary to prepare the trimethylsilyl ether derivative of the metabolite. Isolated metabolite (30 μg) was taken to dryness under a stream of dry N_2. A few drops of a reagent (14 ml of dry pyridine, 4 ml of hexamethyldisilazane, and 2 ml of trimethylsilyl chloride) was added and flushed with N_2, and the mixture was allowed to stand in the dark at room temperature for 3 hr.; 3 ml of Skelly B was added followed by 2 ml of 10% H_2SO_4. The mixture was vigorously mixed and then allowed to separate. The Skelly B phase was dried over a small amount of anhydrous Na_2SO_4. It was then chromatographed on silicic acid column as described by Lund *et al.* (1967). In tubes 19–27 the disilyl ether derivative was located (15 μg) and was found to be essentially pure by gas–liquid partition chromatography. It was then used for determination of mass spectra.

Identification of 25-Hydroxyergocalciferol. As already pointed out, the ultraviolet spectra and the gas–liquid partition chromatographic data clearly suggest that the triene structure remains unchanged in the metabolite structure. The mass spectrum of the metabolite (Figure 6) shows intense peaks at *m/e* 136 ($C_9H_{12}O$) and 118 (C_9H_{10}) deriving from ring A of the molecule including C-6 and -7 (see Figure 7) and are present also in the spectrum of vitamin D_2 (Figure 6).

The mass spectrum of the metabolite (Figure 6) indicated a molecular ion of *m/e* 412, the increase of 16 mass units over the molecular weight of vitamin D_2 (Figure 6), suggesting the incorporation of an additional oxygen atom. Confirmation was provided by the high-resolution mass spectrum, since the exact mass of the molecular ion, 412.3341, required the composition of $C_{28}H_{44}O_2$ (calcd 412.3341). The location of the additional oxygen substituent in the side chain is apparent from comparison of the spectra of vitamin D_2 (Figure 6) and its metabolite (Figure 6). Both show peaks at *m/e* 271 ($C_{19}H_{27}O$ 271.2065, by high-resolution mass spectrometry) resulting from loss of the entire side chain by cleavage of the C_{17}–C_{20} bond. In addition the high-resolution spectrum of the metabolite contains minor peaks which are probably due to the side chain itself, *m/e* 141.1255 ($C_9H_{15}O$) and 123.1181 (C_9H_{15}).

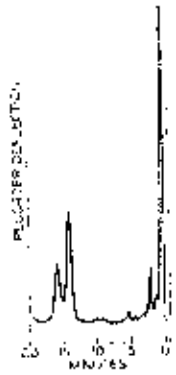

FIGURE 5: Gas–liquid partition chromatogram of 25-hydroxyergocalciferol.

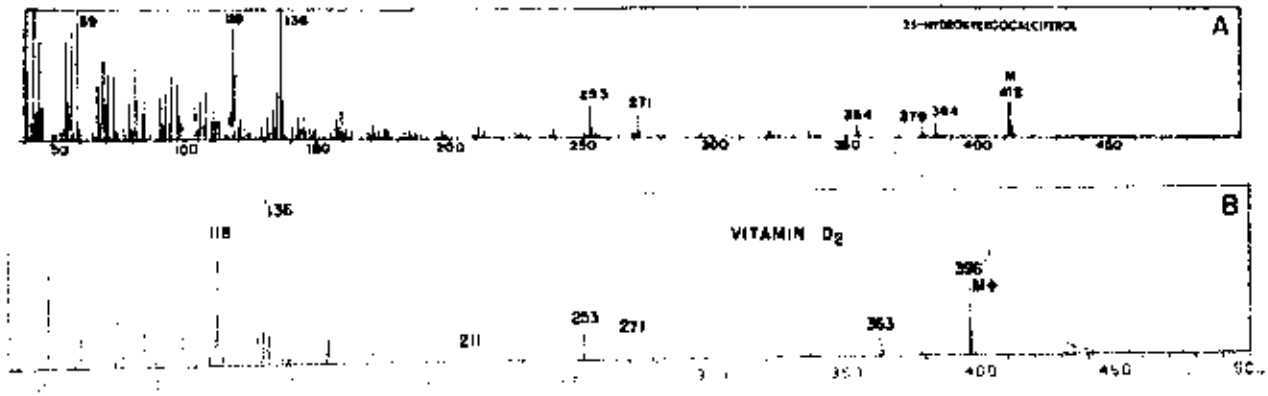

FIGURE 6: Mass spectra of 25-hydroxyergocalciferol and ergocalciferol.

A fragment of mass 59 (C_3H_7O, as determined by high-resolution mass spectrometry) and the elimination of 58 mass units from the molecular ion to give a peak at m/e 354 (354.2900 $C_{25}H_{38}O$), both present in the mass spectrum of the metabolite but absent in that of vitamin D_2, provided evidence for a hydroxyl function at C-25. The former peak would arise by simple cleavage of the C-24 C-25 bond to give the ion $(CH_3)_2C=O^+H$, the latter by hydrogen rearrangement with elimination of the elements of acetone, a process not uncommon for homoallylic alcohols.

Proof of the position of the hydroxyl grouping was obtained from the mass spectrum of the silyl ether derivative of the metabolite which exhibited a molecular ion at m/e 556, as required for a dimethylsilyl ether, and the expected intense peak at m/e 131 (base peak), shown to correspond to C_3H_7SiO, $(CH_3)_2C=O^+Si(CH_3)_3$, by high-resolution mass spectrometry. This peak, which also occurs in the spectra of the trimethylsilyl ether derivatives of 25-hydroxycholesterol and 25-hydroxycholecalciferol (J. W. Blunt, H. F. DeLuca, and H. K. Schnoes, unpublished data), but not in the spectra of the silyl ethers of vitamin D_2 or D_3, thus requires C-25 as the position of the additional hydroxyl function.

The nuclear magnetic resonance spectrum of the metabolite (Figure 8) fully corroborated these deductions and provided independent structural evidence: an intense singlet at δ = 1.24 ppm can be assigned to the two methyl groupings at C-25, while two doublets at δ = 0.79 (J = 6.5 cps) and 0.81 ppm (J = 7.0 cps) must be due to the remaining methyl substituents of the side chain (C-21 and -28). The spectra of 25-hydroxycholesterol and 25-hydroxycholecalciferol exhibit a singlet for the C-26, 27 methyl groups at essentially the same position (δ = 1.20 ppm). It should be noted that two doublets at δ 0.87 (J = 7.0 cps) and 0.98 (J = 6.0 cps) originally present in the ergocalciferol spectrum (Figure 9) are absent in that of the metabolite. This elimination of two doublets which must correspond to two methyl groups can be explained only by hydroxyl substitution at C-25. The remaining identifiable feature is the singlet due to the C-18 angular methyl group. These data, therefore, establish the structure of the metabolite as 25-hydroxyergocalciferol (Figure 10).

The isolated metabolite was finally assayed for antirachitic activity in rats (U. S. Pharmacopoeia, 1955) and by other techniques. Routinely it assayed at 60–64 IU of antirachitic activity per micrograms of metabolite. Thus by this test its activity is essentially that of 25-hydroxycholecalciferol and 1.4–1.5 times more active than ergocalciferol or cholecalciferol.

Discussion

There is now mounting evidence that vitamin D_3 (cholecalciferol) must be converted into its metabolically active form

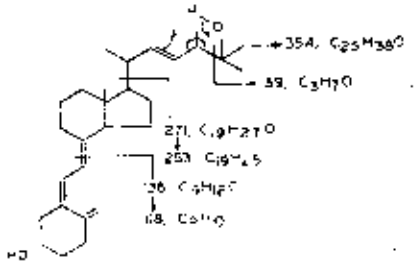

FIGURE 7: Schematic representation of bond ruptures leading to prominent ions in the mass spectrum of 25-hydroxyergocalciferol.

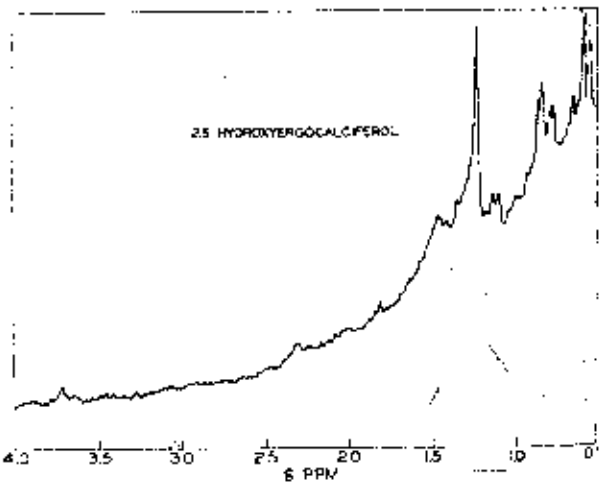

FIGURE 8: Nuclear magnetic resonance spectrum of 25-hydroxyergocalciferol. The 0.54-ppm peak is a spinning side band of the trimethylsilane while the 0.58 ppm is due to the C-18 protons.

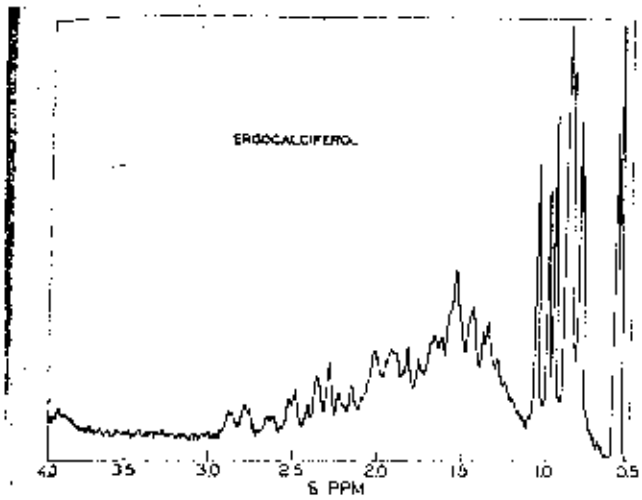

FIGURE 9: Nuclear magnetic resonance spectrum of ergocalciferol. The 0.54-ppm peak is a spinning side band of trimethylsilane while the 0.58 ppm is due to the C-18 protons.

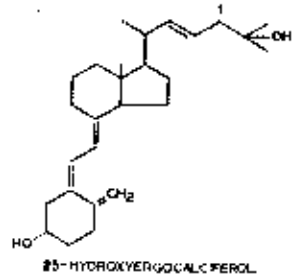

FIGURE 10: Structure of 25-hydroxyergocalciferol.

before it can produce its characteristic physiologic responses (Blunt *et al.*, 1968c; DeLuca, 1969; Trummel *et al.*, 1969). In this laboratory it has been shown that radioactive vitamin D_2 (Drescher *et al.*, 1969), vitamin D_3 (Lund and DeLuca, 1966; Blunt *et al.*, 1968a,b; DeLuca, 1967), and vitamin D_4 (H. F. DeLuca, 1969, unpublished data) are converted into polar, highly biologically active metabolites. Recently it was possible to isolate in milligram quantities the metabolite derived from vitamin D_3 and identify its structure as 25-hydroxycholecalciferol (Blunt *et al.*, 1968a,b). The structure was unequivocally confirmed when 25-hydroxycholecalciferol was synthesized chemically and shown to be identical in all respects with the isolated material (Blunt and DeLuca, 1969). This metabolite has been shown to stimulate intestinal Ca^{++} transport and bone mobilization more rapidly than does vitamin D_3 when given to D-deficient animals (Blunt *et al.*, 1968c). It also induces bone mobilization in tissue culture (Trummel *et al.*, 1969) and calcium transport in perfused intestine (E. B. Olson and H. F. DeLuca, 1969, unpublished data) while vitamin D_3 itself has little or no effect. Evidence is therefore strong that 25-hydroxycholecalciferol is the metabolically active form of vitamin D_3. We have now been able to show the production of the analogous 25-hydroxyergocalciferol from vitamin D_2 in pigs. This metabolite is also more biologically active than either vitamin D_4 or D_2 in curing rickets in rats. It is therefore likely that the metabolically active form of vitamin D_2 is 25-hydroxyergocalciferol and that all D vitamins are converted into the 25-hydroxy derivative before they can act.

The identification of the 25-hydroxyergocalciferol bears some comment. It was first of all necessary to feed higher levels of vitamin D_2 to pigs before adequate peak IV metabolite could accumulate in the plasma than was the case with vitamin D_3. Next, another metabolite (peak IVa) was difficult to separate from the peak IV and could best be achieved by a multibore silicic acid column. When the metabolite was finally purified, the nmr data were more difficult to interpret because it was difficult to assign positions of the C-21, C-28, C-26, and C-27 doublets of ergocalciferol. Because two doublets were absent from the metabolite nuclear magnetic resonance spectrum (as compared to ergocalciferol) (δ = 0.87 ppm, J = 7.0 cps; δ = 0.98 ppm, J = 6.0 cps) and an intense singlet appeared at δ = 1.24 ppm, it was apparent that only an hydroxyl at the 25 position could satisfy this shift. This suggests that δ = 0.79 ppm (J = 6.0 cps) and δ = 0.81 ppm (J = 7.0 cps) represent the 28 and 21 methyl groups of ergocalciferol.

Acknowledgments

We are indebted to Dr. E. J. Briskey of the Meat and Animal Science Department, University of Wisconsin, for the pigs and facilities used in this study, to Miss Martha Petrie, Department of Chemistry, University of Wisconsin, for her excellent technical production of the nuclear magnetic resonance results, Dr. H. Whitlock, also of the Chemistry Department, University of Wisconsin, for his help in nuclear magnetic resonance interpretation, and to Drs. A. L. Burlingame and D. H. Smith, Space Sciences Laboratory, University of California, Berkeley, for use of the mass spectrometer and their help in obtaining high-resolution mass spectra.

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Pseudohypoparathyroidism: Responsiveness to Parathyroid Extract Induced by Vitamin D_2 Therapy

SE MO SUH, DONALD FRASER, AND SANG WHAY KOOH

The Departments of Paediatrics and Physiology, University of Toronto, and the Research Institute, The Hospital for Sick Children, Toronto, Canada

ABSTRACT. Response to bovine parathyroid extract (PTE) was tested in an 11 8/12-yr-old girl with pseudohypoparathyroidism before and during treatment with large doses of vitamin D_2. Prior to vitamin D_2 therapy, there was a slight but subnormal calcemic response and no phosphaturic response to large doses of PTE. Response to the same dose of PTE was tested again while the patient was receiving sufficient vitamin D_2 (100,000 IU/day) to restore serum calcium, serum phosphate and the renal handling of phosphate to normal ranges. The serum calcium rose promptly from 10.0 to 14.4 mg/100 ml in response to intramuscular PTE injection and there were marked increases in urinary phosphate excretion rate and phosphate clearance and a decrease in % TRP. The hypercalcemic response to PTE was also demonstrable when the patient received a very low intake of calcium, indicating that bone calcium was mobilized in response to the PTE administration in the vitamin D_2 treated patient. We conclude that large doses of vitamin D_2 improved the responsiveness of our patient with pseudohypoparathyroidism to PTE, an effect not hitherto reported. The findings indicate that the parathyroid hormone response test may be unreliable as a means of diagnosing pseudohypoparathyroidism if the patient is already receiving vitamin D_2 therapy. (*J Clin Endocr* **30:** 609, 1970)

PSEUDOHYPOPARATHYROIDISM is characterized by hypocalcemia, hyperphosphatemia and, usually, certain somatic and mental abnormalities. The essential feature that distinguishes the condition from hypoparathyroidism is end-organ refractoriness to parathyroid extract (1-3).

We have recently reported (4) a study refuting the hypothesis that hypocalcemia in pseudohypoparathyroidism was due to excessive circulating calcitonin. On completing the study, the patient received appropriately adjusted doses of vitamin D_2 which corrected her severe hypocalcemia and hyperphosphatemia, and improved the renal handling of phosphate.

In the present paper, we compare the responsiveness of the patient to bovine parathyroid extract (PTE) before and during vitamin D_2 therapy. The studies lead us to conclude that large doses of vitamin D_2 improved the responsiveness of the patient to PTE, an effect not hitherto reported.

Case Report

CC, HSC #480684, born August 23, 1955, was an 11 8/12-yr-old girl with untreated pseudohypoparathyroidism. She had generalized convulsions and tetany, short stature, mental deficiency and intracranial calcification. When studied in May 1967, serum calcium averaged 4.1 mg/100 ml, serum inorganic phosphate averaged 8.5 mg/100 ml and serum alkaline phosphatase activity was 24 King-Armstrong units. Radiographs of the skeleton showed normal architecture and bone density. Fecal fat excretion was normal. Paper chromatography of urine showed a normal amino acid pattern. Antirachitic activity, equivalent to 0.2 U vitamin D/ml, was demonstrated in the serum by the rat tibia line test.[1]

Thyroidectomy was carried out on June 27, 1967 as a part of the "calcitonin study" (4). Response to parathyroid extract was assessed before operation, and again immediately postoperatively. Commencing shortly after surgery, the patient received thyroid replacement

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[1] Performed by Wisconsin Alumni Research Foundation Laboratories, using method USP XV 889, 1955.

therapy. From July 1967, vitamin D_2 was administered at a dose of 100,000 IU/day and her serum calcium was maintained at approximately 10 mg/100 ml and inorganic phosphate at 4.5 mg/100 ml. Radiographs of the skeleton remained normal. She was hospitalized again 3 months and 9 months later for reassessment of response to parathyroid extract.

Materials and Methods

The studies were carried out in the Clinical Investigation Unit of The Hospital for Sick Children, Toronto. During all 3 admissions, the patient consumed a diet containing 1200 mg calcium and 1200 mg phosphorus/day except during one study, when she received 80 mg calcium and 675 mg phosphorus/day. As the test of responsiveness to parathyroid hormone, the patient was given 200 USP U of PTE[2] intramuscularly every 6 hr for varying periods, and on one occasion she received, at the conclusion of the course of PTE administration, 1000 U purified bovine parathyroid hormone (PTH)[3] every 6 hr for four doses. Response was assessed by serial determinations of serum calcium and serum inorganic phosphate, and assessment of the renal handling of phosphate [urinary phosphate excretion rate, urinary phosphate clearance (Cp) and the percentage of filtered phosphate reabsorbed by the renal tubules (% TRP)].

Serum and urine specimens were analyzed by the methods described in the previous report (4). A balance study was conducted by standard techniques. Daily calcium and phosphorus intakes during the balance study were 80 and 675 mg, respectively. Feces were pooled for 3 consecutive 3-day periods without markers. Urine specimens were collected for 24-hr periods. Aliquots of feces and diet were dry-ashed at 400 C for phosphorus and 600 C for calcium determinations.

Results

1. *Response to PTE prior to vitamin D_2 therapy.* Responsiveness to PTE prior to vitamin D_2 therapy was tested on two occasions—before and after total thyroidectomy. Results of these studies have been reported in detail in our previous paper (4). Serum calcium and inorganic phosphate concentrations and urinary phosphate excretions during the last four days of these tests are summarized in Fig. 1, A and B.

The serum calcium increased only slightly above base line values during prolonged intramuscular administration of PTE but the patient remained severely hypocalcemic (6 mg/100 ml or less). During the PTE administration, renal phosphate clearance remained low and % TRP high. The slight and inconsistent increase in urinary phosphate excretion observed during this period was attributed to the rise in serum phosphate level. It was concluded that no phosphaturic response occurred.

Thyroidectomy did not alter the responsiveness to PTE.

2. *Response to PTE during vitamin D_2 therapy.* While the patient was receiving vitamin D_2, 100,000 IU/day, serum calcium was approximately 10 mg/100 ml and inorganic phosphate 4.5 mg/100 ml. The phosphate excretion rate, phosphate clearance and % TRP improved to the normal ranges (6). PTE responsiveness was reassessed under these conditions at two levels of dietary calcium intake.

Calcium intake, 1200 mg/day (Fig. 1C). Fig. 1C indicates the response of the patient to PTE after four months of vitamin D_2 therapy. Within 48 hours of commencing PTE, serum calcium increased from 10.0 to 14.4 mg/100 ml, and PTE was dis-

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[2] Para-Thor-Mone, Eli Lilly Co.; all tests were carried out using the same batch of extract (Lot #ORU 64E). This batch of extract was shown to possess calcium-mobilizing and phosphaturic activities in a patient with idiopathic hypoparathyroidism. Intramuscular injection of 200 USP U every 6 hr raised the serum calcium from 6.6 to 12.1 mg/100 ml in 3 days. Serum phosphate decreased from 9.4 to 3.6 mg/100 ml. Urinary phosphate excretion increased from 33.0 to 59.3 mg/hr, and TRP decreased from 93.7 to 77.2%.

[3] Purified bovine parathyroid hormone was prepared by the procedure of Aurbach and Potts (5). The powder had a potency of 3000 USP U/mg as determined by the Munson bioassay method. The hormone was dissolved in 3 ml saline, cysteine HCl was added to a concentration of 0.12M, and the pH of the solution was adjusted to 4.0. One ml of the patient's serum was added to the solution and the entire mixture was filtered through a Swinny Millipore filter holder (0.22 μ pore membrane) into a sterile flask. Sterility was confirmed before use. No febrile reaction was observed when the material was administered to the patient.

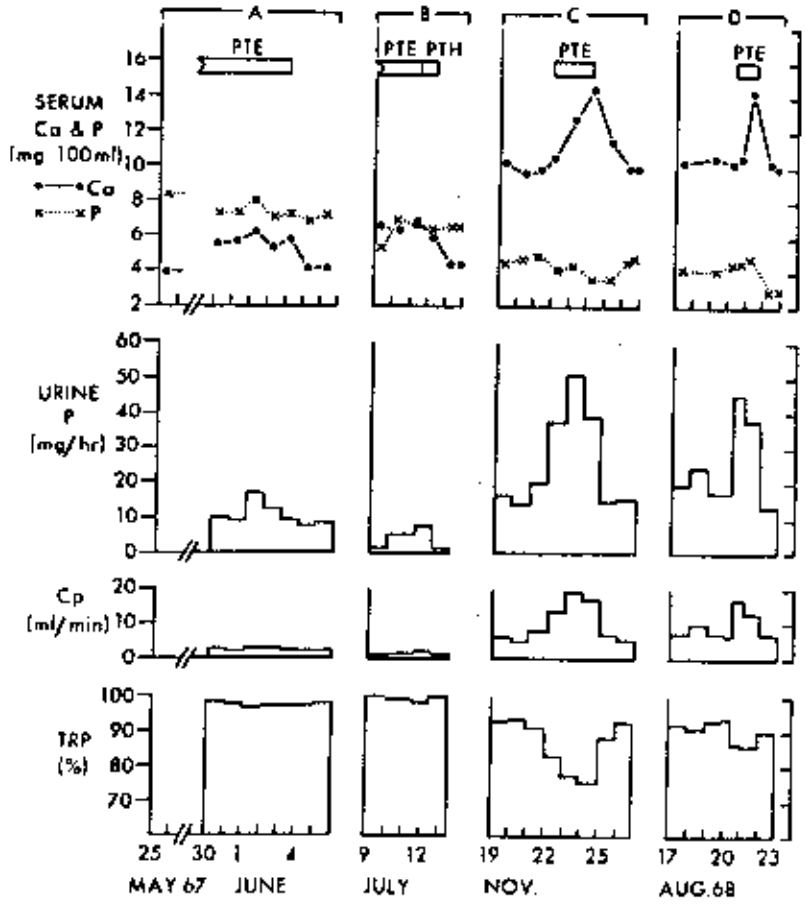

FIG. 1. Serum Ca and P concentrations and urinary P excretion in response to intramuscular PTE, 200 USP U every 6 hr, in 4 different circumstances: A. Before thyroidectomy, before vitamin D_2 therapy, oral calcium intake 1200 mg/day. B. After total thyroidectomy, before vitamin D_2 therapy, oral calcium intake 1200 mg/day. C. After total thyroidectomy, receiving vitamin D_2 therapy, oral calcium intake 1200 mg/day. D. After total thyroidectomy, receiving vitamin D_2 therapy, oral calcium intake 80 mg/day.

continued because of hypercalcemia. Serum calcium concentration returned promptly to the pre-injection level.

Urinary phosphate excretion rate and phosphate clearance increased more than 2-fold during PTE administration, and % TRP decreased considerably. The increased urinary phosphate excretion rate was not explained by a change in filtered load of phosphate since neither the serum phosphate concentration nor creatinine clearance was altered by PTE administration (average creatinine clearance without PTE 74 ml/min and with PTE 79 ml/min).

Calcium intake, 80 mg/day (Fig. 1D). Fig. 1D indicates the response of the patient to PTE on a low-calcium diet for 12 days. The patient had been treated with vitamin D_2 for 13 months. Serum calcium and phosphate averaged 10.5 and 4.1 mg/100 ml, respectively, during the control period. When PTE was given, serum calcium rose in 24 hours to 14.3 mg/100 ml, and, as before, returned promptly to control levels on discontinuing PTE administration. The hypercalcemic effect of PTE was similar to that observed when the vitamin D-treated patient was ingesting 1200 mg calcium.

Balance studies were conducted before and during this period of PTE administration (Fig. 2). Before PTE administration, fecal calcium averaged 42 mg/day and urinary calcium averaged 158 mg/day, denoting a negative calcium balance of 120 mg/day. When PTE was given, fecal calcium decreased to 19 mg/day, urinary calcium increased to 169 mg/day and a negative balance persisted. Because the calculated amount of calcium required to raise

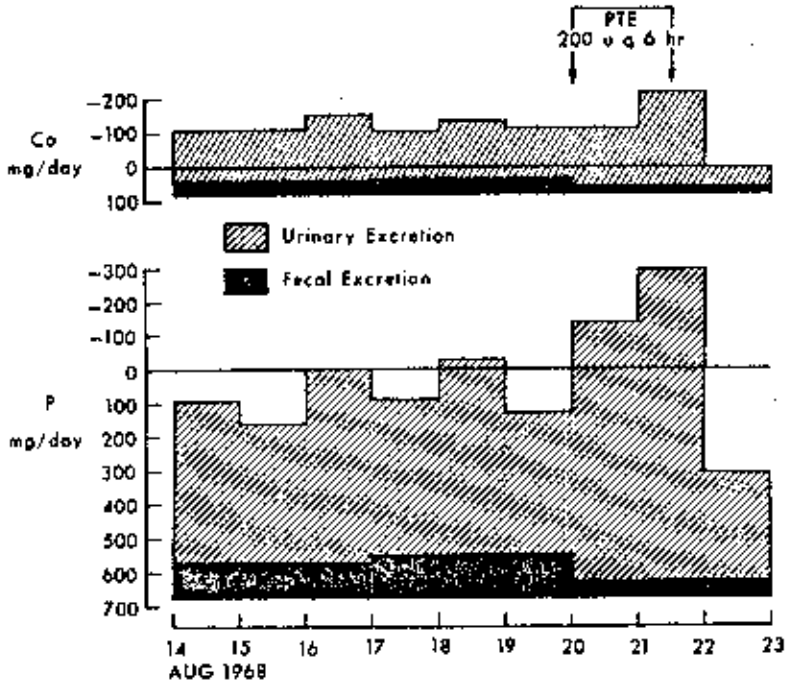

FIG. 2. Ca and P balances. The patient ingested 80 mg of calcium, 675 mg of phosphorus and 100,000 IU vitamin D_2/day. The areas above the zero line indicate negative balances. During the period of intramuscular PTE administration, negative calcium balance persisted, whereas serum Ca rose from 10.5 to 14.3 mg/100 ml. Note also the increase in urinary P excretion during this period.

the extracellular calcium level from 10 to 14 mg/100 ml would be approximately 320 mg in this patient (body weight 40 kg, estimated extracellular fluid volume 8 liters), and because the response occurred within 24 hours, the small increase in intestinal calcium absorption (23 mg/day) could not have contributed importantly to the hypercalcemic response.

A phosphaturic response was again noted during PTE administration (Fig. 1D and 2). The urinary phosphate excretion rate increased 2-fold, and there was an increase in phosphate clearance and a decrease in % TRP.

Discussion

Our study of a patient with pseudohypoparathyroidism indicates that responsiveness to PTE was dependent upon the vitamin D status of the patient. Before commencing the large doses of vitamin D_2 necessary to control hypocalcemia and hyperphosphatemia, repeated large intramuscular doses of bovine PTE caused only a small increase in serum calcium and no phosphaturic response. Since this refractoriness was not ameliorated in our patient by total thyroidectomy (4), or in another by thyroid irradiation (7), we had concluded that the refractoriness to PTE was not attributable to excessive secretion of thyroidal calcitonin, as proposed by some investigators (8).

After correction of the levels of serum calcium and phosphate by administration of large doses of vitamin D_2, the responsiveness of the patient to exogenous PTE was greatly increased. A pronounced hypercalcemic response occurred promptly when PTE was given, and a definite phosphaturic effect was observed. The hypercalcemic response took place whether the patient was receiving an adequate calcium intake or one that was markedly restricted. The studies carried out in the latter situation demonstrated that, in the vitamin D-treated patient, PTE was capable of stimulating calcium mobilization from bone.

While receiving vitamin D_2 therapy, serum calcium was within the physiological range, and this level was maintained during drastic curtailment of dietary calcium intake. The markedly negative bal-

ance of calcium during this period gives a measure of the rate at which calcium was being mobilized from the skeleton. However, it is not possible to state whether calcium mobilization was attributable to high levels of vitamin D_2 acting directly on tissues of the treated patient, or whether this process was caused by increased responsiveness of the vitamin D_2-treated patient to endogenous parathyroid hormone. Parathyroid hormone has been demonstrated in serum of patients with pseudohypoparathyroidism (4, 9, 10).

During vitamin D_2 therapy, the renal handling of phosphate showed improvement, as was reported by Halden *et al.* (11) in hypoparathyroidism. Eisenberg (12) attributed this alteration in phosphate handling in hypoparathyroidism to establishment of normal serum calcium levels. In our pseudohypoparathyroid patient, however, elevation of serum calcium to normal ranges by intravenous calcium infusion did not induce phosphaturia. It is therefore unlikely that the phosphaturic effect was caused by an improved serum calcium level in our patient.

When PTE was given during vitamin D_2 therapy, the patient showed a definite phosphaturic response, which was in marked contrast to the refractoriness observed before vitamin D_2 therapy.

Studies in rachitic animals (13, 14) have shown that the calcium mobilizing action of PTE is dependent upon the presence of vitamin D, and there is evidence from clinical studies of rickets (15–17) that vitamin D has a similar permissive action of PTE responsiveness in humans. Absence of rickets in our patient and the demonstration by bioassay of detectable antirachitic activity in her serum before treatment eliminate vitamin D deficiency as the cause of PTE refractoriness. We conclude that the hypercalcemic and phosphaturic actions of PTE are dependent upon massive, as opposed to physiological, intakes of vitamin D. Whether this situation is determined by a specific disorder in vitamin D metabolism remains to be investigated.

Our studies give no indication of the mechanism by which the large doses of vitamin D exert this "permissive" effect. Large doses of vitamin D raise the serum calcium levels in both hypoparathyroidism and pseudohypoparathyroidism. The fact that the calcium ion is important in transport phenomena and cell function (18–21) raises the question whether the induction of PTE responsiveness by vitamin D therapy may have resulted from improvement in the calcium environment of the cell. However, the concentration of calcium in serum and extracellular fluid does not necessarily give an indication of calcium ion activity in the intracellular fluid or at the cell membrane. Correction of serum calcium by parenteral calcium administration did not induce PTE responsiveness (ref. 4; Fig. 2); on the other hand, in hypoparathyroidism severe hypocalcemia does not impede the calcium-raising effect of exogenous PTE.

Chase *et al.* (10) demonstrated little or no increase in the urinary excretion of 3′,5′-AMP in response to the administration of PTE to individuals with pseudohypoparathyroidism, whereas a significant increase in 3′,5′-AMP excretion resulted from similar treatment of patients with idiopathic hypoparathyroidism. These authors stated that the metabolic defect of pseudohypoparathyroidism could be accounted for by a lack of or defective form of parathyroid hormone-sensitive adenyl cyclase. On the other hand, other studies suggest that the action of parathyroid hormone may not be mediated by 3′,5′-AMP (22, 23). Our findings raise the question whether or not induction of PTE responsiveness causes a corresponding enhancement of 3′,5′-AMP production in our patient.

Although, in typical cases, certain physical features are helpful in the diagnosis of pseudohypoparathyroidism, the generally accepted diagnostic criterion is lack of response to PTE. Before treatment, our pa-

tient showed this feature. By contrast, however, when receiving effective vitamin D_2 therapy, she responded to PTE. Therefore, responsiveness to the administration of PTE does not rule out the diagnosis of pseudohypoparathyroidism if a patient is receiving vitamin D_2 therapy at the time of study.

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Parathyroid Hormone Effects in Rats Treated with Diphosphonate

Abstract. *The ability of disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP; 40 milligrams per kilogram of body weight per day) to reduce the hypercalcemic effect of parathyroid hormone in thyroparathyroidectomized rats was confirmed. However, treatment with this large dose of EHDP enhanced the hypophosphatemic effect of a low dose of parathyroid hormone (10 international units per 100 grams of body weight), apparently by promoting the renal excretion of phosphate. The data suggest that EHDP may have a direct effect on the renal action of parathyroid hormone and, in this way, may also affect vitamin D metabolism by the kidney.*

Diphosphonates have been reported to inhibit bone resorption (*1, 2*) and to prevent soft tissue calcification (*3*). At least part of the skeletal effect has been attributed to the binding of the pyrophosphate analog on the surface of apatite crystals (*4*). In rats, disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP), at a daily dose of 40 mg per kilogram of body weight for 3 days, reduced the hypocalcemic response to calcitonin but did not affect the hypophosphatemic action of the hormone (*5*). A similar reduction in the hypercalcemic response of EHDP-treated rats to parathyroid hormone (PTH) also has been reported (*6*). The studies reported here suggest that the renal response of the rat to PTH is not inhibited by pretreatment with this dose of EHDP, but in fact actually may be enhanced.

Male rats weighing between 140 to 200 g (Zivic-Miller strain) were injected subcutaneously with EHDP, 40 mg kg^{-1} day^{-1} (I. Y. Rosenblum, Procter & Gamble Co.), between 9 and 10 a.m. for five successive days. This dose level may have been toxic since it reduced the gain in weight of these growing rats by 25 percent. On the afternoon of the fifth day, all rats were thyroparathyroidectomized (TPTX), and food and water were continuously supplied. The morning of the sixth day, a single subcutaneous injection of PTH, either 10 or 20 international units (I.U.) per 100 g of body weight (Wilson Laboratories, 250 units per milligram of trichloroacetic acid powder), was administered. Blood samples (from tail vein) were obtained prior to hormone injection and at 1 and 3 hours after injection. In one experiment the rats were bilaterally nephrectomized 2 hours prior to injection of PTH. Plasma calcium (Ca) and inorganic phosphate (P_i) were determined by standard methods (*7, 8*). Statistical comparisons were made by Student's *t*-test.

In preliminary experiments the following reported effects of EHDP treatment in rats were confirmed: (i) Ca and P_i fluxes into bone were markedly reduced, as were the rates of removal of previously deposited radionuclides; (ii) after 5 days of treatment, the concentration of plasma Ca was slightly elevated, while the concentration of plasma P_i was significantly lowered; and (iii) following TPTX or parathyroidectomy, the rate of fall in plasma Ca and the rate of rise in plasma P_i were significantly reduced by treatment with EHDP. The inhibition of the ability of PTH (10 to 20 I.U. per 100 g of body weight) to raise plasma Ca concentrations in TPTX rats was also confirmed; however, these experiments suggested that the hypophosphatemic action of PTH was enhanced by 5 days of treatment with EHDP, despite the fact that plasma P_i concentrations at the time of PTH injection were between 1.0 and 1.5 mg/100 ml lower than in controls. The experiments described below confirmed this finding.

Using the standardized procedures (18 hours after TPTX; animals either fasted or fed), we made a comparison of the immediate effects of PTH on plasma Ca and P_i in control rats and in those treated with EHDP. The data, summarized in Fig. 1, demonstrate that in EHDP-treated rats the hypercalcemic action of PTH was reduced while its hypophosphatemic effect was augmented. The increased hypophosphatemia in EHDP-treated animals after injection of PTH was more apparent at the lower dose of the hormone and at 1 hour after its injection than at the higher dose or at 3 hours after injection of PTH. A similar sequence of changes was observed in plasma ^{45}Ca and ^{32}P activities when the radionuclides were injected 3 to 6 days prior to PTH administration.

Preliminary data on the effect of EHDP on renal excretion of Ca and P_i have been obtained. Based on the values of both P_i and ^{32}P in urine, the increase in P_i excretion following administration of PTH was greater in EHDP-treated rats than in nontreated animals. Changes in the concentration of Ca in urine followed a different pattern. In EHDP-treated TPTX rats, urinary Ca was increased almost ten-

fold over controls not treated with EHDP. Following injection of PTH, this value dropped to approximately that of the controls. Quantitative urinary measurements to verify these changes have not yet been reported.

In order to study further the role of the kidney in this increased sensitivity of EHDP-treated rats to PTH, 20 I.U. of this hormone (per 100 g of animal body weight) was injected into rats, previously treated for 3 days with EHDP, 2 hours after nephrectomy. The hypophosphatemic response to PTH was negated by nephrectomy in the EHDP-treated TPTX rats as well as in those not treated with the diphosphonate. Nephrectomy did not abolish the relative hypercalcemic effects of PTH on plasma calcium in the two groups of rats (Table 1).

The time course of development of the effects of this dosage of EHDP on the action of PTH was examined by repeating the experimental procedure except that the rats were TPTX 24 or 48 hours after the first injection of EHDP. While inhibition of the hypercalcemic effect of PTH was apparent by 24 hours after the initial administration of the drug, the hypophosphatemic effect of PTH was unaffected for 3 days of treatment, indicating that the renal effect of EHDP is slower in developing. The first significant decrease in plasma P_i also occurred between the fourth and fifth day of drug treatment.

Table 1. Plasma phosphate and calcium values after nephrectomy. The initial bleeding was performed on all rats 2 hours after nephrectomy and 18 hours after TPTX. Concentration of PTH injections was 20 I.U. per 100 g of body weight. Values are given as means ± the standard error of the mean.

Treatment	Animals (No.)	Before PTH	After PTH: 1 hour	After PTH: 3 hours
		Phosphate		
TPTX (no PTH)	4	10.6 ± 0.3	11.5 ± 0.5	12.5 ± 0.5
TPTX plus PTH	5	10.1 ± 0.5	10.8 ± 0.6	11.5 ± 0.6
EHDP-TPTX (no PTH)	5	9.6 ± 0.2	10.1 ± 0.2	11.2 ± 0.3
EHDP-TPTX plus PTH	5	10.0 ± 0.2	10.6 ± 0.3	11.8 ± 0.4
		Calcium		
TPTX (no PTH)	4	8.3 ± 0.6	8.5 ± 0.8	7.5 ± 1.2
TPTX plus PTH	5	8.9 ± 0.6	9.5 ± 0.6	10.3 ± 0.9
EHDP-TPTX (no PTH)	5	10.0 ± 0.1	10.6 ± 0.3	10.5 ± 0.2
EHDP-TPTX plus PTH	5	9.8 ± 0.4	10.2 ± 0.4	10.7 ± 0.4

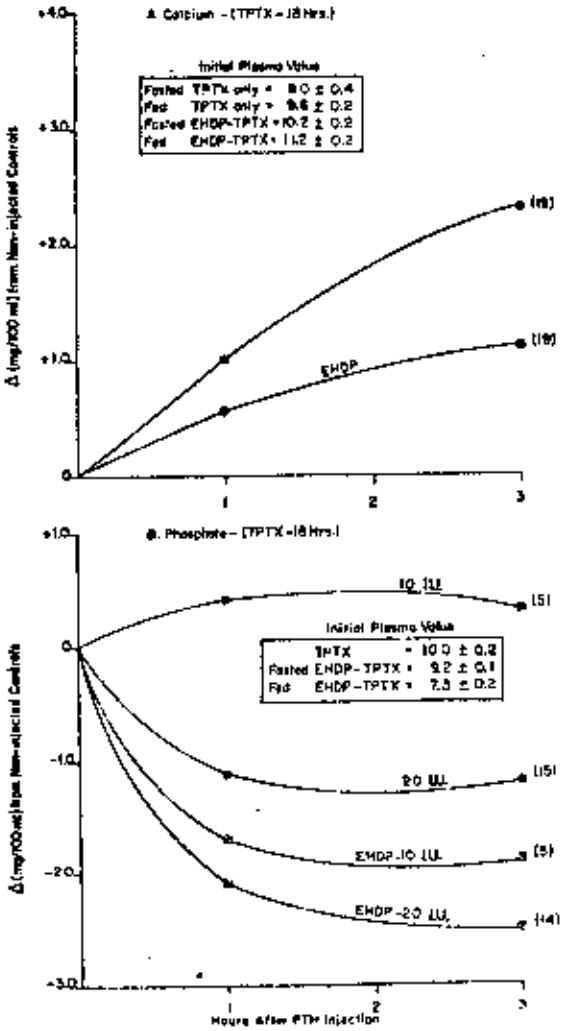

Fig. 1. Plasma calcium and phosphate changes (Δ) in milligrams per 100 ml of plasma after PTH injection in TPTX rats. All data from EHDP-treated rats were statistically different ($P < .005$) from respective controls. Rats administered PTH were injected with either 10 or 20 I.U. per 100 g of body weight. In (B) changes in values of plasma calcium from rats receiving two levels of PTH have been combined. Numbers in parentheses indicate the number of animals. Closed symbols refer to mean values of control rats; open symbols, to mean values of EHDP-treated rats.

The contrasting effect of this drug in man and rat is difficult to explain. Administration of EHDP lowers concentrations of plasma P_i in the rodent while raising them in humans (*9*). In addition, Recker *et al.* (*9*) have reported no significant change in the phosphaturic effect of the administration of PTH in humans who were maintained on oral doses of EHDP. However, they concluded that the EHDP-induced rise in plasma phosphate values was the result of a renal effect of the drug. Plasma P_i concentrations in man are normally low (3 mg/100 ml) whereas those in the young rat are high (7 to 9 mg/100 ml); but in rats maintained on a rachitogenic diet, which lowers concentrations of both plasma Ca and P_i, EHDP raises rather than lowers the P_i (*10*). It is possible, therefore, that the opposite net effects on plasma P_i concentrations might be due to the difference in the starting values of plasma P_i rather than to different effects of the drug on renal metabolism.

It has recently been reported that adult human subjects treated for long periods of time with EHDP develop widened osteoid seams and signs of osteomalacia (*2, 11*). Rats administered large doses of EHDP absorb less calcium from the gut (*12*) and, even though receiving adequate or excess vitamin D, may develop rickets (*13*). Whereas most of these bone changes can be attributed to a direct action of EHDP on bone, an effect of the drug on renal hydroxylation of 25-hydroxycholecalciferol also has been reported (*10, 14*). Whether this is a direct effect of EHDP on renal 1-hydroxylase or an indirect result of changes in endogenous hormone levels is open to question. However, based on the results of the experiments described here, we conclude that the administration of EHDP in sufficiently large doses may

have a direct effect on the renal action of PTH, an agent which by itself has been implicated in the control of vitamin D metabolite hydroxylation (*15*).

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Mechanism of Action of 1,25-Dihydroxycholecalciferol on Intestinal Calcium Transport

(rat/vitamin D/actinomycin D/kidney/"peak V" metabolite)

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ABSTRACT **The prior administration of actinomycin D prevents the metabolism of [^{3}H]25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol, a metabolite of vitamin D_3 that is effective in the stimulation of intestinal calcium transport. In this paper, the question of whether the response of intestinal calcium transport to 1,25-dihydroxycholecalciferol is sensitive to actinomycin D was examined. While the response of intestinal transport to physiological amounts of 25-hydroxycholecalciferol is blocked by actinomycin D, the response of intestinal calcium transport to 1,25-dihydroxycholecalciferol is insensitive to the antibiotic. These results suggest that 1,25-dihydroxycholecalciferol, or a further metabolite thereof, is the metabolically active form of vitamin D in the intestine, that it functions by a process not involving transcription of DNA, and that the step sensitive to actinomycin D in the action of vitamin D on the intestine does not occur in the intestine, but is the conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol in the kidney.**

In 1965, Zull *et al.* (1) conclusively demonstrated that actinomycin D given prior to, but not after, vitamin D prevents completely the action of the vitamin in increasing intestinal calcium transport and bone mineral mobilization. At the same time, Norman (2) reported that actinomycin D blocks vitamin D-stimulated intestinal absorption of calcium in chickens. Further studies (3) provided very strong evidence that the mechanism of vitamin D action both in the intestine and in bone must involve transcription of DNA and protein synthesis. The concept was further strengthened when it was demonstrated that nuclear RNA labeling by [^{3}H]orotic acid or [^{3}H]uridine could be stimulated by vitamin D in both rats (4) and chicks (5). Since that time, it has been assumed that vitamin D must initiate these actions by activating a gene(s) in the intestine that in turn provides the messenger RNA for the synthesis of protein components required for calcium transport or bone mineral mobilization.

Lund and DeLuca (6), following still another lead with regard to the mechanism of vitamin D action, were able to demonstrate quite convincingly the existence of a metabolite(s) of vitamin D more active than the parent vitamin itself. Furthermore, this metabolite acted more rapidly in initiating intestinal calcium transport than did vitamin D_3 itself (7), leading to the concept of metabolically active forms of vitamin D. Subsequently, this metabolite was isolated in pure form from the plasma of pigs and was identified as 25-hydroxycholecalciferol 25-(OH)D_3 (8). Strong evidence has since then been provided that 25-hydroxylation represents the initial step in the functional metabolism of the vitamin (9). Still other work has demonstrated that 25-(OH)D_3 represents the major circulating active form of the vitamin and thus may be considered a hormonal form (9, 10). With radioactive 25-(OH)D_3, Cousins *et al.* (11, 12) demonstrated the accumulation of a metabolite more polar than 25-(OH)D_3 in intestine and bone, as well as other tissues, that could well represent a metabolically active form of vitamin D (10–13, 14). Haussler *et al.* (13), and more recently Myrtle *et al.* (15) and Kodicek *et al.* (16), have provided evidence that this metabolite has potent biological activity in stimulating intestinal calcium transport. This metabolite acts more rapidly in initiating intestinal calcium transport than does 25-(OH)D_3 (17–19), but it is less effective in curing rickets in rats than 25-(OH)D_3 (18) and is no more active than 25-(OH)D_3 in stimulating bone mineral mobilization (18). In any case, the present evidence would strongly suggest that this polar metabolite ("peak V") represents the metabolically active form of the vitamin in the intestine. This metabolite was recently isolated in pure form from intestine and identified conclusively as 1,25-$(OH)_2D_3$ (20), in agreement with a structure deduced (21) from a preparation of 30% purity.

Of significance is the recent finding (22) that actinomycin D or cycloheximide given prior to the injection of radioactively labeled 25-(OH)D_3 prevents the metabolism of 25-(OH)D_3 to 1,25-$(OH)_2D_3$; the 25-(OH)D_3 then accumulates in the intestine. When actinomycin D or cycloheximide is given after 25-(OH)D_3, these agents are no longer able to block its metabolism to 1,25-$(OH)_2D_3$. These results suggest that 25-(OH)D_3 itself induces the formation of an enzyme or enzymes that are responsible for the production of 1,25-$(OH)_2D_3$. It is possible that the actinomycin D-sensitive step in the metabolic function of vitamin D is the conversion of 25-(OH)D_3 to the polar metabolite, which in turn functions in the intestine to initiate intestinal calcium transport.

This report provides unequivocal proof that the 1,25-$(OH)_2D_3$, or a further metabolite thereof, represents the metabolically active form of the vitamin in the intestine. Furthermore, we show that this metabolite initiates intestinal calcium transport by a process that does not involve transcription of DNA into RNA; thus, the actinomycin D-sensitive step in vitamin D function in the intestine is the conversion of 25-(OH)D_3 to 1,25-$(OH)_2D_3$.

Abbreviations: 25-(OH)D_3, 25-hydroxycholecalciferol; 1,25-$(OH)_2D_3$, 1,25-dihydroxycholecalciferol.

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MATERIALS AND METHODS

Young male weanling rats were obtained from the Holtzman Co., Madison, Wis. They were housed individually in hanging wire cages and fed an adequate calcium (0.47%) and phosphorus (0.3%) diet deficient in vitamin D (23). For all of the intestinal calcium transport work, the rats were fed the adequate calcium and phosphorus diet for 4–5 weeks, at which time they ceased to grow and exhibited a severe hypocalcemia (about 4–5 mg calcium/100 ml of plasma); these rats were used for the everted intestinal sac experiments.

Radioactive Compounds. The [1,2-^{3}H]vitamin D_3 was synthesized as previously described (24), and had a specific activity of 0.6 Ci/mmol. The [26,27-^{3}H]25-(OH)D_3 had a specific activity of 1.3 Ci/mmol (25).

Preparation of 1,25-(OH)$_2$D$_3$. 25-(OH)$_2$D$_3$ was kindly supplied in crystalline form by the Philips-Duphar Co. of The Netherlands. 1,25-(OH)$_2$D$_3$ was isolated from intestinal mucosa of chicks given [1,2-^{3}H]vitamin D_3 (18, 20). Vitamin D_3 is a commercial product purchased from the Philips-Roxane Co. of the United States.

Intestinal calcium transport measurements

The vitamin D–deficient rats were maintained on the 0.47% calcium, 0.3% phosphorus diet for 5 weeks and divided into an appropriate number of groups. Where indicated, 1 μg of actinomycin D per g of body weight was injected intrajugularly 2 hr before the animals were injected with the appropriate metabolite in the jugular vein. Control animals received the vehicle alone at the appropriate times. The animals were killed either 8 or 12 hr later. Intestinal calcium transport was measured by the everted sac technique (26).

Metabolism experiments with [26,27-^{3}H]25-(OH)D_3

Rats fed the appropriate diet were given actinomycin D (Nutritional Biochemicals, Inc., Cleveland, Ohio) (1 μg/g body weight) intrajugularly 2 hr before they received 65 pmol of [^{3}H]25-(OH)D_3. The control animals received the appropriate volume of vehicle at the indicated times. The rats were killed by decapitation and the intestine or kidneys were immediately excised. The intestine was slit lengthwise and rinsed in ice-cold isotonic saline, and the mucosa were scraped from the intestine, which rested on a stainless-steel plate embedded in ice. A tissue homogenate (25% in distilled water) was prepared and then immediately extracted with methanol and chloroform (6). The chloroform extracts thus obtained were chromatographed on 2 × 20 cm Sephadex LH-20 gel columns in a solvent of 65% chloroform in petroleum ether (bp 67–69°C) (27) for the resolution of polar metabolites of vitamin D. Fractions were collected from the chromatographic columns and dried directly under a stream of air in liquid-scintillation counting vials. To each vial was added 15 ml of a toluene-based counting solution (23), and the tritium content was determined by means of a Packard liquid scintillation counter, model 3375, equipped with external standardization for the determination of disintegrations per minute.

RESULTS

We had previously demonstrated that accumulation in the intestine of 1,25-(OH)$_2$D$_3$ reached a plateau about 8 hr after injection of [^{3}H]25-(OH)D_3 to vitamin D–deficient rats (11, 12, 22). It is evident from the present experiments that 8 hr after injection of vitamin D–deficient rats with [^{3}H]25(OH)D_3 there is a marked accumulation of the 1,25-(OH)$_2$ D_3 in the intestinal mucosa. In agreement with previous results, the prior administration of actinomycin D to these vitamin D–deficient rats markedly inhibited the accumulation of 1,25-(OH)$_2$D$_3$ in the intestine (Fig. 1). Because it appears that the kidney is the primary, if not the sole, site of synthesis of 1,25-(OH)$_2$D$_3$ (28, 29), it was of interest to determine whether inhibition of the accumulation of 1,25-(OH)$_2$D$_3$ could be observed in this tissue. Clearly, the actinomycin D also prevented the appearance of 1,25-(OH)$_2$D$_3$ in the kidney after injection of 25-(OH)D_3 (Fig. 1). These findings, and those suggesting that 1,25-(OH)$_2$ D_3 represents the metabolically active form of 25-(OH)D_3 in the intestine, raise the question of whether the actinomycin D–sensitive step in the vitamin D–induced increase in intestinal calcium transport might not be the formation of 1,25-(OH)$_2$D$_3$ in the kidney.

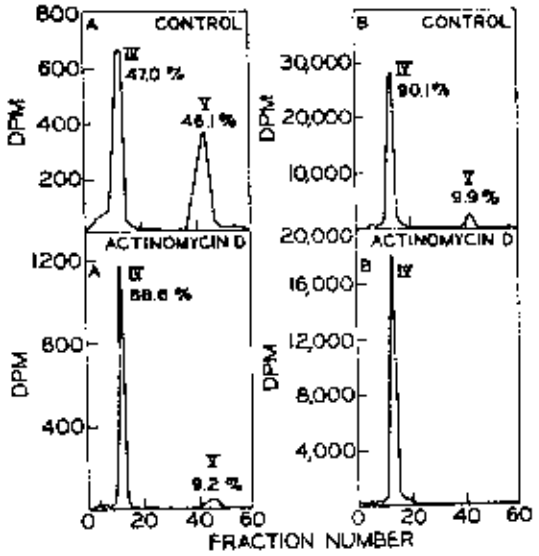

FIG. 1. Actinomycin D inhibition of the metabolism of 25-(OH)D_3 to 1,25-(OH)$_2$D$_3$. Vitamin D–deficient rats were given 1 μg/g body weight of actinomycin D 2 hr prior to a 65-pmol dose of [^{3}H]25-(OH)D_3 (0.05 ml, i.v., 95% ethanol). Controls received ethanol only. All rats were killed 8 hr after the administration of [^{3}H]25-(OH)D_3. Chloroform extracts were prepared of (*A*) intestinal mucosa and (*B*) kidney. Chromatography was on a LH-20 column (2 × 20 cm) with a solvent of 65% chloroform in Skellysolve B (bp 67–69°C) (26). Peak IV = unchanged 25-(OH)D_3, while peak V = biologically active 1,25-(OH)$_2$ D_3 (18). The figures near the peaks represent the % of chromatographed radioactivity.

The results shown in Table 1 provide the necessary evidence that the action of 25-(OH)D_3, like that of vitamin D (1, 3), in inducing intestinal calcium transport is blocked by the prior administration of actinomycin D, although at doses higher than 65 pmol of 25-(OH)D_3 per rat the block is incomplete. This may be related to the incomplete block in the production of 1,25-(OH)$_2$D$_3$ by actinomycin (Fig. 1) (22). These results appeared to be consistent with the previous data concerning the block of vitamin D action by actinomycin D, since 65 pmol of 25-(OH)D_3 can be considered to be a physiological dose. Of central importance is the finding that the rise in intestinal calcium transport that is induced by 65 pmol of

TABLE 1. *Failure of actinomycin D to inhibit the rise in intestinal calcium transport induced by 1,25-(OH)$_2$D$_3$*

Treatment		Transport
Actinomycin D	Vitamin D metabolite	^{45}Ca inside/^{45}Ca outside
−	—	1.7 ± 0.2*
−	25-(OH)D$_3$	2.7 ± 0.1†
+	25-(OH)D$_3$	1.8 ± 0.2
−	1,25-(OH)$_2$D$_3$	2.9 ± 0.2†
+	1,25-(OH)$_2$D$_3$	3.1 ± 0.3†

Rats were fed the 0.47% calcium, 0.3% phosphorus diet deficient in vitamin D for 3 weeks. Where indicated, each rat received 1 μg/g body weight of actinomycin D intrajugularly in 0.05 ml of ethanol. 2 hr later, where indicated, each rat received 65 pmol of 25-(OH)D$_3$ or 1,25-(OH)$_2$D$_3$ intrajugularly in 0.05 ml of ethanol. Control rats received the appropriate amount of ethanol in each case at the appropriate time. All rats were killed 12 hr after 25-(OH)D$_3$ or 1,25-(OH)$_2$D$_3$ administration. Intestinal calcium transport was measured as described by Martin and DeLuca (25).

* Standard deviation. There were four rats in each group.

† Significantly different from control group ($P < 0.01$ by Student's *t*-test).

1,25-(OH)$_2$D$_3$ is insensitive to actinomycin D (Table 1). These results are of fundamental importance in two regards. One, they provide firm evidence that 1,25-(OH)$_2$D$_3$, or a further metabolite thereof, must be the metabolically active form of the vitamin in the intestine. Even more important, they provide evidence that the intestinal calcium transport initiated by 1,25-(OH)$_2$D$_3$ does not involve transcription of DNA into RNA.

DISCUSSION

Our results demonstrate that 1,25-(OH)$_2$D$_3$, or a further metabolite thereof, must be the metabolically active form of vitamin D$_3$ in the intestine. This conclusion is based entirely on the fact that in the presence of actinomycin D, 25-(OH)D$_3$ does not act, whereas the 1,25-(OH)$_2$D$_3$ is able to initiate intestinal calcium transport. This finding, taken together with the fact that actinomycin D prevents the metabolism of 25-(OH)D$_3$ to 1,25-(OH)$_2$D$_3$, can only suggest that 25-(OH)D$_3$ is the precursor of the metabolically active form of vitamin D in the intestine. The results presented here also provide a new insight into the mechanism of vitamin D action in the intestine, namely that 1,25-(OH)$_2$D$_3$ can initiate intestinal calcium transport in the presence of actinomycin D. This finding suggests that transcription of DNA into RNA is not involved in vitamin D$_3$ action once the correct, metabolically active, form of the vitamin is provided.

Whether protein synthesis is required for the action of vitamin D$_3$ cannot be deduced from the present experiments. Our attempts to use cycloheximide to examine this question have not as yet proven fruitful. The results in the present report also support the previous conclusion that 25-(OH)D$_3$ probably induces the formation of an enzyme or enzymes involved in the production of 1,25-(OH)$_2$D$_3$. Recently, it has been clearly demonstrated (28, 29) that the kidney is the primary, if not the sole, site of synthesis of 1,25-(OH)$_2$D$_3$. This finding raises the question of why tritium derived either from vitamin D$_3$ or from 25-(OH)D$_3$ in the intestine is localized in cell nuclei (4, 13).

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Prostaglandins, calcium metabolism and cancer[1,2,3]

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ABSTRACT

The transplantable mouse fibrosarcoma, $HSDM_1$, produces a potent bone resorption-stimulating factor. The factor can be extracted from the tumor tissue and harvested from the medium of clonal strains of $HSDM_1$ cells grown in culture. Using serological methods, we have shown that $HSDM_1$ cells synthesize and secrete large quantities of prostaglandin E_2 (PGE_2). Indomethacin and 5,8,11,14-eicosatetraynoic acid, potent inhibitors of PGE_2 synthesis in $HSDM_1$ cells, also inhibit production by the cells of the bone resorption-stimulating factor. Mice bearing the $HSDM_1$ tumor have elevated concentrations of calcium and PGE_2 in serum. Administration of indomethacin (100–125 μg/mouse per day) to tumor-bearing mice lowers serum calcium and PGE_2 concentrations, reduces in parallel the tumor content of bone resorption-stimulating activity and PGE_2, and diminishes tumor size. We conclude that the $HSDM_1$ bone resorption-stimulating factor is PGE_2, and that excessive secretion of this prostaglandin by the tumor produces in the mouse a hypercalcemic syndrome with several characteristics similar to those of the unexplained ectopic humoral hypercalcemias observed in certain patients with malignant disease.—TASHJIAN, A. H., JR., E. F. VOELKEL, P. GOLDHABER AND L. LEVINE. Prostaglandins, calcium metabolism and cancer. *Federation Proc.* 33: 81–86, 1974.

Hypercalcemia occurs frequently in the presence of neoplastic disease, and in certain cases it appears to have a humoral etiology. On occasion, it can be shown that the tumor produces parathyroid hormone (18, 20) or a parathyroid hormone-like substance (17). In other cases, however, it has not been possible to measure parathyroid hormone either in the patient's plasma or in tumor extracts, despite the fact that highly sensitive immunological procedures were used (16, 19). In these cases, the cause or causes of the hypercalcemia remains obscure.

In the course of a broad investigation of a variety of factors that stimulate the resorption of bone in organ culture, we have uncovered a tumor system in the mouse which serves as a new model for studies of the hypercalcemia of cancer. The tumor is a transplantable fibrosarcoma ($HSDM_1$) which synthesizes and secretes large amounts of prostaglandin E_2 (PGE_2) and produces an elevation of serum calcium concentration in animals bearing the tumor. In this report, we review the evidence that links the hypercalcemia observed in the tumor-bearing animals to PGE_2 production by the tumor.

MATERIALS AND METHODS

Origin of the $HSDM_1$ tumor

Induction of the $HSDM_1$ fibrosarcoma in a Swiss albino mouse has been described by Goldhaber (6, 8). The tumor has been passed serially in mice of this strain for more than a decade (22).

Establishment of $HSDM_1$ tumor cells in monolayer culture

The methods for establishing primary cultures of $HSDM_1$ cells have been described (22), and 5 clonal strains (designated $HSDM_1C_1$, C_2, C_3, C_4 and C_5) have been isolated by single cell plating techniques. In the experiments reported here, the cells were grown in plastic petri dishes (Falcon) in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal calf serum. In certain experiments, the cells were grown to high density in the complete medium described above and then washed extensively and incubated in serum-free F10 medium for 24 hours. Cultures were incubated at 37 C in a humidified atmosphere of 5% CO_2 and 95% air.

Assays for prostaglandins

Prostaglandins in extracts of tumor tissue, in culture medium, and in mouse serum were assayed immunologically using the technique of Levine et al. (13).

At the termination of each experiment, tumors were excised, weighed, and extracted in Gey's balanced salt solution (3 ml/g). Protein was precipitated by heating, and the supernatant solution was extracted 3 times with diethyl ether, dried under nitrogen gas, and the residue dissolved in Gey's solution as described by Tashjian et al. (22) for prostaglandin radioimmunoassay and for biological assay of bone resorption-stimulating activity.

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[4] American Cancer Society Professor of Biochemistry, Award PRP-21.

Cell culture medium was assayed directly or after extraction and concentration with diethyl ether (22).

For analysis of prostaglandins in serum, the extraction and concentration procedure of Gutierrez-Cernosek et al. (10) was used. We have described the application of this technique to mouse serum (22).

Assay for bone resorption-stimulating activity

One-day-old mice were injected with 10 μCi of ^{45}Ca. Four days later the calvaria were removed and fixed to cover slips and cultured as described by Goldhaber (9). After a 24-hour washout period (22), fresh medium containing control, standard, or test substances was added to groups of 4 to 5 bones. Twenty-four hours later an aliquot of medium was taken for determination of ^{45}Ca by liquid scintillation counting. The experiment was terminated at 48 hours (after 2 days of treatment) and the total accumulated ^{45}Ca in the medium determined. The results are expressed as counts per minute ^{45}Ca per milliliter of medium at 24 and 48 hours, and a mean value and standard error were calculated for each time interval.

Analysis of serum calcium

Serum from individual control and tumor-bearing mice was obtained as described previously (21). The concentration of calcium in serum was determined by the method of Copp (3).

Statistical method

Serum calcium and ^{45}Ca data in each experiment were subjected to analysis of variance. The standard errors (SE) were calculated from the residual error term of each analysis.

RESULTS AND DISCUSSION

Initial observations

When Goldhaber cultured fragments of the HSDM$_1$ tumor in the same culture vessel with mouse calvaria, he observed marked stimulation of bone resorption (5, 7). Fragments of HSDM$_1$ tumor could be cultured alone, and the medium from such explants also stimulated bone resorption when it was added to calvaria in organ culture. These findings suggested that the tumor produced and secreted a bone resorption-stimulating factor.

In 1971, Voelkel and his co-workers

Figure 1. The appearance of stained (von Kossa) bones (4 per group) at the end of a 7-day period in culture. Control bones on the left show little loss of mineral (black stain). Fractions 93-III and 93-V are materials from gel filtration on a column of Sephadex G-10. Fraction 93-III is from the void volume of the column and did not stimulate bone resorption at a dose level of 30 μg of solid/ml culture medium. Fraction 93-V was from the retarded portion of the chromatogram and enhanced bone resorption at 4.0 and 0.4 μg of solid/ml. (Reproduced from Voelkel et al. (23) with permission.)

(23) showed that it was possible to extract bone resorption-stimulating activity from the tumor tissue. Figure 1 shows the effects of three dose levels of a partially purified extract of HSDM$_1$ tumors on the mineral content of cultured calvaria. In these early experiments, two significant new observations were made. First, the specific biological activity of the HSDM$_1$ factor was very high—that is, only small amounts were required to stimulate bone resorption in vitro. Second, and somewhat unexpectedly, it was found that the biological activity was extracted essentially quantitatively into organic solvents such as diethyl ether and ethyl acetate. Table 1 gives the relative specific activities of several types of bone resorption-stimulating factors.

TABLE 1. Specific biological activity of bone resorption-stimulating factors

Factor[a]	Approximate concentration required for half-maximum bone resorption, M
Parathyroid hormone PGE$_1$, PGE$_2$ 1,25(OH)$_2$ vitamin D HSDM$_1$ factor	10^{-7} to 10^{-9}
PGA$_1$, PGA$_2$ 25(OH) vitamin D	10^{-8}
dibutyryl cyclic AMP vitamin D	10^{-6}
PGF$_{1\alpha}$, PGF$_{2\alpha}$, PGB	$<10^{-5}$

[a] Abbreviations used here and elsewhere in this report for prostaglandins of the A, E, F, and B series are PGA, PGE, PGF and PGB, respectively.

Characterization of the HSDM$_1$ factor and cell culture studies

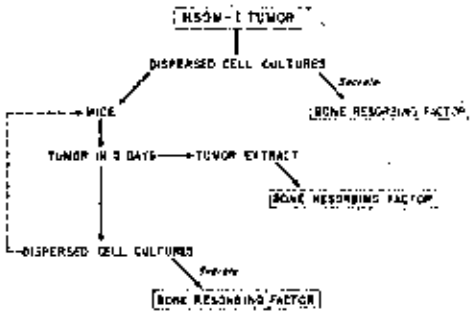
At the same time that Voelkel was extracting bone resorption-stimulating activity from the tumor tissue, experiments were initiated to establish dispersed cell cultures of HSDM$_1$ cells. The initial goals of these experiments were to develop homogeneous populations (clones) of cells which produced the bone resorption-stimulating factor, and to use such cultures as a source for the substance and to study the control of its production.

Such cultures were established and 5 clonal strains of cells obtained (22). Each clone secreted the bone resorption-stimulating factor into the culture medium. Furthermore, the cultured cells could be injected back into host mice, where they produced tumors that contained the HSDM$_1$ factor, and the cycle from mouse to culture could be repeated, apparently indefinitely (Fig. 2).

Because the material secreted into the medium of HSDM$_1$ cells in culture had both biological and chemical properties of certain prostaglandins, we examined the medium of HSDM$_1$ and control cells for prostaglandins using the serological methods developed by Levine and his colleagues. This approach seemed especially reasonable in view of the findings of Klein and Raisz (12) who showed that certain prostaglandins were potent stimulators of bone resorption in vitro. The results of our experiments led us to conclude that the HSDM$_1$ cells did synthesize and secrete large amounts of prostaglandins, and that the predominant prostaglandin produced by the cells was PGE$_2$ (14). The evidence supporting this conclusion follows.

1) In serum-free culture medium, HSDM$_1$ cells produce rela-

Figure 2. Cycle of tumor to culture and back to animal for HSDM$_1$ (HSDM-I) tumor cells. At each stage at which the bone resorption-stimulating factor could be extracted from the culture medium or tumor tissue so also could PGE$_2$ be measured serologically.



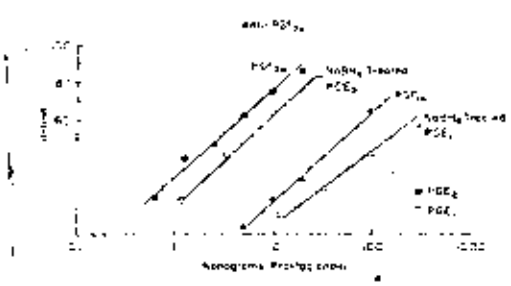
tively large amounts of material which inhibit the binding of [^{3}H]PGB$_1$ to anti-PGB$_1$. Its serological activity was enhanced more than 100-fold by treatment with alkali, a behavior characteristic of PGE but unlike the serological activity of PGA, which is enhanced only 10- to 15-fold by alkaline treatment (14, 15).

2) PGE$_2$, after sodium borohydride reduction, is converted to a mixture of PGF$_{2\alpha}$ and PGF$_{1\beta}$ (PGF$_{2\beta}$ cross-reacts < 0.5% with anti-PGF$_{2\alpha}$) (15). With our antiserum, after reduction of known quantities of PGE$_2$, the yield of serologically active products, when assayed with anti-PGF$_{2\alpha}$, was, as expected, 50% (Fig. 3). Similar reduction can be carried out with PGE$_1$ and assayed with anti-PGF$_{1\alpha}$. PGA and PGB treated with sodium borohydride did not react with anti-PGF. Thus, an unknown sample containing either PGE$_2$ or PGE$_1$ can be reduced with sodium borohydride and the products assayed serologically with anti-PGF$_{2\alpha}$ and anti-PGF$_{1\alpha}$. The relative specificities of anti-PGF$_{2\alpha}$ and anti-PGF$_{1\alpha}$ make it possible to identify the original prostaglandin (before reduction) as either PGE$_2$ or PGE$_1$.

This strategy was applied to serum-free HSDM$_1$ culture medium. After reduction with sodium borohydride, the HSDM$_1$ factor was assayed with anti-PGF$_{2\alpha}$ and anti-PGF$_{1\alpha}$. The relative activities of the reduced product with these two antisera identified the PGE produced by HSDM$_1$ cells as PGE$_2$ (14), the most active bone resorption-stimulating prostaglandin (22). Consequently, the results obtained with HSDM$_1$ cells are expressed as PGE$_2$.

3) While HSDM$_1$ cells do produce PGF, these prostaglandins have little or no bone resorption - stimulating activity (12, 22).

Figure 3. Inhibition of [^{3}H]PGF$_{2\alpha}$ anti-PGF$_{2\alpha}$ binding by PGF$_{2\alpha}$, PGF$_{1\alpha}$, PGE$_1$, PGE$_2$, PGE$_3$ and PGE$_2$ after treatment with sodium borohydride. (Reproduced from Levine (15) with permission. Copyright 1973, The Williams & Wilkins Co., Baltimore.)



The amounts of PGE$_2$ produced by HSDM$_1$ cells are given in Table 2. HSDM$_1$C$_1$ cells produce PGE$_2$ during both the logarithmic and early plateau phases of the growth cycle (Fig. 4). The synthesis of prostaglandins by HSDM$_1$C$_1$ cells was inhibited by indomethacin, aspirin, and 5,8,11,14-eicosatetraynoic acid (1, 4). Indomethacin is a potent inhibitor, causing a 50% reduction in the synthesis of PGE$_2$ at a concentration of 1 ng/ml (3×10^{-9} M) (Fig. 5). The effects of eicosatetraynoic acid are shown in Table 3. These results demonstrate that prostaglandin synthesis can be inhibited in HSDM$_1$ cells by agents which do not produce general toxic effects on the cells as measured by the rate of cell growth, total cell protein, and protein synthesis (data not shown). It, therefore, was of major interest to examine the effects of inhibitors of prostaglandin synthesis on the production

TABLE 2. Prostaglandin production by cells in culture

Cell type	Medium	Production of PGE$_2$,[a] (µg/mg cell protein per 24 hours)
HSDM$_1$	Complete F10	1.12
HSDM$_1$	Complete F10 (ether soluble)[b]	1.16
HSDM$_1$	F10, no serum[c]	0.25
HSDM$_1$	F10, no serum (ether soluble)[b]	0.22
HSDM$_1$C$_1$[d]	Complete F10	2.04
HSDM$_1$C$_2$	Complete F10	1.28
HSDM$_1$C$_3$	Complete F10	2.00
HSDM$_1$C$_4$	Complete F10	0.60
HSDM$_1$C$_5$	Complete F10	0.72
Mouse fibroblasts (C1-1D)	Complete F10	<0.03
Mouse neuroblastoma (N1A$_3$)	Complete F10	<0.01
Rat glial (C$_6$)	Complete F10	<0.01
HeLa	Complete F10	<0.01
Mouse adrenal (AT$_{1P}$)	Complete F10	<0.01

[a] Medium samples were 3-day collections that were assayed after alkaline treatment. [b] Medium was extracted with ether and the reconstituted ether extract was assayed. [c] Cells were grown in complete F10 medium. Cells were washed twice with medium lacking serum, and then were maintained for 3 days in F10 medium without serum. [d] Five clones of HSDM$_1$ cells (C$_1$ through C$_5$).

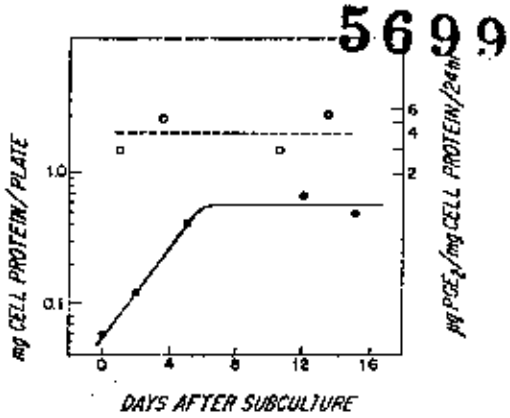


Figure 4. Production of PGE$_2$ by HSDM$_1$C$_1$ cells. Cells were plated in replicate dishes 12 hours before the start of the experiment. At zero time fresh medium was added to each dish. Every 2–4 days medium was collected for prostaglandin assay and fresh medium was added. At each collection duplicate dishes were washed and collected for cell protein determination. (Reproduced from Levine et al. (14) with permission of Academic Press, Inc.)

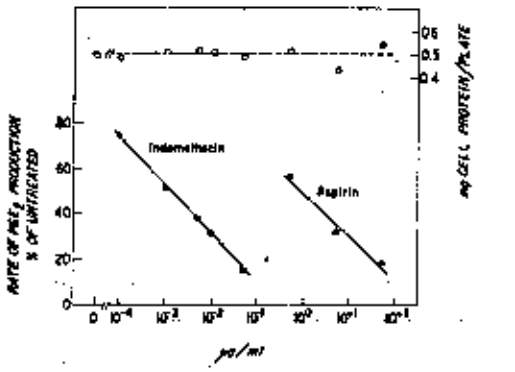


Figure 5. Inhibition of PGE$_2$ synthesis by indomethacin and aspirin. Fresh medium containing either no additions or the indicated concentrations of indomethacin or aspirin was added to duplicate dishes. Three days later the medium was collected for prostaglandin assay and dishes were washed and frozen for cell protein determination. Cells from untreated control dishes produced 1.94 µg PGE$_2$/mg cell protein per 24 hours. Results for dishes treated with indomethacin and aspirin are expressed as percentages of this control value. It should be noted that at the dose levels used neither indomethacin nor aspirin affected total cell protein. (Reproduced from Levine et al. (14) with permission of Academic Press, Inc.)

TABLE 3. Inhibition by 5, 8, 11, 14-eicosatetraynoic acid of PGE_2 synthesis by $HSDM_1C_1$ cells in culture

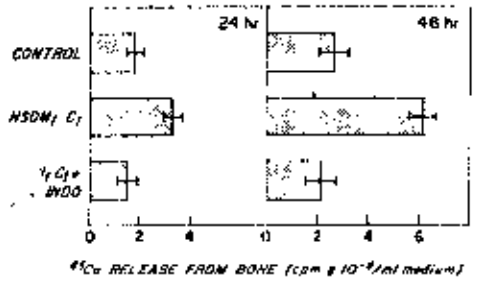
Treatment	Dose, μg/ml	PGE_2, ng/mg cell protein per 24 hours	% inhibition
None		152	
Eicosatetraynoic acid	10	7.5	95
Eicosatetraynoic acid	1.0	22	86
Eicosatetraynoic acid	0.10	90	40
Eicosatetraynoic acid	0.01	150	0
Eicosatetraynoic acid	0.001	150	0

of the bone resorption-stimulating factor by $HSDM_1$ cells.

Figure 6 shows that medium from $HSDM_1C_1$ cells stimulates the release of ^{45}Ca from mouse bone in organ culture and that, if the cells are grown in the presence of indomethacin, there was no stimulation of bone resorption. The effect of indomethacin is not to antagonize the action of PGE_2 or the $HSDM_1$ factor in the bone culture system. Figure 7 shows that indomethacin does not affect the basal release of ^{45}Ca from bone, nor does it inhibit the resorption stimulated by added PGE_2. Likewise, indomethacin does not cause the $HSDM_1$ cells to produce an inhibitor of bone resorption, for when medium from $HSDM_1C_1$ cells treated with indomethacin is supplemented with added PGE_2 the expected stimulation of ^{45}Ca release is observed (Fig. 8).

Figure 9 again shows that medium from $HSDM_1C_1$ cells stimulates the release of ^{45}Ca; it also shows that, if the cells are grown in the presence of eicosatetraynoic acid, there is a dose-related inhibition of the appearance of the bone resorption-stimulating factor in the cell culture medium. As with indomethacin (Fig. 7) eicosatetraynoic acid does not affect the basal release of ^{45}Ca from bone, nor does it inhibit the resorption stimulated by PGE_2 (Fig. 10). Furthermore, eicosatetraynoic acid does not inhibit the response of the calvaria to added $HSDM_1C_1$ medium (Fig. 11).

Figure 6. Effect of indomethacin on the production of bone resorption-stimulating activity by $HSDM_1$ cells. Cells were grown for 4 days in medium without or with indomethacin (100 ng/ml). The medium was extracted with ether and tested for 24 and 48 hours at dose levels equivalent to 0.10 ml of original medium for untreated cells and 0.20 ml for indomethacin-treated cells. Each bar gives the mean value of 4 bones ± SE.



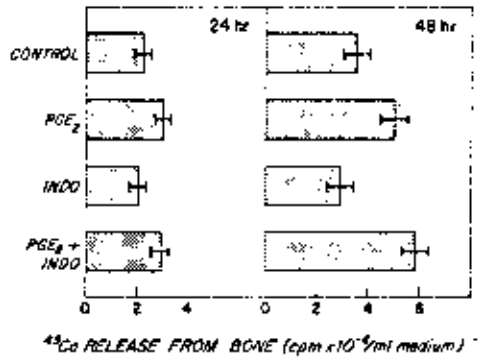


Figure 7. Effects of indomethacin on ^{45}Ca release from labeled bone. Labeled bones were cultured in control medium and in medium containing PGE_2 (100 ng/ml), indomethacin (500 ng/ml), and PGE_2 (100 ng/ml) plus indomethacin (500 ng/ml) for 24 and 48 hours. Each bar gives the mean value of 4 bones ± SE.

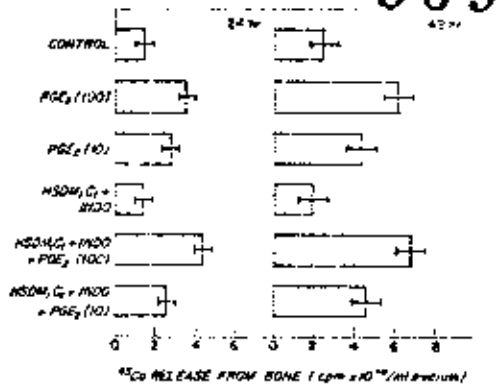


Figure 8. Demonstration that medium from indomethacin-treated $HSDM_1C_1$ cells does not inhibit the effect of PGE_2 on bone resorption. Cells were treated with indomethacin (100 ng/ml) for 3 days and the medium collected and extracted with ether for assay of bone resorption-stimulating activity either without or with added PGE_2 (100 or 10 ng/ml). $HSDM_1C_1$ medium was tested at a dose level equivalent to 0.10 ml of original medium. Each bar gives the mean value of 4 bones ± SE.

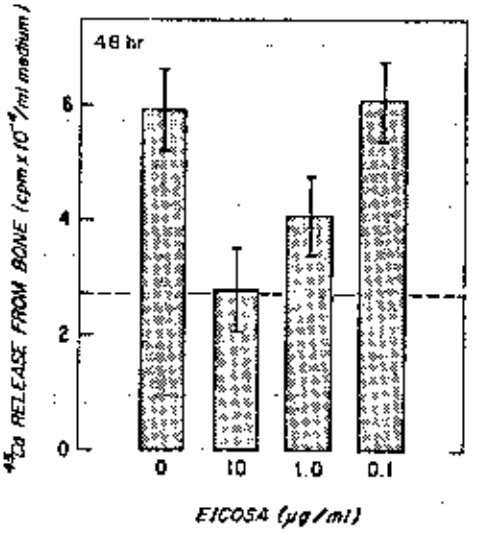


Figure 9. Effect of 5, 8, 11, 14-eicosatetraynoic acid (eicosa) on the production of bone resorption-stimulating activity by $HSDM_1C_1$ cells. Cells were grown for 4 days in medium without (zero) or with eicosatetraynoic acid at the dose levels shown. There were no effects of eicosatetraynoic acid at these dose levels on total cell protein. The medium was extracted with ether and tested for 48 hours at dose levels equivalent to 0.10 ml of original medium for untreated cells and 0.20 ml for eicosatetraynoic acid-treated cells. Each bar gives the mean value of 4 bones ± SE. The dashed line gives ^{45}Ca release in control bone cultures.

Studies in tumor-bearing animals

The above results obtained with the $HSDM_1$ cell culture system reveal that inhibition of PGE_2 synthesis was associated with the loss of appearance of the bone resorption-stimulating factor in the cell culture medium, a finding consistent with the hypothesis that the $HSDM_1$ factor is PGE_2. These in vitro data suggested to us that it would be feasible to extend these observations to the intact animal model (21).

Table 4 shows that mice bearing the $HSDM_1$ tumor have elevated concentrations of calcium in serum. These findings have been extended to include eight consecutive experiments in which the serum calcium concentrations were higher in tumor-bearing mice than in control mice (mean difference was + 0.76 ± 0.08 mg/100 ml, $P < 0.001$).

Mice bearing the $HSDM_1$ tumor also had elevated concentrations of PGE_2 in serum (Table 5).

These results show that tumor-bearing mice have elevated concentrations of both calcium and PGE_2 in serum. To demonstrate a causal relationship between the elevated PGE_2 levels and the hypercalcemia, we took advantage of the knowledge gained from the cell culture experiments and examined the effects of the

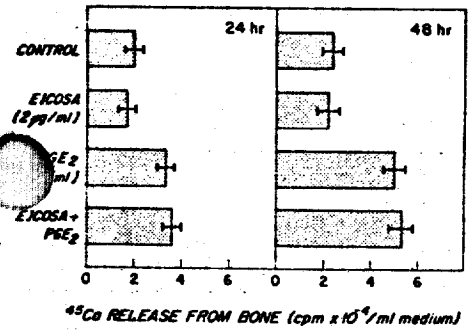


Figure 10. Effects of 5,8,11,14-eicosatetraynoic acid (eicosa) on ^{45}Ca release from labeled bone. Labeled bones were cultured in control medium or in medium containing PGE_2 (50 ng/ml), eicosa alone (2 μg/ml), or PGE_2 (50 ng/ml) plus eicosa (2 μg/ml) for 24 and 48 hours. Each bar gives the mean value of 4 bones ± SE.

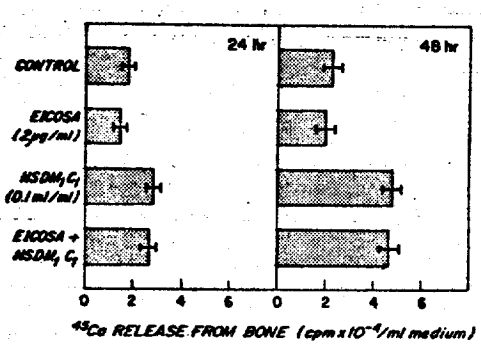


Figure 11. Demonstration that 5, 8, 11, 14-eicosatetraynoic acid (eicosa) does not inhibit the effect of $HSDM_1C_1$ medium on bone resorption. Labeled bones were cultured in control medium or medium containing eicosa (2 μg/ml), $HSDM_1C_1$ medium (dose equivalent to 0.10 ml/ml bone culture medium), or eicosa plus $HSDM_1C_1$ medium, at the same concentrations used above, for 24 and 48 hours. Each bar gives the mean value of 4 bones ± SE.

TABLE 4. Serum calcium concentrations in control and $HSDM_1$ tumor-bearing mice

Expt no.	Treatment	No. of mice	Serum calcium[a] (mg/100 ml)
I	Control	22	9.6 ± 0.10
	Tumor-bearing[b]	24	10.3 ± 0.10
II	Control	23	9.8 ± 0.08
	Tumor-bearing[b]	35	11.1 ± 0.16

[a] Mean values ± SE. [b] Animals were bled 18–24 days after inoculation of tumor cells.

administration of indomethacin to tumor-bearing mice. In these experiments, treatment with indomethacin was begun on the day of the tumor transfer or within 48 hrs, and the time from transfer until termination of the experiments was 19–23 days. Indomethacin was administered orally in an average dose of 100–125 μg/mouse per day. The drug was incorporated in a known amount of ground diet which the mice consumed completely each 24 hours.

TABLE 5. Concentrations of PGE_2 in the serum of control and $HSDM_1$ tumor-bearing mice

Expt no.	Treatment	Serum PGE_2, pg/ml
I[a]	Control	245 ± 35
II[b]	Control	185
	Tumor-bearing 1	680
	Tumor-bearing 2	520
	Tumor-bearing 3	500
	Tumor-bearing 4	500
III[b]	Control	275
	Tumor-bearing 1	720
	Tumor-bearing 2	840

[a] Mean value ± SE; six separate pools of control sera, each pool derived from 5–10 mice. [b] Each value is the mean of duplicate determinations of a separate pool of serum from 6–20 control or tumor-bearing mice.

The data from a single experiment are presented in Table 6. The increase in serum calcium concentration in tumor-bearing mice was accompanied by an increase in serum PGE_2 levels, and tumor contents of PGE_2 and bone resorption-stimulating activity were high. Administration of indomethacin caused a reduction in serum calcium and PGE_2 concentrations compared to those measured in control mice treated with indomethacin alone. There was also a marked reduction in indomethacin-treated mice of tumor PGE_2 content and of bone resorption-stimulating activity. In four additional experiments, administration of indomethacin to tumor-bearing mice reduced the elevated serum calcium concentrations to control or near-control levels (mean difference was −0.50 ± 0.08 mg/100 ml, $P < 0.001$). Indomethacin administration to control mice (not bearing tumors) did not affect the serum calcium concentration (mean difference was + 0.20 ± 0.11 mg/100 ml, $P > 0.05$).

The relationship of the decreases in serum calcium and PGE_2 concentrations and of bone resorption-stimulating activity and PGE_2 contents in tumor tissue gave quantitative evidence to support the hypothesis that the bone resorption-stimulating factor is PGE_2. The strength of this quantitative relationship is summarized in Table 7. The effects of indomethacin treatment on PGE_2 concentrations in serum and tumor tissue and on bone resorption-stimulating activity are clearly evident. Furthermore, the quantitative similarity between the decrease in the bone resorption-stimulating activity and of PGE_2 content in the tumors makes

TABLE 7. Summary of tumor contents of bone resorption-stimulating activity and of PGE_2 concentrations in experimental animals

Exptl group	Tumor content of bone resorption-stimulating activity, cpm × 10^{-4}/mg fresh wt	Tumor content of PGE_2,[a] ng/g fresh wt	Serum PGE_2,[b] pg/ml
Control			270 ± 53
Indo. alone			117 ± 75
Tumor alone	24.1[c]	136 ± 27	516 ± 53
Tumor + indo.	0.86[d]	4.9 ± 1.8	242 ± 53
Ratio of tumor/tumor + indo.	28	28	

Indo. = indomethacin. [a] Mean values ± SE for 7 separate experiments. [b] Mean values ± SE for 6 separate experiments (except indo. alone, 3 experiments only). [c] Mean value for 5 separate experiments (total range of values was 7 to 36 cpm × 10^{-4}/mg fresh wt). [d] Mean value for 5 separate experiments (total range of values was 0.39 to 1.2 cpm × 10^{-4}/mg fresh wt).

TABLE 6. Serum calcium, concentrations of PGE_2 in serum and tumor extracts, and bone resorption-stimulating activity in tumor extracts from a single experiment

Exptl. group	Serum calcium,[a] mg/100 ml	Concentration of PGE_2[b]		Tumor content of bone resorption-stimulating activity,[c] cpm × 10^{-4}/mg fresh wt
		serum, pg/ml	tumor, ng/g fresh wt	
Control	9.9 ± 0.16	250		
Indo. alone	10.1 ± 0.17	120		
Tumor alone	10.8 ± 0.13	475	76	6.7 ± 0.70
Tumor + indo.	10.2 ± 0.15	140	2.3	0.39 ± 0.07

Indo. = indomethacin. [a] Mean values ± SE, 10–14 mice/group. [b] Mean values of duplicate determinations of pooled samples. [c] Mean values ± SE for ^{45}Ca released from labeled bone into culture medium.

TABLE 8 Summary of body weights and tumor weights

Expt group	No. of mice	Weight of mice,[a] g	Weight of tumors,[b] g
Control	58	26 ± 0.35	
Indo. alone	37	25 ± 0.44	
Tumor alone	83	29 ± 0.30	4.4 ± 0.24
Tumor + indo.	64	27 ± 0.34	3.2 ± 0.27

Indo. = indomethacin. [a] Mean values ± SE from 4 separate experiments. Tumor-bearing mice were heavier than control or indomethacin-treated mice ($P < 0.001$). Tumor-bearing mice treated with indomethacin weighed less than tumor-bearing mice alone ($P < 0.001$) but more than control mice or mice treated with indomethacin alone ($P < 0.05$). [b] Mean values ± SE from 5 separate experiments. Tumors in mice treated with indomethacin weighed less than tumors from untreated mice ($P < 0.001$).

it unlikely that the $HSDM_1$ tumor contains a bone resorption-stimulating factor other than PGE_2 and lends strong support for our conclusion that the $HSDM_1$ factor is PGE_2.

Finally, we wish to point out that the dose levels of indomethacin used (100–125 μg/mouse per day) did not have overt toxic effects on the mice as measured by rates of body weight gain (Table 8), but the tumors from indomethacin-treated mice were smaller than those from untreated mice (mean difference was -1.2 ± 0.36 g, $P < 0.001$, Table 8). This finding is of particular interest in relation to the report of Hammarström et al. (11), who described elevated levels of PGE_2 in cultures of BHK cells transformed with polyoma virus. If there is a relationship between high PGE_2 synthesis and lack of cellular growth control in certain neoplastic cells, the studies reported here with $HSDM_1$ cells form a useful background against which additional experiments with inhibitors of prostaglandin synthesis may be pursued in both the host animal and with the neoplastic cells in culture.

COMMENT

The results of the experiments summarized here demonstrate that the transplantable mouse fibrosarcoma, $HSDM_1$, produces large amounts of PGE_2 in cell culture as well as in the host animal. In the mouse, the tumor also produces a syndrome of hypercalcemia which bears a resemblance to certain of the unexplained humoral hypercalcemias of malignancy in man (16, 19). The hypercalcemia in the mouse is eliminated, at least temporarily, by treatment of the tumor-bearing animals with indomethacin, a powerful inhibitor of PGE_2 synthesis in the $HSDM_1$ tumor. These findings suggest that certain patients with malignant disease and hypercalcemia should be studied in such a way as to determine whether prostaglandins play a role in the etiology of the hypercalcemia. For if they do, then inhibitors of prostaglandin synthesis or antagonists of prostaglandin action might have beneficial effects against some of the metabolic abnormalities observed in these patients.

We are aware that attempts to induce hypercalcemia in rats (12) and dogs (2) by the acute administration of prostaglandins have been unsuccessful. This is not surprising in view of the known high rate of clearance of PGE_2 from the circulation. We presume, in $HSDM_1$ tumor-bearing mice, that the constant high rates of PGE_2 synthesis and secretion are sufficiently large so as to sustain a blood level, measured as 2–3 times normal, which is sufficiently high to produce the observed biological response. It is also possible that the effects might be produced by even higher concentrations of PGE_2 on local skeletal sites close to the tumor rather than systemically. In this regard, histological examination of the long bones closest to the site of tumor implantation has not shown metastatic tumor nor overtly greater evidence of bone resorption than noted in contralateral bones. FP

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A STUDY OF THE ACTION OF IRRADIATED ERGOSTEROL AND OF ITS RELATIONSHIP TO PARATHYROID FUNCTION

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Part I

In a preliminary report published last year the effects upon dogs of overdosage with irradiated ergosterol were described and their similarity to the effects of the parathyroid hormone were pointed out.[1] We employed this as a basis for the thesis that irradiated ergosterol acted through the intermediary of the parathyroid glands. We have since then amplified our experiments upon the actions of ergosterol and parathormone, and have obtained, we believe, additional evidence to offer in support of this explanation for the hypercalcæmia and other manifestations characteristic of overdosage with irradiated ergosterol.

Several observations made prior to our researches suggested the possibility of such an interaction of the two substances. It has been established, for instance, that infantile tetany is relieved by parathormone, and prevented, ameliorated or cured by vitamin D in the form either of cod liver oil or of artificially irradiated ergosterol. The similarity between the symptoms of the infantile condition, which has been recognized within recent years to result from vitamin D deficiency, and the tetany of parathyroidectomized animals is very close. Noel Paton[2] considered the two disturbances to be identical in origin. Block and Faber[3] were among the first to suggest that irradiation with ultra-violet light relieved the tetanic symptoms by stimulating parathyroid function. Grant and Gates[4] reported that hyperplasia of the parathyroids occurred in rabbits as a result of ultra-violet irradiation. Higgins and Sheard,[5] on the other hand, observed hyperplasia of the parathyroids followed by degeneration in chickens fed a diet deficient in vitamin D and deprived of sunlight. Swingle and Rinkhold[6] found that irradiation with a quartz mercury-vapour lamp ameliorated but did not abolish the effects of parathyroidectomy in dogs. Hess and Lewis[7] induced, by means of a calcium deficient diet, a low serum calcium and latent tetany in a monkey, and then gave large amounts of irradiated ergosterol by mouth. This raised the calcium to normal. The parathyroids were then removed, when the calcium fell again to the tetany level, and repeated doses of irradiated ergosterol failed to raise it. Similar results were obtained in parathyroidectomized dogs by Hess, Weinstock and Rivkin.[8] The obvious conclusion derived from these experiments was that the vitamin acted through the parathyroids. Later, however, the latter observers[9] appear to have reversed this decision, since they were able to elevate the serum calcium of dogs from which the parathyroids had been removed. The possible explanation of this conflict of results which suggests itself is that accessory parathyroid tissue existed in different amounts in the animals of the respective experiments. Jones[10] found that the administration of 20 c.c. of cod liver oil daily for some days before parathyroidectomy either prevented the onset of tetany or had a favourable influence upon its course. Cod liver oil if given only after operation failed to ameliorate the tetanic symptoms.

Brougher[11] reported in 1928 that animals given cod liver oil for a period before the removal of the parathyroids developed tetany after the lapse of a longer time (in 8.2 days) than control animals (in 2.5 days) that received no oil. Wade[12] published somewhat similar results. Greenwald and Gross[13] have criticized the results of the two preceding observers, and could observe little virtue in cod liver oil in protecting parathyroidectomized animals from tetanic symptoms. In fact they state that if their judgment had not been influenced by the results of others, so slight was the effect in their own experiments that they would have concluded that the vitamin was devoid of any anti-tetanic effect in their animals. Even enormous doses (300 mg.) of irradiated ergosterol, they report, were unable to influence the course of tetany. They believe, nevertheless, that their experiments furnish evidence of parathyroid stimulation, since there was a tendency for the serum calcium to rise under the influence of cod liver oil, while the excretion of calcium and phosphorus was increased. An increased excretion of these elements is considered by these observers to be characteristic of parathyroid action. The increased excretion of calcium in their animals, however, occurred practically entirely by the intestinal route, and increased excretion of calcium into the bowel as a result of parathormone administration could not be demonstrated by Stewart and Percival,[14] nor by Aub *et al.*,[15]

and the subsequent gradual decline which we have observed upon several occasions after a *single* dose had been given, suggest that the material acts through the stimulation of, or possibly by inducing actual hyperplasia in, some glandular tissue. This mode of action is also suggested by the long period for which the calcium curve is sustained when ergosterol is given daily for a time and then discontinued.

When repeated doses of irradiated ergosterol are given and very severe effects of overdosage are induced the calcium curve in most instances shows a pronounced drop some hours before death. This terminal decline in the curve was also remarked upon by Collip[25] and by Macleod and Taylor[25] after parathyroid overdosage.

Inorganic phosphorus of whole blood.—Estimations of blood phosphorus were made by the Briggs[20] method. During the early stages of parathormone administration the inorganic phosphorus is inconstant. Collip found it to be not far from normal; usually there was a slight drop for the first few hours followed by a period in which the value gradually rose. Toward the end of life, usually about the third or fourth day, there was a sharp upturn to the curve, which reached a remarkably high level (over 20 mg. per 100 c.c. of serum) shortly before death. This sharp ascent appeared about the same time that the terminal fall in the calcium curve occurred, so that the two curves crossed.

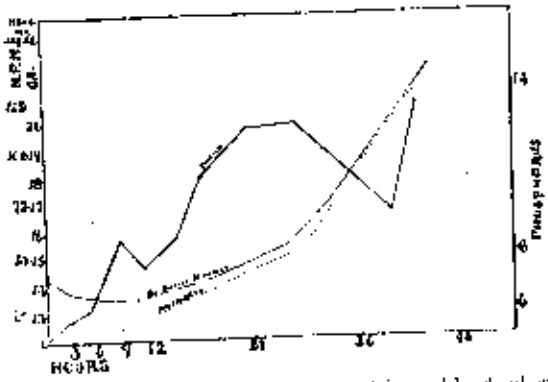

Chart II.—Curves of serum calcium, blood phosphorus and non-protein nitrogen reproduced from Collip's article describing the effects of parathyroid extract. The curve representing the non-protein nitrogen is not from the same animal as the curves of serum calcium and blood phosphorus.

Chart II reproduces typical calcium, phosphorus and non-protein nitrogen curves from an article of Collip's in *Medicine*.[25] These characteristic changes in the blood phosphorus value have been found by us in all cases in which death occurred from ergosterol poisoning and in which the phosphorus was estimated. Ten animals have been investigated in this way. In one adult dog the concentration of the inorganic phosphate was 26 mg. per 100 c.c., and in a puppy it was 15 mg. a few hours before death. Chart III

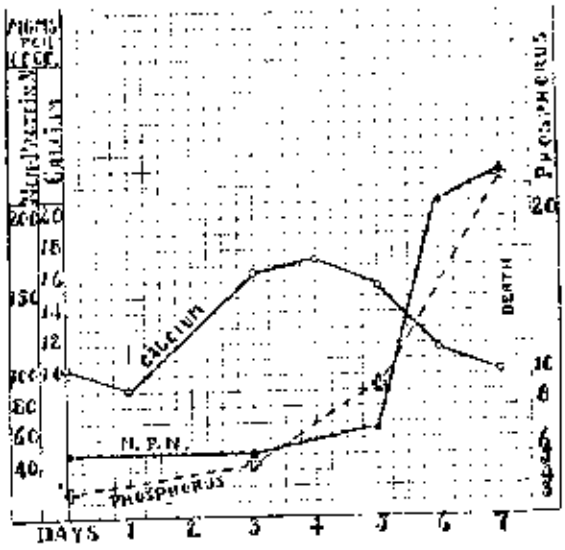

Chart III.—Curves of serum calcium, blood phosphorus and non-protein nitrogen from a dog receiving large doses of irradiated ergosterol. Compare these curves with those in Chart II.

shows the calcium, phosphorus and non-protein nitrogen curves characteristic of ergosterol poisoning. These are closely comparable with the curves in Chart II. The curves in the latter figure cover a period of about 42 hours, whereas those derived from the ergosterol experiments extend over a period of several days. This disparity in the time relations is simply a matter of dosage, or of individual susceptibility. Forty hours is an exceptionally short time for an animal to survive after overdosage with parathormone. Collip produced the effect by "pyramiding", that is, by repeating the dose at 3 hour intervals. A similar effect can be induced by irradiated ergosterol, though on account of the long latent period the survival time is usually a little longer with this substance than with parathormone. The real difference between the two calcium curves will not have escaped the notice of the reader. This is the small but very distinct initial fall in the calcium curve of Chart III. No such decline is seen in the corresponding curve in Chart II

This initial drop in the serum calcium which has already been remarked upon, we have found to be an almost invariable result of ergosterol administration. Such a dissimilarity in the effects of the two substances is not however a feature incompatible with our view that *excessive* doses of ergosterol stimulate parathyroid activity. The early depression of the serum calcium level is, we believe, the physiological action of the sterol which might be expected while small amounts only are coming into play.

The non-protein nitrogen.—This was found by Collip to be increased by from 200 to 400 per cent as a result of parathyroid overdosage. The rise in the non-protein nitrogen curve commenced about the same time as the rise in phosphate. The values of the two also reached their maxima practically simultaneously. We have found a very pronounced rise in the non-protein nitrogen toward the end of life, resulting from excessive doses of irradiated ergosterol in all animals in which this feature was investigated. In some instances the value rose to 200 mg. or more per 100 c.c. of blood. This represents an increase of about five times the normal. The blood-urea-nitrogen was increased proportionately. The urinary urea was found to fall decidedly in some animals at the time the blood urea was rising. In others, however, a reduction in the urea excretion did not occur. It is thought probable, therefore, that increased production as well as reduced elimination of this substance is responsible for its increase in the blood. In some instances there appeared to be a definite rise in the excretion of urea after ergosterol had been administered, but before the blood urea had risen.

The source of the excess serum calcium responsible for the hypercalcæmia.—The work of Hunter and Aub,[30] of Hunter[31] and of Bauer, Aub and Albright,[32] has established the fact that the cancellous tissue of the bones is an important, if not the chief, source of the excess serum calcium following parathormone administration. Hunter and Aub showed that in lead poisoning the metal is deposited in the bones in the form of a tertiary lead phosphate. Parathormone causes an increased concentration of both lead and calcium in the blood and the excretion of both these elements in the urine.* Bauer, Aub and Albright[32] have brought forward evidence for the view that the trabeculæ of the long bones in cats and rabbits serve as readily available reserves of calcium. Parathormone, it was shown, mobilizes these calcium stores and reduces the richness of trabeculation in the bones of rabbits. These observers were unable to induce hypercalcæmia in cats by means of the hormone, and in these animals decalcification of the bones was not observed after its use. On the contrary, Stewart and Percival[33] and Caven and Taylor[34] found independently that parathormone raised the serum calcium in cats within six hours after the entire gastro-intestinal tract had been removed. The latter observers gave 10 units repeated at half hour intervals for 4 doses. Removal of the gastro-intestinal tract alone, without the administration of the hormone, leaves the serum calcium unaffected. That the rise in the serum calcium after parathormone is derived, in part at least, from sources within the body, is proved by these experiments. Ninety-seven[11b] per cent of the calcium of the body is held by the skeleton. This fact, taken in conjunction with the work of Hunter and Aub and his associates, points to the bones as the most probable source of the greater part of the excess calcium in the serum which is found after parathyroid administration.

Convincing evidence can be cited for the belief that the hypercalcæmia of irradiated ergosterol overdosage is also dependent, to a large extent at any rate, upon the release of calcium from storehouses within the body. Taylor and Weld[34] found that when animals were placed upon a calcium deficient diet, and thyroid extract given to induce a negative calcium balance, hypercalcæmia was pronounced within six days after the commencement of ergosterol administration. In one such experiment the total quantity of calcium taken in the food plus thyroid over a six-day period was 51 mg., the calcium excreted during the same period in the urine and fæces was 713 mg., yet

* The effect of irradiated ergosterol upon the excretion of lead has been investigated in two animals (cats). They were each given 50 mg. of lead acetate daily for 10 days. The administration of the drug was then discontinued. Two weeks after the drug had been withdrawn, and when the daily excretion of lead in the urine had reached a steady state, maximal doses of irradiated ergosterol were given. A striking and prompt increase in the excretion of the metal resulted.

the serum calcium rose to 19.2 mg. from a normal of 10.7 mg. per 100 c.c. The estimated amount of serum in this animal was 300 c.c., so despite the fact that it had a negative balance of over 662 mg. some 25 mg. of excess calcium were derived from the body tissues to raise the level of this mineral in the blood. Subsequently it was found that irradiated ergosterol itself caused the excretion of such large amounts of calcium in the urine that it was unnecessary to give thyroid for the purpose of inducing a pronounced negative calcium balance. When animals were given a diet containing only a few milligrams of calcium, or were starved, a relatively enormous quantity of calcium was lost in the urine at the time that the calcium of the serum was 80 to 90 per cent above normal. Also it has been shown that the calcium of the blood can be raised by the intravenous administration of ergosterol after the gastro-intestinal tract has been removed.[34] Hess, Weinstock and Rivkin[35] also found that the serum calcium of young rats fed on a calcium deficient diet could be elevated from a subnormal to a normal level by ergosterol administration. Brown and Shohl[36] have shown that large doses of irradiated ergosterol cause a loss of calcium from the body and a reduction in the mineral constituents of the bones. The experiments of Ashford[37] lead to the same conclusion regarding the source of the excess calcium. Though ergosterol administration can raise the serum calcium independently of the food, the hypercalcæmic effect evidently is intensified by the presence of soluble calcium in the gastro-intestinal tract. Bills and Wirick[38] have shown this for rats, and Jones, Rapoport and Hodes[39] for dogs. The latter observers go further and state that ergosterol is unable to elevate the serum calcium in an animal placed upon a calcium free diet. They however used smaller doses than Taylor and Weld, and the experiments were of shorter duration. Furthermore, the excretion of calcium in the urine was not determined. Judging from our experience this certainly must have been increased. Consequently the failure of the serum calcium to rise to any significant height did not necessarily imply that excess calcium had not been mobilized from the tissues. We have found that irradiated ergosterol is certainly less effective in elevating the serum calcium of dogs upon a calcium deficient diet—about double the dose being required than when the diet is adequate in calcium. Harris,[40] from his results with high calcium diets, and Dixon and Hoyle[41] suggest that increased absorption is responsible for the hypercalcæmia.

All experiments with diets in which the calcium is in excess merely show that the calcium of the food accentuates the action of the sterol, but fail to prove that the calcium reserves of the body are not drawn upon or that the absorption of calcium is chiefly concerned in raising the serum calcium in animals upon a normal diet. The results of the calcium balance experiments cited above and the rise in the serum calcium after the administration of irradiated ergosterol to eviscerated animals furnish evidence of a more direct nature. Collip,[26] it will be recalled, found that ingested calcium also enhanced the hypercalcæmic effect of parathormone in dogs, yet there appears to be no doubt that mobilization of the tissue calcium is, to a large extent, responsible for the rise in serum calcium resulting from parathyroid excess.

That the bones are the source of the excess calcium after ergosterol overdosage is attested to by several independent observations. Decalcification of the bones by excessive doses has been observed by Kreitmair and Hinzelmann,[42] Baumgartner, King and Page,[43] and György.[44] The results of Brown and Shohl in this regard have been mentioned. Similar observations have been made recently by Branion and Kay.[45]

The source of the excess serum calcium is a question of more than passing interest, for, *if the calcium rise following ergosterol administration is the result of increased absorption of the mineral from the food, then hypercalcæmia may be looked upon as a favourable effect of this administration. If on the other hand the tissues of the body furnish the excess calcium the reverse may be true.* A rise in the serum calcium above the normal as a result of treatment with viosterol may then indicate excessive dosage and a deleterious action.

The excretion of calcium and phosphorus.—The excretion of calcium in the urine is greatly increased after parathormone administration, as has been shown by Hunter and Aub[30] for the human subject, and by Greenwald and Gross[46] for dogs. We have obtained similar results in dogs as a result of overdosage with the hormone.

Watchorn[46] observed a striking increase in the urinary calcium of rats as a result of *ergosterol* feeding. The fæcal calcium was reduced, though an increase over the normal occurred after the administration of ergosterol was discontinued. This shift of the calcium from the fæces to the urine by large doses of irradiated ergosterol was also shown for rats by Brown and Shohl.[36] Similar effects of the vitamin upon the urinary excretion of calcium have been reported by Ashford[37] for rabbits.

We have observed the urinary calcium of dogs to increase from a few milligrams in 24 hours before the administration of ergosterol to as much as 240 milligrams or more after hypercalcæmia had been induced. The fæcal calcium, according to Albright, Bauer, Ropes and Aub,[15] is not increased in the human subject after the administration of parathormone. Stewart and Percival,[14] and Taylor and Fine[10] were unable to demonstrate that parathormone caused an increase in the excretion of calcium into the intestinal lumen of cats. If anything there was a slight reduction. Greenwald and Gross,[45] on the other hand, reported an increased output of calcium in the fæces of dogs injected with parathormone. It need scarcely be pointed out, however, that the amount of calcium recovered from the fæces is simply a balance of excretion over absorption, and can give no sure measure of the absolute amount excreted. In the experiments of Stewart and Percival and of Taylor and Fine the method was such that it was possible to determine the excretion of the element independently of its absorption.* We have found that the fæcal calcium is reduced by ergosterol at the time that the urinary calcium rises.

Phosphorus.—The data concerning the excretion of phosphorus in ergosterol and parathyroid overdosage respectively in different species are not entirely unequivocal. The urinary phosphorus has been reported by Greenwald and Gross[45] to be increased in dogs after the administration of parathormone, and by Albright, Bauer, Ropes and Aub[15] in the human subject. In our experiments upon dogs an increase of 100 per cent in the urinary phosphorus has been observed under the influence of irradiated ergosterol. Urinary phosphorus was found to be increased in rabbits by Ashford[37] after ergosterol feeding. A similar effect was observed by Brown and Shohl[36] in rats. Watchorn,[46] on the other hand, and Hoyle and Buckland,[47] employing rats, found no significant change in the excretion of phosphorus by the kidney as a result of ergosterol adminstration. The latter observer, and Brown and Shohl, found the phosphorus of the fæces, like the calcium, to be reduced during ergosterol feeding. As was the case with the calcium of the fæces, Watchorn found an increased fæcal excretion of phosphorus in the period of recovery following ergosterol administration.

TABLE II

EFFECT OF OVERDOSAGE WITH PARATHYROID EXTRACT AND WITH IRRADIATED ERGOSTEROL ON THE PHOSPHATASE CONTENT OF THE TISSUES

Animals	*Irradiated ergosterol*			*"Parathormone"*		
	Plasma	*Bone*	*Kidney*	*Plasma*	*Bone*	*Bone*
Rats	Reduced*	Reduced	Reduced	Reduced	Reduced[51]	Reduced
Rabbits	Reduced[49]	Reduced[48, 49]	Reduced[49]	Apparently reduced[50]	–	–
Dogs	Reduced (Table III)	–	Reduced	Reduced; (Table III)	–	
Fowls	Reduced	Reduced	Reduced	Unchanged	–	

* When no numerical suffix appears, the findings are our own.

From the foregoing two paragraphs it may be seen that so far as the facts are known the effects of parathormone upon the urinary calcium resemble very closely those produced by excessive doses of irradiated ergosterol. The fæcal excretion of this element is apparently reduced by both irradiated ergosterol and parathormone. The urinary excretion of phosphorus is, in dogs at any rate, very strikingly increased by either agent.

* A number of cases of parathyroid enlargement in man have been reported,[31, 116, 120] in which decalcification of the bones (osteitis fibrosa cystica) and hypercalcæmia were the outstanding features. In this condition increased excretion of calcium occurs in the urine rather than in the fæces.

Tissue and plasma phosphatase.—It has been shown during the past eight years by a number of workers that there is an enzyme, phosphatase, present in several tissues in the body which will hydrolyse phosphoric esters, and is probably intimately concerned in calcium and phosphate metabolism. A method has recently been described by one of us[48] for measuring the phosphatase content of the blood plasma of man or other animals, and it has been shown that in cases of generalized bone disease the phosphatase value of the plasma is very much increased. It is of interest to collect findings of our own and of other workers to show the effect on the phosphatase content of certain tissues (plasma, bone, kidney) in several species of animal, of experimental overdosage with both parathyroid extract and irradiated ergosterol. (See Table II).

An example of the effect of administering excess of parathyroid extract and of irradiated ergosterol on the plasma phosphatase of the dog is shown in Table III.

TABLE III

Irradiated ergosterol			*"Parathormone"*		
Date	*Serum Ca.*	*Plasma phosphatase*	*Date*	*Serum Ca.*	*Plasma phosphatase*
6th	9.1	0.244	11th	9.4	0.095
7th	9.8	0.255	12th	9.8	0.109
Administration of ergosterol begun					
8th	9.2	0.239	15th		0.090
9th	9.25	0.189	Parathormone administr. begun		
			16th	9.95	0.075
10th	10.4	0.062	17th	11.4	0.067
12th	12.2	0.083	18th	15.9	0.056
13th	10.6	0.074	19th	14.4	0.055
14th	12.6	0.108	20th	16.7	0.037
15th	12.8	0.109			

Variations in volume and reaction of the urine.—In 1926 Taylor[52] reported that during the first 24 to 36 hours after the administration of parathormone to dogs the volume of the urine increased from 100 per cent to 200 per cent above the normal. The titratable acidity of the urine rose also at this time to double the normal value or more. A corresponding increase in the excretion of ammonia occurred. Brehme and György[43] reported in 1927 similar changes in the titratable acidity and ammonia of the urine as a result of parathormone administration. (Aub[54] was unable, nevertheless, to demonstrate any increase in the acidity of the urine of human subjects receiving parathyroid extract). During the initial outpouring of acid in the urine following the administration of parathormone the animal appears to be in good health and is in high spirits, though the serum calcium is rising and may have almost reached a maximal value. Soon, however, the urinary volume and titratable acidity fall to normal and then below, until finally in many instances only a few cubic centimetres of urine alkaline to phenolphthalein (pH 8.2) are passed in the twenty-four hours. This phase is accompanied by severe symptoms of overdosage and is terminated by the death of the animal.

We have found that these changes in the urine induced by parathormone are duplicated almost precisely by the administration of excessive doses of irradiated ergosterol to dogs. Hoyle and Buckland[47] reported the occurrence of diuresis in rats as a result of treatment with large doses of ergosterol, but stated that the phosphorus excretion was not increased. Watchorn,[46] on the other hand, states that in rabbits diuresis was not observed to be a sequel to the administration of irradiated ergosterol, nor did Ashford[37] in his experiments upon rats find that this substance induced any diuretic effect.

(To be continued)

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A STUDY OF THE ACTION OF IRRADIATED ERGOSTEROL AND OF ITS RELATIONSHIP TO PARATHYROID FUNCTION

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PART II*

THE EFFECTS OF PARATHORMONE AND IRRADIATED ERGOSTEROL RESPECTIVELY IN DIFFERENT ANIMAL SPECIES

PROBABLY the most remarkable resemblance between the actions of parathormone and irradiated ergosterol in excessive doses comes to light when the reactions of the two substances in different species are considered. Dogs and human subjects are the most susceptible of all to the influence of both these agents. In either species hypercalcæmia is induced with very great facility by parathormone. Maximal doses of irradiated ergosterol after a relatively long latent period raise the serum calcium in dogs with equal readiness. This substance in therapeutic doses induces a decided rise in the serum calcium of the human subject, and there is every reason to believe that dangerous hypercalcæmia would follow its administration in excessive doses. Hess and Lewis[7] reported the occurrence of hypercalcæmia in infants as the result of therapy with irradiated ergosterol. In one instance 5 mg. of activated ergosterol were given daily for 8 weeks to an infant 15 lbs. in weight. The serum calcium was elevated from 9 to 16.2 mg. per 100 c.c. This latter value was not reached until two weeks after the administration had been discontinued. The calcium then fell gradually, but was still above normal four weeks later. In another instance the serum calcium rose to 17.6 mg., and symptoms which we have found so characteristic of parathormone or ergosterol overdosage in dogs began to appear.

Cats are less responsive than dogs, but do react by a rise in calcium to large doses of parathormone. They also respond somewhat less readily than dogs to poisoning with irradiated ergosterol, but eventually succumb to excessive dosage in from four to six days. The gastro-intestinal tract shows features similar to, but much less intense than, those found in dogs. Deep flushing of the mucosa rather than actual hæmorrhage is the rule. The lungs are hæmorrhagic.

Rats can withstand relatively enormous doses of both parathormone and irradiated ergosterol. Bills and Wirick[16] found that 100 times the antirachitic dose of activated ergosterol was without any deleterious effect upon these animals, 1,000 times the prophylactic dose was just perceptibly harmful, 4,000 times definitely injurious, and 40,000 times overdosage was lethal. These doses were given daily over a period of weeks. Dixon and Hoyle[41] reported that rats fed upon a normal diet withstood massive doses of the sterol. Calculi were observed in the urinary tract, but none of the pathological changes described by German workers. Rosenheim and Webster[55] concluded that at least 10,000 times the prophylactic dose was not lethal to rats. Light, Miller and Frey[56] found the lethal dose to be 100,000 times the prophylactic requirement. Harris and Moore[112] gave somewhat similar figures.

With regard to the action of parathormone in these animals, Bauer *et al.*[32] found that 875 units administered over a period of 110 days were insufficient to raise the serum calcium of rats. This is more than double the dose required to elevate the serum calcium of a 10 kilo dog. Calculating, then, on a weight basis a 300-gram rat is resistant to a dose at least 100 times that which will produce a definite

* For Part I see this journal, 1931, 24: 763.

response in a dog. Collip also had reported previously that rats were immune to the action of the hormone. Tweedy and Chandler,[57] however, state that a rise in serum calcium and death may result from the administration of parathormone to these animals. The doses which these observers used were colossal, and consequently their experiments in our opinion do not demonstrate a specific effect of the extract. Rats weighing 194 grams were given 10 c.c. of parathormone (Lilly) subcutaneously over a period of seventeen hours. Two out of the four of these treated animals died. The dose which was given is equal in bulk to the total blood volume of the animal and equivalent to the administration of half a litre (about 10,000 units) of the material to an average sized dog. It must be recalled that parathormone is a relatively crude extract of the parathyroid glands, having neither a pH nor osmotic pressure similar to that of the body fluids. It also must contain many substances other than the parathyroid hormone which in large amounts may be toxic. We have found that a rise in the serum calcium is a phenomenon associated with other states, *e.g.*, intestinal obstruction, severe skin injuries and adrenal deprivation, in which grave disturbances of an unknown and fatal nature occur. It must be clear then that such a high dosage of parathormone is quite unphysiological and a rise in serum calcium need not imply a specific action.

The great resistance of rats to parathormone might be only apparent, and due to more efficient excretion of the element in the urine. Two independent observations seem to eliminate this possibility. Bauer, Aub and Albright[32] found no reduction in the bone trabeculæ of rats subjected to prolonged treatment with parathyroid extract. The density of the bone, on the contrary, was definitely increased. Day[56] gave enormous daily doses to a group of young rats over a period of more than 30 days, and then estimated the calcium and phosphorus of their ashed bodies. The total amounts of these elements were the same in the treated as in the control rats, in other words, no loss of calcium or of phosphorus had occurred.

Mice were found by Macleod and Taylor[28] to be immune to parathyroid overdosage. Tentative experiments carried out by us suggest that the lethal dose of activated ergosterol is somewhat less than that for rats. Fourteen young mice (6 to 7 grams), fed upon a Steenbock antirachitic diet, were given 1 drop of irradiated ergosterol (10,000 D) daily for three days. Six of these animals died within a week from a hæmorrhagic condition of the lungs. The remainder survived and grew to adult size.

This result indicates that a *total* dose of about 15,000 times the prophylactic rat dose of irradiated ergosterol, given over a short period, is about the lethal dose for young mice. On a weight basis the daily dose was about double that found by Bills and Wirick to be lethal for young rats. Death of the mice however occurred in only a small fraction of the time that was required to kill the animals in the experiments of the former observers. We are unacquainted with any experiments that have determined the antirachitic dose of irradiated ergosterol for a mouse, and so are unable to determine the ratio between the prophylactic and lethal doses of the sterol for this species. The large bulk of parathyroid extract and its uncertain composition preclude the possibility of determining by direct experiment the lethal dose of the hormone for a mouse, since if, as is suggested, resistance to it and to ergosterol is of about the same order, it would require a relatively enormous amount of parathormone administered subcutaneously over a short period to cause death.

Rabbits.—The data available by which one can compare in rabbits the effects of parathyroid overdosage with those caused by excess of irradiated ergosterol are somewhat incomplete. It is undoubted, however, that, as compared with dogs, rabbits are resistant to both these substances. With regard to the action of the parathyroid hormone, it was thought at first that rabbits were quite unaffected by it. Collip found that these animals were practically immune. Macleod and Taylor[28] gave large doses to adult rabbits over a period of two weeks without observing any significant rise in serum calcium. Some of the animals died, but none of those symptoms so characteristic of overdosage in the dog preceded death. Davies, Dickens and Dodds[59] showed later that a certain rise in calcium occurred when relatively huge doses were given. This finding was confirmed by Collip, who states

that though "enormous" doses do cause hypercalcæmia, the signs of overdosage observed in the dog are absent. Moritz[60] has also remarked upon the high resistance which rabbits offer to the administration of parathormone.

The observations of Kreitmair and Moll[7] indicate that the rabbit possesses a fairly high resistance to irradiated ergosterol. They found that these animals survived for 10 days though at least 10,000 times the antirachitic rat dose was given daily. Though loss in weight and death may occur in rabbits after large doses of parathormone or of irradiated ergosterol no very definite symptoms which might afford a basis for comparison of the degree of resistance which these animals offer to the two agents follow the administration of either. Perhaps our most reliable criterion for gauging the effects of either substance is the influence upon the serum calcium level. To produce a rise in the serum calcium in rabbits, massive doses of parathormone are required. Similarly, it is only with difficulty that the serum calcium level of these animals may be raised by irradiated ergosterol. Smith and Elvove[62] obtained a rise in the serum calcium of 20 or 25 per cent after 30 doses of irradiated ergosterol, each of which had a potency of 10,000 rat units. Even with amounts 20 times greater than this (200,000 rat units) given for 30 doses a rise of only 40 per cent in the serum calcium was observed. When this amount had been given for 10 doses over a period of 2½ weeks the calcium rise was less than 7 per cent. These amounts, when the weights of the two species are taken into account, are approximately 10 times the dose that will cause the death of a dog within 4 days. Smith and Elvove state that repeated doses not exceeding 20,000 rat units are tolerated well by rabbits. Harris and Stewart[63] gave as much as 10 mg. of irradiated ergosterol over a period of six weeks, without obtaining a significant rise in the serum calcium, and without causing loss of weight in their animals. (Nevertheless, calcification of the tissues had occurred.) Ashford[37] gave similar amounts which were followed by a rise in serum calcium from 13.9 to 18.8 in one animal and from 15.2 to 21 in another after 49 and 27 days respectively. No mention was made of the potencies of the preparations of irradiated ergosterol which the latter observer and Harris and Stewart employed, and it is possible that the material used by Ashford had a much higher content of antirachitic vitamin than that employed by Harris and Stewart. The preparation of viosterol (10,000 D) which we have employed in our experiments was undoubtedly of a different potency again from the solid material used by any of these observers. It is difficult for this reason to know precisely the relative resistance of rabbits and dogs to irradiated ergosterol. There seems no doubt, however, that rabbits so far at any rate as hypercalcæmia is concerned, are, compared with dogs, refractory to its action. Our experiments indicate that the lethal dose for the rabbit is, weight for weight, about a twenty-fifth of that for the rat, as determined by Bills and Wirick.[38] The results of the latter observers upon the rat and of ourselves upon rabbits are closely comparable, since the same preparation of activated ergosterol was employed in both investigations. It should be pointed out that the above figure comparing the resistance of the two species applies to rabbits fed upon an oats diet. The constituents of the diet, particularly the Ca:P ratio, are known to have a profound influence upon the toxicity of irradiated ergosterol. Rabbits fed upon green food were found by us to be very much more resistant.

Guinea-pigs were found to be from twice to three times as resistant to the action of irradiated ergosterol as rabbits. Macleod and Taylor[28] found that this species was also highly resistant to the action of parathormone. They appear to be even more resistant to the hormone than rabbits. Bodansky, Blair and Jaffe[64] have found recently, however, that guinea-pigs are not entirely unresponsive, since mobilization of the skeletal stores of calcium and the development of "experimental osteitis fibrosa" follow when very large doses are administered to young animals.

Chickens.—Massengale and Nussmeier,[65] gave 2,000 times the antirachitic dose of irradiated ergosterol to chickens upon diets with low and high calcium-phosphorus ratios respectively. In the former instances no significant effect upon the serum calcium was induced, even though the doses were continued daily for five weeks. When 6 per cent $CaCO_3$ was added to the diet and the Ca:P ratio increased to 8, a

rise in the serum calcium was observed. The calcium rise, however, was not great and no injurious effects upon the animals were noted. Hypercalcæmia was not caused by ergosterol with any of the other diets employed. Hall and King[66] found the antirachitic dose for chickens to be much less than that determined by the former observers. One drop of viosterol (100 D) every other day was found to be sufficient. An experiment was carried out by us for the purpose of comparing the resistance of the fowl to parathormone* and irradiated ergosterol overdosage respectively. Twelve chickens (5 months old and weighing 1.5 kilos.) were divided into three groups of four. One-half c.c. of irradiated ergosterol (10,000 D) was given daily by mouth to group 1, and 0.5 c.c. parathormone subcutaneously each day to group II. The individuals of group III served as controls. The diet consisted of 1 part of yellow corn and 1 part of wheat, with 2 per cent oats. At the end of thirteen days no signs of overdosage were noticeable in any of the eight birds. The doses of both the irradiated ergosterol and the parathormone were then doubled. At the end of ten days' treatment with 1 c.c. of irradiated ergosterol and of parathormone to the respective groups of birds no appreciable effect was produced. One individual of each of these groups, as well as a normal bird, were then sacrificed for serum calcium and blood phosphorus estimations. No rise in the serum calcium of any of the birds was found. The value was actually considerably lower in the serum of the bird which had received the irradiated ergosterol.

The dosage of irradiated ergosterol and of parathormone to the remaining chickens was increased from this time on to 2 c.c. daily. At the end of a week all the birds were killed. The ergosterol and parathormone birds were found to have lost an equal but small proportion of their weights. The bones of neither group contained less calcium than did those of the controls. On the contrary, there was an increase of from 7 to 8 per cent in the bone-calcium of both the ergosterol and parathormone birds. It may be remarked in this connection that Bauer *et al.*[32] observed an increase in the trabeculæ of rats' bones treated with parathormone. There was no rise in the serum calcium of any of the treated birds. The sera of those receiving the ergosterol showed a definitely lower calcium content. The average values of the calcium in the sera of the normal, parathormone and ergosterol birds respectively are given in Table IV. There was no significant difference in the blood phosphorus between the treated animals and the controls.

TABLE IV

Animals	*Serum calcium*	*Serum phosphorus*
Normal	14.2	6.3
Irradiated ergosterol ...	11.8	6.9
Parathyroid extract	14.7	5.7

The ergosterol birds received daily during the final week 700,000 rat units, or about 25,000 times the dose that would be required for antirachitic purposes, yet a fall, rather than a rise, in the serum calcium occurred. The other group of fowls received daily during the same period 40 units of parathormone. This is double the quantity that will cause the death of a dog weighing 8 kilos or more. In another experiment still larger doses of ergosterol were given over a period of 7 weeks to a group of growing chickens. The serum calcium at the end of this time, though a little higher than that of the controls, was within the normal range (13.8). Daily doses of from 10 to 40 units of parathormone given to a group of fullgrown hens over a period of ten days produced no definite effect upon the serum calcium. These findings have led us to the conclusion that fowl when on a diet of which the Ca-P ratio is approximately normal are practically immune to parathormone and to the toxic effects of irradiated ergosterol. Kreitmair and Moll[61] came to a similar decision with regard to the latter substance. Hall and King[66] gave up to 8,000 times the daily antirachitic requirement to fowl over a period of 40 days. When the birds were sacrificed at the end of this time, decalcification of the bones was observed, accompanied by a diminution in the bone phosphatase, but beyond these no changes were found, either by gross or microscopical examination, which could be definitely ascribed to the treatment.

It will, we think, be agreed from the foregoing paragraphs that the effectiveness of parathormone in producing toxic manifestations in

* We wish to express our thanks for the supplies of parathormone which the Eli Lilly Co. have so generously given us for our several experiments.

TABLE V

LETHAL DOSES OF IRRADIATED ERGOSTEROL AND OF PARATHORMONE COMPARED IN SEVERAL DIFFERENT SPECIES

	Irradiated Ergosterol		*Parathormone*	
	Dose in rat units per 100 grams of animal*		*Units (Collip) per 100 grams of animal*	
Species	*Total*	*Daily*	*Total*	*Daily*
Rats	3,000,000 – 4,000,000	40,000	Resistance too high to determine toxic dose	
Mice (Young)	150,000 – 200,000	50,000 – 75,000†	Resistance too high to determine toxic dose	
Chickens (5 mos. old)	600,000 – 800,000	25,000	30	2.5
	causes no appreciable effect		causes no appreciable effect	
Guinea-pigs	150,000 – 200,000	8,000 – 10,000	100 – 200	5 – 10
Rabbits	40,000 – 60,000	3,000 – 5,000	100 or more	3 or more
Cats	8,000 – 10,000	600 – 1,200	1 – 2 causes hypercalcæmia; lethal dose unknown	
Dogs	6,000 – 8,000	400 – 1,000	0.5 – 1.0	0.1 – 0.2
Infants	†	†	†	†

* A standard rat unit is taken as the minimal daily amount (75 mg.) of a good average cod liver oil which will induce healing in a 60 gram rachitic rat in 10 days. The potency of a preparation of irradiated ergosterol is computed upon this basis. Thus irradiated ergosterol having a potency of 100 or 250 D has weight for weight 100 or 250 times respectively the antirachitic value of cod liver oil.

† The toxicity of irradiated ergosterol depends to a large extent upon the size of the daily dose as well as upon the total amount given. In the experiment which we have undertaken with this species a relatively large daily dose was given. The survival time was consequently probably shorter than if smaller doses had been given. The daily lethal dose is probably considerably less than the figure given here and the total lethal dose may be much greater.

TABLE VI

CURATIVE DOSES OF IRRADIATED ERGOSTEROL, COD LIVER OIL AND PARATHORMONE IN DIFFERENT SPECIES

	Irradiated Ergosterol	*Cod Liver Oil*	*Parathormone*
Species	*Rat units per 100 grams animal per day*	*Rat units per 100 grams animal per day*	*Units (Collip) per 100 grams animal per day*
Rats	1.3	1.7	†
Chickens	1 – 2	1 – 2	†
Dogs	†	1 – 2	0.03
Infants	Dose prescribed varies from 2 – 10	1 – 2	0.03

The values in both the foregoing tables are of necessity only approximate.

any species in which the action of the hormone has been investigated runs closely parallel with that of large doses of irradiated ergosterol. Nevertheless, it is, we believe, a significant fact that for those species in which this has been studied, namely, rats, chickens, dogs and the human subject, the antirachitic dose is of the same order of magnitude in all. See Tables V and VI.

In other words, whereas in the different species the physiological action of irradiated ergosterol appears to be induced by comparable amounts of this substance, the tolerance to excessive doses varies enormously. The latter exert a toxic effect upon the calcium metabolism of a given species in a manner and to a degree which is dependent upon a susceptibility common to the sterol and to parathormone. The main points of similarity between the actions of parathormone and irradiated ergosterol are given in Table VII.

TABLE VII

THE EFFECTS OF IRRADIATED ERGOSTEROL (LARGE DOSES) AND OF PARATHORMONE COMPARED

1. *Effect upon the course of tetany.*—Rapid and complete cure by a single dose of irradiated ergosterol (1 c.c., 10,000 D) or of parathormone (20 units).
2. *Symptoms.*—The psychic changes, lassitude, anorexia, vomiting, loss of weight, weakness, the passage of blood in the stools and vomitus and other symptoms are identical in the two conditions when the acute type of poisoning is induced by large doses.
3. *Duration of life with maximal doses.*—Usually about 24 hours longer with irradiated ergosterol.
4. *Post mortem.*—The macroscopic and histological findings are identical.
5. *Blood changes.*—Hypercalcæmia, rise in N.P.N., late rise in inorganic phosphate and terminal fall in calcium occur at the same time and are of the same magnitude in both conditions.

6. *Excretion of calcium and phosphorus.*—So far as the data are available both agents affect calcium and phosphorus excretion similarly.
7. *Source of the excess calcium.*—From within the body in both conditions and most probably from the bones.
8. *Phosphatase.*—Both agents cause a pronounced fall in plasma phosphatase.
9. *Urinary changes.*—Both substances affect the volume, titratable acidity, phosphorus and calcium in the same way.
10. *Species susceptibility.*—Those species resistant to parathormone overdosage are also resistant, to about the same degree, to overdosage with irradiated ergosterol.
11. *Calcium deposition in the soft tissues.*—Metastatic calcification results from the prolonged administration of either substance.[42, 61, 62, 63, 67, 68, 69, 116, 121]

THE ADMINISTRATION OF ERGOSTEROL BY DIFFERENT ROUTES

Intravenous.—In the great majority of our experiments ergosterol has been administered by mouth. Shortly after the intensely toxic effect of irradiated ergosterol was first observed and the remarkable similarity between the gastro-intestinal changes induced by this substance and by parathormone was recognized, the question naturally arose whether the haemorrhagic extravasations were due to a local action of the sterol upon the mucous membranes or, as with parathormone, they would follow upon parenteral administration. One c.c. doses of viosterol (10,000 D) were therefore injected daily into the saphenous vein of a large dog (No. 47). This animal developed the characteristic symptoms and died in five days. The typical pulmonary and gastro-intestinal signs of overdosage were found post mortem. Since then a sufficient number of animals have been injected with the material intravenously to demonstrate that it is at least just as effective when injected directly into the blood stream. We are unaware that the intravenous action of viosterol has been previously recorded.* Ten normal animals altogether have been given irradiated ergosterol intravenously. In all a pronounced rise in serum calcium followed, and in each of those to which a dose equivalent to the oral dosage was given death occurred at the usual time, and typical signs were found post mortem. In one pup of about three months of age death occurred from intussusception. When a single large dose, or a series of doses is given over a short period, the effect upon the serum calcium can be observed for several days. There is a gradual rise to a maximum and then a gentle decline to normal or below. The initial fall in the serum calcium has been remarked upon elsewhere. See Fig. 1.

Subcutaneous.—Perhaps the most interesting phenomenon associated with this mode of administration is the fact that irradiated ergosterol is much less effective, in so far as the hypercalcaemia and other signs of overdosage are concerned, when administered subcutaneously. The first adult animal which was treated by subcutaneous injection received 1 c.c. doses twice daily for 16 days before the signs of over dosage appeared and death occurred. The usual signs in the gastro-intestinal tract characteristic of overdosage were then found. In another experiment six pups of approximately 2½ months of age and weighing about 2 kilos were divided into three pairs. The first pair was given 0.5 c.c. of irradiated ergosterol daily by mouth. The second and third pairs were given 1 c.c. intramuscularly and subcutaneously respectively. Those receiving the material by mouth lost weight rapidly and died in 5 and 8 days respectively. The pair receiving 1 c.c. doses by subcutaneous injection survived for 12 and 18 days respectively. The reduced susceptibility of the latter animals was in reality greater than the lengthened survival time implies, since they were very late in showing any symptoms whatever of overdosage. Even after a week or more of treatment they appeared bright and lively, whereas the animals which received the substance by mouth showed the characteristic symptoms within 48 hours. Though the possibility was considered, there was no indication, nor is there any reason to believe, that the relative ineffectiveness of the material in these instances was the result simply of non-absorption. To test this point specifically 20 c.c. of corn oil were injected beneath the skin. The swelling resulting from the injection had completely disappeared within a few hours. The animals receiving the irradiated material in the muscles of the thigh also were more resistant than those which received it orally, but were no less so than those into which it was injected subcutaneously. A single maximal dose by mouth in many instances

* After this paper had been written our attention was drawn to the communication of Reed and Thacker[70] in which the toxic effects of the intravenous injection of ergosterol were reported.

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produces a greater effect upon the serum calcium than a series of such doses given subcutaneously or intramuscularly. The initial fall in the calcium curve was very pronounced in the animals receiving the doses by the subcutaneous and intramuscular routes. A drop of from 1 to 3 mg. occurred and the subnormal level persisted for from 2 to 3 days. In rabbits we have found that subcutaneous injections of irradiated ergosterol extending in some instances over a period of weeks are ineffective, whereas when the material is given by mouth, the symptoms of overdosage have appeared at the usual time. We have had no experience with the intramuscular administration of the sterol to these animals. Smith and Elvove have stated, however, that oral and intramuscular administration are equally effective. But, in examining the figures of these investigators, some indications of a greater effect upon the serum calcium by oral than by intramuscular administration were found when the dosage in both instances was comparatively small (0.5 to 2 mg.). The loss in weight was less when the intramuscular route was employed, though the total dose was larger. A rise in the serum calcium of from 20 to 25 per cent was caused by the administration of 0.5 mg. by mouth for 30 doses, whereas 1 mg. given intramuscularly over the same period was followed by a fall of from 3 to 16 per cent in the serum calcium. This depression in the serum calcium is suggestive in view of our own findings recorded in the first part of this paper, that the first effect of the administration of excessive doses of irradiated ergosterol is a lowering of serum calcium.

What is the explanation for the fall in the serum calcium? Does it indicate the passage of calcium from the blood into the solid tissues, *e.g.*, the bones? We believe this to be the most probable explanation. This view is consonant with several observations relating to the action of vitamin D in physiological dosage. Taylor and Weld[34] have found that irradiation of the body surface or the administration of small doses of the sterol invariably causes a decrease in serum calcium.

The relative refractoriness of animals to the subcutaneous administration of irradiated ergosterol is an observation which possibly is not without physiological significance. Natural foods are poorly supplied with the antirachitic vitamin. The latter is produced presumably *in vivo* as *in vitro*, from its precursor ergosterol, by the action of ultra-violet light. In the animal body this action can only take place in or upon the skin. Hence the latter is believed to be the main route by which the vitamin enters the body under natural conditions. It is perhaps not too speculative to suggest that any vitamin entering the body by the cutaneous route in unnecessarily large amounts as a result of over-irradiation of the body surface may be stored in the subcutaneous or intramuscular tissues. Or it may be that some mechanism exists in these situations whereby quantities of vitamin excessive to the body's needs are rendered inert. We have in the pigmentation of the skin a mechanism allied to that suggested which shields the deeper layers of the skin from injury by over-irradiation with ultra-violet light. The latter mechanism alone, however, could not protect against the absorption of ergosterol secreted and irradiated upon the surface of the skin or in the layers of the epidermis superficial to the pigment layer.

Injection.—We have found that irradiated ergosterol administered by this means acts the same way as by subcutaneous injection.

DISCUSSION

We have offered, we believe, substantial evidence for a close association between the action of *large* doses of irradiated ergosterol and parathyroid function. The nature of the association may possibly be other than that of one which our experiments have suggested, namely, a direct stimulation of parathyroid tissue by the vitamin.* We also admit that

* Our experiments furnish no evidence that vitamin D, or irradiated ergosterol, in *small doses* stimulate the parathyroids. It very well may be that parathyroid function comes into play as a compensatory mechanism to withdraw calcium from the bones and increase the excretion of calcium only when an abnormal amount of the vitamin enters the body. The evidence points to small doses of irradiated ergosterol having an action contrary to that of excessive doses. The former cause the deposition of calcium in the bones, a fall in the serum calcium and a positive calcium balance. The latter decalcifies the bones, induces hypercalcemia and a negative calcium balance. We can only compare the excessive dosage of ergosterol with large doses of parathormone. Little is known of the effect of small doses of the latter substance. Whether or not these can cause an increase in calcium retention and a deposition of this mineral in the bones is unknown. Whether the parathyroids are concerned in the etiology of rickets is also

Footnote continued on page 27

the final and direct proof of our contention has yet to be furnished. The fact that many animals possess accessory parathyroid tissue is the chief difficulty in the way of deciding the question by direct experiment. Though we have attempted to overcome this difficulty by making a free dissection of the neck tissues, the experiments are not as conclusive as we would desire. The duration of life has been greatly prolonged when the complete operation has been performed. Nevertheless, on account of the number of variables, age, diet, weight, etc., which must be controlled, the results of a large number of experiments would be required before a truly decisive conclusion could be arrived at.

The findings (a) that tetany is so very promptly relieved by irradiated ergosterol in an animal from which the thyroparathyroid apparatus alone has been removed, and (b) that an animal upon which the complete operation has been performed fails to respond to the treatment, provide, we think, important evidence that parathyroid tissue is concerned in the reaction of the body to ergosterol overdosage. The only explanation which we can offer for the development of any overdosage effects at all in the animals upon which the complete operation had been performed, but which did not develop tetany, is the same as for the fact that the serum calcium did not fall to the tetany level in these animals after the operation, namely, that parathyroid tissue exists in inaccessible regions, such as the thorax. Unfortunately, anatomical studies which might inform us of the extent to which accessory parathyroid tissue is possessed by various species have not been exhaustive. Reports of a number of investigations upon this question can however, be found in the literature.

To-day the view seems to be very commonly held that parathyroid function is served practically exclusively by those parathyroid glands found in close association with the thyroid. This appears to be far from the truth and the observations of those most conversant with this field of investigation are directly opposed to such a view. So general is the former conception, however, that the practice of invoking the possible existence of accessory parathyroid tissue in order to explain otherwise intractable experimental results is looked at askance. Yet the evidence at our disposal is sufficient for us to decide with certainty that the four discrete collections of parathyroid tissue generally known as the parathyroid glands are, in a large proportion of animals at any rate, merely a part of the parathyroid apparatus. Even of these glands, only one pair may have such a close relation with the thyroid that they are removed when the latter gland is ablated. The performance of a parathyroidectomy or even of a thyroparathyroidectomy and the study of the effects of this presumably complete operation appears to be a simple and clear cut experi-

FIG. 4.—Section from dog (No. T11) showing a parathyroid gland surrounded by thyroid tissue. We are indebted to Prof. E. A. Linell for taking this microphotograph.

a question which cannot be definitely answered. Erdheim,[73] it may be recalled, reported hyperplasia of the parathyroids in rats suffering from rickets. He also found defective calcification of the teeth and bones of these animals after parathyroidectomy. Iselin[72] observed osseous changes resembling rickets in parathyroidectomized rats. Erdheim's findings were criticized by Korenchevsky[73] on the ground that the vitamin content of the diet was unknown. The latter observer was unable to duplicate Erdheim's results but produced rickets in rats with diets deficient in vitamin D. Others since, however, have experienced great difficulty in producing rickets in these animals by deficiency in vitamin D alone, and there is no reason to believe that the diets used by Erdheim were otherwise rachitogenic. It may be mentioned that Pappenheimer and Minor[74] and Sitter[75] have described hyperplasia of the parathyroids in human rickets.

ment. Yet as a matter of fact, unless serial sections of the tissues of the neck and thorax be made at autopsy, any conclusion concerning parathyroid function which is based upon the assumption that all parathyroid tissue has been removed must be invalid, since it is never possible to know that active parathyroid tissue has not been left behind. In the dog it has been our experience that the parathyroids associated with the thyroid may be of almost microscopical size and unable to be detected at operation (they may be embedded in the thyroid; see Fig. 4), and the only way to remove these with certainty is to remove the latter gland. Even then, the lower pair may be aberrant and left in place. Conversely we have found that in the dog or cat it is utterly impossible to remove the thyroids alone and leave the minute upper pair of parathyroids, or the lower pair, if these are in close relationship with the thyroid, undisturbed. Sandström,[79] the discoverer of the parathyroids, believed that, though man possessed two pair of glands, cats and dogs had only one pair, since in all the individuals of the latter species which he had examined only the upper or internal pair were found in association with the thyroid. It will also be recalled that Gley,[17] who first demonstrated the physiological importance of the parathyroids, was able to furnish the final proof of this importance by showing that in the rabbit the lower pair of parathyroids lay below the thyroid lobes and often hidden from view by adjacent structures. Their presence was revealed in many instances only after a careful search had been made. Prior to Gley's work rabbits were believed to enjoy an immunity to tetany not possessed by other animals. Gley's predecessors in this field had formed this opinion from animals in which the lower pair of glands had not been removed.

Gley also reported finding tissue in the dog in excess of that represented by the four glands of Sandström. Vassale and Pianca[78] found accessory parathyroid tissue extending into the thorax. Forsyth,[79] who made very careful studies in a large number of human subjects and in various animal species, states that the parathyroids vary very widely in anatomical position and in number. One person may have 10 or 20 times the amount of parathyroid tissue possessed by another. In mammals generally, as well as in birds, these glands were found to occupy no constant position, but the superior pair of glands were more usually found in the expected situation. Forsyth after a searching histological examination of the structures of the neck found 1, 8 and 1 collections of parathyroid tissue, respectively, in three green monkeys. A fruit bat, on the other hand, possessed 10. He concludes that parathyroid tissue may be found anywhere from the jaw to the interior of the thorax. Mousseu[80] reported finding a parathyroid attached to the submaxillary gland. Collip[123] found one in the centre of a lymph gland near the angle of the jaw in a sheep. They have also been observed in lymphatic glands and in the thymus by Forsyth. In several of the animals of our series only two parathyroids could be found in the extirpated thyroids. In one animal none was found in the latter though a careful histological search was made. Greenwald and Gross[13] state that the majority of dogs possess accessory parathyroid tissue. Marine[81] observed parathyroid tissue in the thymus in 6 per cent of dogs. Harvier[82] found it so situated in 50 per cent of cats. Nicholas and Swingle[83] found accessory parathyroid tissue in the thymus in 35 per cent of cats, Shapiro and Jaffe[84] in 12 per cent after a routine examination of merely a single section removed from the thymus of each animal, and Farner and Klinger[85] discovered it in the thymus of practically every cat in which a serial section of the gland was made. The presence of parathyroid tissue was demonstrated in the thymus of all the rabbits (twenty) examined by Haberfeld and Schilder.[86] Cowdry[87] states that parathyroid tissue may be found lying against the œsophagus or in the thorax. From the close embryological association of the parathyroids and thymus it is not surprising that accessory parathyroid should be found so frequently in the latter situation. Even the lower pair of parathyroids may be in this situation in adult life, having become included and dragged down with the thymic anlagen. Klotz[88] discovered an accessory gland at the tracheal bifurcation, and in the neck of one dog 12 separate collections of parathyroid cells were found. Horsley[89] removed the thyroids in young monkeys and described convulsive seizures and other nervous manifestations which we know now were due to parathyroid deficiency. Forsyth, it has been mentioned, found in the green monkey the parathyroids associated with the thyroid to be very variable in number. Swale Vincent[90] states that, though he made a thorough search for parathyroid tissue in thyroidectomized monkeys, none was discoverable. These results are pertinent to Hess's experiment upon a monkey already referred to in which ergosterol, though effective as an anti-tetanic agent before operation, had no appreciable action after parathyroidectomy. Adami and Nicholls (quoting Freedman)[91] found in the human subject "a very wide variation both in the number of parathyroids in normal relationship to the larger gland (thyroid) and in the accessory parathyroids."

The variability in the development of tetany after parathyroidectomy is also best explained by the inconstancy of the parathyroids. We have found, as have many others, that it is never possible to predict the time of onset or the severity of tetany in dogs after thyroparathyroidectomy. Twenty-four, 48 or 72 hours may elapse before convulsions ensue. In other animals only mild symptoms appear, such as twitchings and stiffness of the hind limbs. Not uncommonly the animal remains quite normal. In cats the proportion which develops tetany is much smaller. In fact in our experience it is unusual to see severe tetany develop in these animals. The serum calcium shows as a rule a very moderate drop. Ed-

mands[92] removed four parathyroids from each dog in a series of nine. Three animals died within a week, one in 4 weeks and five survived. Adami and Nicholls[91] quote Shepherd, who had a large experience, as stating that he had never observed tetany to follow a complete thyroidectomy in the human subject, yet it was well known at that time that this accident did occur, and cases of post-operative tetany were being recorded in various parts of the world. It must also be admitted that in only a small proportion of the patients upon whom Kocher[93] and Reverdin[94] performed a complete thyroidectomy did tetanic seizures develop. The obvious conclusions to be drawn from these observations upon the development of tetany in animals and man is that the variability of the onset and severity of the condition depends upon the variable amounts of parathyroid tissue possessed by the different species and by different individuals of the same species. This variability which we refer to is quite independent of age, sex, diet or any other known difference.

The similarity between the effects of ergosterol overdosage and those of hyperparathyroidism, so far as these have been investigated and recounted in the present paper, is undoubtedly remarkably close, and this evidence alone makes it extremely probable that the irradiated sterol in large doses exerts its toxic action by a mechanism which calls parathyroid function into play. (Table VII). We are not unaware, however, that comparisons of the actions of two agents, no matter how closely parallel these actions may run, can lead to a fallacious conclusion. For instance, it is comprehensible that some single physiological reaction might be common to two quite independent and distinct substances. This reaction might then be the starting point for an inevitable train of disturbances. It might be argued on such grounds that the diverse effects resulting from overdosage were simply the result of the hypercalcæmia, and that mobilization of calcium from the bones was the key factor. It has been pointed out that irradiated ergosterol and parathormone have this latter action in common. Yet, though thyroid extract and acids,[15, 64, 95] cause decalcification of the bones, these agents do not cause the other manifestations characteristic of overdosage with parathormone or irradiated ergosterol.

On the other hand, Caven and Taylor[96] found a moderate hypercalcæmia, unaccounted for by blood concentration, after adrenalectomy. This has been confirmed by Rogoff and Stewart.[97] Adrenal deprivation in animals is also associated with gastro-intestinal lesions of a hæmorrhagic and ulcerative nature. Yet there is no evidence that the calcium rise in this condition has any causative relationship with the gastro-intestinal condition. Furthermore, in our experiments with ergosterol we have found that though hypercalcæmia is an index of overdosage and is always pronounced at some time during the course of a fatal case it should be looked upon as an associated phenomenon and not the primary one. In some instances the serum calcium has been very high for weeks and no gastro-intestinal changes have been found after death. In other animals, though the calcium has been high in the early stages of poisoning, it has been normal or not greatly elevated for some days before death. It has moreover been shown by others, Salveson,[98] Collip,[25] and Taylor and Fine,[16] that large quantities of calcium may be injected intravenously, and, after a transient hypercalcæmia, the serum calcium rapidly falls again to normal and no untoward effects are induced. It must be mentioned, however, that Collip has reported toxic effects simulating parathyroid overdosage by the combined injections of phosphorus (acid sodium phosphate) and calcium. The question then naturally arises are the manifestations of overdosage with irradiated ergosterol due to a combination of hypercalcæmia with hyperphosphatæmia. We cannot believe that this is the correct explanation. A rise in the inorganic phosphorus of the blood is preceded, often by several days and in the long period experiments by weeks, by symptoms of the gravest nature. The phosphate may be subnormal while diarrhœa, loss of weight, weakness and other signs of overdosage are pronounced.

In connection with the question of an interrelationship between vitamin D and the parathyroids it may be mentioned that fish, the only class of vertebrates which does not possess parathyroid glands,[87] are also exceptional in having relatively enormous supplies of vitamin D in their livers. This fact, in our opinion, is significant.

Another possibility to be considered concerning the association of the actions of the two substances is that irradiated ergosterol and parathormone act in common upon some endocrine or other organ. The greater susceptibility to ergosterol of normal animals as compared with those deprived of parathyroid tissue militates against this possibility.

Morgan and Garrison[18] observed that vitamin D rendered animals (dogs) more susceptible to overdosage with parathormone. Animals on vitamin D deficient diets were stated to be tolerant to parathormone administration. This has not been our experience, though it is well known that a certain proportion (Collip, Aub *et al.* and Moritz) of dogs for some unexplained reason are resistant and apparently immune to ordinary doses of the hormone. This is particularly true if the doses are small to start with and are gradually increased. As mentioned elsewhere, a similar tolerance to ergosterol overdosage has been observed in some animals.*

The results of Morgan and Garrison[18] would at first sight suggest that ergosterol and parathormone were complementary to one another in their actions in a manner similar perhaps to the interaction of an enzyme and its co-enzyme. We believe however that the apparent susceptibility to parathormone of dogs given irradiated ergosterol is simply the result of an additive effect of the two agents. From our experience with the actions of the two substances this appears to us to be the most probable explanation. An experiment which we performed supports this view. Two animals of approximately the same age and weight were given daily 0.5 c.c. of irradiated ergosterol and 20 units of parathormone respectively. To a third animal the same doses of the two agents, each on alternate days, were given. The survival period and the general development of the symptoms were not significantly different in this last animal from those observed in the other two.

In a communication appearing while our first paper was in preparation, Jones, Rapoport and Hodes[19] reported that when hypercalcæmia was produced by the administration of irradiated ergosterol for a period of two weeks, and parathyroidectomy then performed, there followed a milder type of tetany than that which developed in parathyroidectomized animals that had received no ergosterol. Irradiated ergosterol treatment when continued after operation sustained the serum calcium level above normal, and so long as this was so tetany did not recur. In animals to which irradiated ergosterol was given for the first time after operation the course of tetany was favourably influenced. The animals of the latter series received calcium in the form of milk, so it is not possible to decide that the vitamin had been the determining factor in the relief of the symptoms. When the administration of the sterol was continued in these parathyroidectomized animals, and the serum calcium determined over a period of several weeks, hypercalcæmia was observed. These investigators therefore have concluded that stimulation or hyperplasia of parathyroid tissue cannot be concerned in the action of irradiated ergosterol. This conclusion was supported by their observations upon the general symptoms of normal animals receiving excessive doses. They state that concentration of the blood, vomiting, diarrhœa, weakness, etc.—characteristic features of parathyroid overdosage—were not observed. The only explanation which we can offer for these results is that the doses of irradiated ergosterol employed by these observers were much smaller than those employed by us. Bauer and Marble[20] have also come to the decision that irradiated ergosterol does not act through the parathyroids. They found that the bone trabeculæ of cats when reduced in number by calcium starvation were regenerated to a greater extent later when calcium and ergosterol were fed together than when calcium was given alone. This implies that calcium deposition is favoured by the sterol, whereas parathormone is known to cause decalcification. In these experiments small doses (therapeutic) were given, and such an action of small doses is freely admitted. The action of large doses is however quite another matter. These undoubtedly have an opposite effect. In Fig. 5 is a diagram which illustrates our conception of the movement of

* We know of no experiments in which the administration of parathormone was continued for a long period after tolerance had become manifest. It would be interesting to see whether there would follow slow metabolic disturbances, similar in nature to those observed by us in certain dogs (Nos. 5 and 8) which showed a tolerance to irradiated ergosterol so far as the acute manifestations were concerned.

calcium under the influence of either parathormone or of large doses of irradiated ergosterol on the one hand and of small doses of the sterol on the other. The fall in the serum calcium observed by us after the administration of small doses to normal animals, the deposition of calcium in the bones, and the reduction

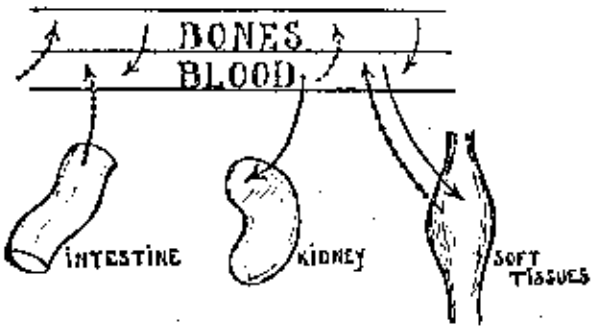

FIG. 5.—Diagram comparing the effects upon calcium metabolism of small doses of irradiated ergosterol with those of excessive doses of this substance or of parathormone.

→ = Small doses of irradiated ergosterol.
→ = Excessive doses of irradiated ergosterol or of parathormone.

in the negative calcium balance as rickets is healing,[99, 100] as well as the precipitation of tetany at this stage in the rachitic process, are among the observations which support our conception.* Ultra-violet light also converts a negative calcium balance in lactating animals to a positive.[104] In non-lactating animals on calcium deficient or on high calcium diets the value of a positive calcium balance is increased by irradiation.[105, 106] The action of excessive doses of irradiated ergosterol upon the calcium balances of normal dogs and the effect of irradiation with ultra-violet light upon the serum calcium have been mentioned.[34, 121] It is the reversal of the sterol's action leading to the withdrawal of calcium from the skeleton when given in excess which has been such a confusing feature in the study of this substance. Bauer and Marble speak of irradiated ergosterol "in therapeutic doses". This and "prophylactic doses" are terms in common usage. It is from these expressions that a very practical question arises. Surely the dosage at which calcium retention and the deposition of calcium in the bones are optimal should be a basis for the determination of the quantity to be given therapeutically. There is little evidence—and our experiments furnish none—to suggest that the parathyroids are stimulated by doses below this level. A dose above this would obviously be of less curative value, and still larger doses, even though not noticeably detrimental, would nevertheless produce an effect upon calcium metabolism which would be undesirable. The magnitude of the dose at which the reversal of action occurs is unknown. Experiments are in progress by which we hope this may be determined.

Beyond the studies of the investigators cited in the foregoing paragraphs there are few reports in the literature of the action of excessive doses of irradiated ergosterol upon dogs or cats, the species of laboratory animal which more closely resemble the human, in so far as calcium metabolism is concerned, than any other. Of those who have employed these animals, with one or two exceptions, none has observed that irradiated ergosterol had any particularly toxic action. Kreitmair and Moll[61] studied the toxic effects in a number of different species and reported upon dogs incidentally, stating that

* It might be expected that tetany would be relieved only when the dosage had reached the magnitude at which the parathormone-like action came into play. Our experiments indicate that this is so, and the failure of others to demonstrate that irradiated ergosterol rapidly cures tetany is probably for the reason that the dosage employed had not been sufficiently great. It may be recalled that several observations reported in the past suggest that small amounts of vitamin D are more likely, at first, to precipitate a latent tetany than to cure the manifest condition. Fluidchinsky[101] reported that ultra-violet light had this effect. Scheerman and Arntzenius[102] were among the first to point out that though in florid rickets there was a normal serum calcium value this might become lowered during the healing process. Bakwin, Bakwin and Gottschall[102] employed cod liver oil for the cure of tetany but found it ineffective relatively to irradiated ergosterol. In several of their patients the serum calcium level was either unaltered or actually lower after a week or more of treatment than before this was instituted. Large daily doses (4 mg.) of irradiated ergosterol on the other hand, abolished the muscular hyperexcitability within a few days, the serum calcium having risen above the tetanic level. Swingle and Rhinhold[6] employed ultra-violet light for the relief of tetany in parathyroidectomized dogs. An ameliorative effect, but not a permanent cure, followed this method of treatment. The serum calcium was not elevated and in some animals it was lower after than before irradiation. Gerstenberger, Hartman, Russell and Wilder[103] have reported the development of tetany as a result of ultra-violet irradiation, but lay emphasis upon the intermittent nature of the antirachitic treatment in the causation of spasmophilia. These observations taken in conjunction with data given in the present paper have furnished substantial aid toward the development of the idea that small doses of vitamin D, whether as irradiated ergosterol or as cod liver oil, cause an effect upon the movement of calcium in relation to the bones, blood and soft tissues, opposite to that of excessive doses.

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death occurred after excessive dosage. Cartland, Speir and Heyl,[107] experimenting upon two dogs, have nevertheless obtained results directly opposed to our own. They reported that a dose 1,000 times the antirachitic requirement for this species was innocuous.

Conclusions regarding the action of irradiated ergosterol have been drawn mainly from experiments upon rats or other species of rodents. These species have been found highly resistant to the toxic action of the sterol. They are, as we have shown, also practically immune, or at any rate so resistant to parathormone that the unit bulk of this material precludes the possibility of producing an effect by an amount which could be considered within physiological limits. *Dogs, on the other hand, react to parathormone in doses of the same magnitude as those which influence the calcium and phosphorus metabolism of the human subject.* Collip and Leitch[105] found that the dose of the hormone necessary to cure infantile tetany was of practically the same magnitude as that required to relieve the symptoms of parathyroidectomized dogs. Hoag and Rivkin[108] found that the number of units required to elevate the serum calcium of the human subject was essentially the same as that employed to raise the serum calcium in the dog. Hunter and Aub[30] observed a serum calcium of nearly 20 mg. per cent and a pronounced elevation of the phosphorus and non-protein nitrogen of the blood after the administration of 120 units of parathyroid extract and calcium lactate to a patient over a period of six days. Petty, Stoner and Schaffer[114] observed a very high serum calcium in adult human subjects who had received parathormone in doses comparable with those which cause hypercalcæmia in dogs. Nausea and vomiting were, in several instances, associated with the elevation of the serum calcium. Though the evidence which we have furnished in this regard is indirect, it has led us to more than a suspicion that irradiated ergosterol acts in the same way upon the calcium metabolism of the human subject as it does upon that of the dog, and that the toxic dose in the two species is comparable. Nor is direct evidence of this similarity of action and magnitude of dosage wanting. Hess and Lewis,[7] in 1928, recorded the following observations upon infants receiving irradiated ergosterol in "therapeutic" doses— "After this infant had received about 5 mg. of irradiated ergosterol for a period of a little over three weeks, it developed a moderate fever and began to lose weight; in the course of twelve days it lost 2 pounds (0.9 kg.). There was some vomiting, marked drowsiness and a *peculiar mental condition.** The calcium rose to 16.2 mg. per hundred cubic centimetres and persisted at a high level, although the phosphorus percentage remained about normal. The other instance which manifested abnormal symptoms was similar, in that there was loss of weight accompanied by fever and peculiar drowsiness, a clinical picture reminding one of dehydration. It is difficult to interpret this clinical phenomenon. . . . Possibly these children were peculiarly sensitive to the action of irradiated ergosterol. In fact, one of them began to develop similar symptoms for a second time. . . . In spite of the hypercalcæmia, the steady gain in weight continued until the twenty-fifth day, when the infant began to lose weight and vomited once. The calcium in the serum was found to be 17.6 mg. per hundred cubic centimetres." This description of the symptoms following the administration of large doses of irradiated ergosterol to infants might be used equally well to depict the early effects of overdosage which we have observed in dogs. Often the most striking of the early signs of overdosage with either ergosterol or parathormone are a strange apathy and a change in the behaviour of the animal which, though difficult to describe in a word, are quite definite and unmistakable to one who has seen them a few times.

The action of irradiated ergosterol has offered great opportunities for the study of certain problems in calcium metabolism and though our findings are mainly of physiological interest they are not without a practical bearing. They show that in a dosage which does not exceed so very much that which is used therapeutically irradiated ergosterol possesses great possibilities for harm. The harmful action, so far as clinical interest is concerned, has been obscured by the results of the administration of the sterol to insusceptible animals. The general tenor of the writings in the recent literature, with a few exceptions, is to the effect that irradiated ergo-

* The italics are ours.

sterol, except in relatively enormous doses, is harmless. To quote Dixon,[110] "Certain preparations of irradiated ergosterol given to rats which are being fed on a synthetic diet act as a poison, but if the rats are fed on bread and milk the effect of the ergosterol is negligible. In real life we do not live on a completely synthetic diet. . . . So the practical value of this type of experiment may be overestimated, though it is of considerable academical interest." Similar views have been recently (1929) expressed by Hess, Lewis and Rivkin.[111] "Too much", they say, "has been made of these studies." Light, Miller and Frey,[96] in view of their experiments upon rats, have suggested that for the infant "the harmful dose would be 1.5 litres per day of a solution of irradiated ergosterol in oil 100 times as potent as cod-liver oil."

Harris[10, 117] has also pointed out that the calcium phosphorus ratio of the diet is a most important factor in the development of toxic features in rats. He found that the effects were greatly intensified by adding calcium phosphate to the diet. Bills and Wiricke[18] have shown a similar susceptibility of rats fed calcium carbonate. Harris[117] would allay the fears of overdosage in these words "the quantity of vitamin D which proves toxic to a rat. . . . is some 100,000 times an adequate physiological dose. The toxic dose varies somewhat from one species to another but there should be little practical danger to humans of ill-effects from excessive vitamin intake." In our experiments the animals were upon a normal diet. Toxic effects, resulting in death, were induced in pups on relatively small doses—from 10 to 20 times the therapeutic dose per kilo—when a diet of whole milk and bread and adequate in the other vitamins was fed.* Death was also caused in adult dogs placed upon a calcium deficient diet, though as mentioned elsewhere, the symptoms were as a rule slower in their development when the calcium intake was low. On the other hand the observations of Hess, Poncher, Dale and Klein[113] upon the effects of excessive doses of irradiated ergosterol given to infants would appear to pronounce that clinically this substance is free from a harmful action. The daily amount of viosterol (100 D) which these investigators found it necessary to give for therapeutic purposes was in some instances as much as 60 drops. The amounts given for the purpose of determining whether or not irradiated ergosterol possessed a toxic action were equivalent to from 210 to 525 drops of 100 D, that is, from 3½ to 8¾ times the maximal therapeutic dose which these authors employed. The latter figure indicates a somewhat lower dosage than we have found to be lethal for young animals. In a very large proportion of those puppies of our experiments which succumbed in the early stages of treatment, that is before loss in weight and metabolic disturbances became very prominent features, intussusception of the bowel was found at autopsy. Julius Hess *et al.* have stated that in two-thirds of the infants to whom excessive doses were given, severe colic, often of a most intractable nature, was a prominent feature. These observations suggest to us that possibly the dose administered to these infants was not far from the danger line. Beyond the high incidence of severe colic there were no other significant features in the group of infants treated by large doses. Hypercalcæmia was absent. The gain in weight was continuous though decidedly less in the treated group, at least for the first three months, than in the control group.

SUMMARY

1. This paper gives a condensed account of a number of experiments from which fresh evidence has been derived for a close relationship between the overdosage effects of irradiated ergosterol and parathyroid function. This relationship is thought to be most probably, a direct one, namely, the stimulation of parathyroid tissue by the sterol.

2. Death of adult dogs follows the administration of irradiated ergosterol when the amount given per kilo has a greater potency than 20 times that of the maximal therapeutic dose. Puppies show a greater susceptibility to overdosage than full grown animals.

3. The symptoms and post-mortem findings in the blood following overdosage with irradiated ergosterol are indistinguishable from those resulting from the administration of lethal doses of parathormone. The chemistry of the

* The results of these experiments, which comprise a large number of animals, will be reported in detail in a subsequent communication.

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blood, in so far as this has been investigated, is affected in an almost identical manner by either substance.

4. The effects of excessive doses of irradiated ergosterol upon calcium and phosphorus metabolism run closely parallel with those resulting from parathyroid overdosage.

5. It is pointed out that those species which show a high resistance to the toxic action of irradiated ergosterol are tolerant to a corresponding degree to the action of parathormone. Since the dog and the human subject are, as contrasted with other species, highly susceptible to the hormone, it is suggested that the human subject may share with the dog a high susceptibility to irradiated ergosterol. Clinical observations are cited as direct evidence for the latter view.

6. Our experiments and those of others indicate that, upon increasing the dosage of irradiated ergosterol from small to very large amounts, its effect upon calcium metabolism becomes reversed, a parathormone-like action becoming manifest. The precise level of dosage at which the reversal of action occurs is unknown.

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Effects of dietary excesses of vitamins A and D on some constituents of the blood of chicks

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1. Groups of chicks were given diets containing four levels of vitamins A and D, 1, 10, 100 and 1000 times the basal level, in all sixteen combinations, with the object of investigating a possible antagonism between the two vitamins.

2. Diets containing 1000 times the basal level (approx. 1700 times the dietary requirements) of one or both vitamins depressed growth and induced changes in the blood.

3. The packed cell volume was substantially reduced from 4 weeks of age in the chicks given the highest level of vitamin A. It is suggested that this was due to an effect of the vitamin on the fragility of the red cells and thus on their life span.

4. Chicks given the toxic level of vitamin D showed an increase in plasma calcium and a decrease in plasma inorganic phosphorus. The highest level of vitamin A depressed plasma Ca without influencing plasma inorganic P. Increasing amounts of vitamin A given in combination with the highest level of vitamin D caused a progressive increase in the plasma inorganic P.

5. The highest level of dietary vitamin A significantly increased the activity in the plasma of three lysosomal enzymes: acid phosphatase, β-glucuronidase and arylsulphatase. Excess vitamin D given in combination with the basal level of vitamin A significantly depressed the plasma acid phosphatase and the activity of this enzyme increased with increasing amounts of vitamin A. Excess vitamin D had no influence on the other hydrolases studied.

6. A marked antagonism between the effects of excessive amounts of the two vitamins occurred only in respect of their actions on the plasma levels of Ca, inorganic P and acid phosphatase, all of which are involved in bone metabolism.

Both vitamin A and vitamin D are toxic when fed in excessive amounts. The pathological effects of excess vitamin A in chicks have been reported by Wolbach & Hegsted (1952) and by Pudelkiewicz, Webster, Olson & Matterson (1964). The most obvious clinical signs are a reduced growth rate, an encrustation of the eyelids and a reddening of the corners of the mouth. The eye and mouth lesions usually disappear at about 10 days of age and thereafter the only clinical manifestations of the toxicity are reduced growth and a general appearance of unthriftiness. Bone fractures, which are among the most striking effects of vitamin A toxicity in rats (Davies & Moore, 1931), do not occur in chicks.

Little work has been carried out on the effects of excess vitamin D in chicks but it is known that birds are far more resistent to vitamin D intoxication than mammals, in which the main lesions are calcareous deposits in the blood vessels and soft tissues, particularly the kidneys.

A number of biochemical effects of excess vitamin A have been attributed to the action of the vitamin on the membranes of cells and subcellular organelles, particularly the lysosomes. Treatment of lysosomes with vitamin A in vitro causes the release

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of their proteolytic enzymes (Dingle, 1961) and other hydrolases [illegible]le, Sharman & Moore, 1966) and the loss of mucopolysaccharides from cartilaginous limb-bone rudiments grown in organ culture in the presence of excess vitamin A (Dingle, Lucy & Fell, 1961) is probably due to the action of acid hydrolases liberated from the lysosomes by the vitamin. Experiments in vivo have shown that the liver lysosomes of rats treated with excess vitamin A are less stable than those of control animals (Dingle *et al.* 1966), but as far as we are aware no work has been published on the plasma activities of acid hydrolases in animals intoxicated with vitamin A, with the exception of that of Baker, Howell & Thompson (1967) who observed an increase in the plasma level of acid phosphatase in hypervitaminotic chicks. There are reports in the literature, reviewed by Nieman & Obbink (1954), that vitamin D may exert a protective influence against some of the harmful effects of excess vitamin A and that large amounts of vitamin A may prevent many of the pathological changes of hypervitaminosis D (Clark & Bassett, 1962). The object of the present experiments was to investigate the possibility of an antagonism between excesses of the two vitamins in chicks, using packed cell volume and the activities in the plasma of some acid hydrolases as measures of the toxic effects of vitamin A; plasma levels of calcium and inorganic phosphorus were used as measures of the severity of hypervitaminosis D.

EXPERIMENTAL

Animals and treatment

Chicks. Male chicks (Thornber '404' strain) were placed in electrically heated tier brooders and fed the experimental diets from 1 day of age. The treatments were randomized throughout the brooders. Food and water were supplied *ad lib.* and the chicks were weighed weekly.

Diets. The composition of the basal diet, in parts by weight, was as follows: maize 55, soya-bean meal 30, dried skim milk 4, dried yeast (unextracted) 5, dried grass 3, limestone flour 1, dicalcium phosphate 1·5, salt 0·5. The calculated Ca and P contents of this diet were 1·0 and 0·8% respectively. To each kg diet were added 200 mg manganese carbonate, 2600 i.u. retinol (Rovimix A325; Roche Products Ltd, Welwyn Garden City) and 500 i.u. cholecalciferol (dissolved in arachis oil). These basal additions represent the Agricultural Research Council (1963) estimates of requirements of chicks for vitamins A and D_3 plus a margin of safety of approximately 70%. Three levels of vitamin excess were fed, providing 10, 100 and 1000 times the basal level of each vitamin and these were added to the basal diet in a number of different combinations and in the same forms as in the basal diet. The four levels of vitamins A and D used were designated 1A, 10A, 100A and 1000A and 1D, 10D, 100D and 1000D, respectively.

Expt 1. This was a 4 × 4 factorial experiment with the four levels of vitamin A and four levels of vitamin D fed in all combinations with twelve chicks on each treatment. Two chicks from each treatment were bled at 4, 5 and 6 weeks of age, and packed cell volume and plasma levels of Ca, P and acid and alkaline phosphatases were determined. Total proteins were determined on the plasma of the 4-week-old chicks.

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Expt 2. The only changes observed in Expt 1 were at the highest levels of dietary vitamin additions (1000 times basal level) and the effects of these diets were studied in greater detail in Expt 2. The eight dietary treatments were: control (1A–1D), 1A–1000D, 10A–1000D, 100A–1000D, 1000A–1000D, 1000A–1D, 1000A–10D, and 1000A–100D. Once again there were twelve chicks on each treatment and blood samples were taken from three chicks from each treatment at 2, 3, 4 and 5 weeks of age. In addition to the determinations made in Expt 1, the activities in the plasma of β-glucuronidase, arylsulphatase and chitobiase (*N*-acetyl-β-glucosaminidase) were determined, although it was not always possible to carry out all enzyme assays on all samples of plasma.

Analytical methods

All determinations were made on heparinized blood obtained from the jugular vein or on plasma derived from it.

Packed cell volume (PCV). Blood was centrifuged in Wintrobe tubes at 3200 *g* for 30 min at 5°. The values obtained in this way were not corrected for occluded plasma.

Plasma total Ca. The method described by Hertelendy & Taylor (1961) was used.

Plasma inorganic P. The procedure of Allen (1940) was carried out on trichloroacetic acid filtrates of plasma.

Total plasma proteins. The biuret method was used.

Plasma enzymes. Enzymes were assayed by the following methods: alkaline phosphatase (*EC* 3.1.3.1), Bell (1960); acid phosphatase (*EC* 3.1.3.2), Bell & Siller (1962); β-glucuronidase (*EC* 3.2.1.31), Fishman, Springer & Brunetti (1948) as modified by Levvy & Marsh (1959); chitobiase (*N*-acetyl-β-glucosaminidase (*EC* 3.2.1.29)), Sellinger, Beaufay, Jacques, Doyen & de Duve (1960), using *p*-nitrophenyl 2-acetamido 2-deoxy-β-D-glucopyranoside as substrate; arylsulphatase (*EC* 3.1.6.1), Roy (1953, 1958), using acetate buffer at pH 4·8 (which we found to be optimum for chicken plasma) and 2-hydroxy-5-nitrophenyl sulphate (3 mM) as substrate: the incubation time was 14 h at 37°. Under these conditions most of the activity measured would have been due to arylsulphatase A (Bleszynski & Dzialoszynski, 1965).

RESULTS

Growth. The only treatments that significantly influenced the growth rate of the chicks in Expt 1 were those in which 1000 times the basal level of either vitamin A or D (or both) were fed, in which a marked depression of growth was observed. An example of this effect is shown in Table 1 in which the mean live weights of the chicks at 5 weeks of age are given. Analysis of variance of these results showed significant differences due to both vitamin A and vitamin D ($P < 0{\cdot}001$) and there was also a significant interaction between the two vitamins ($P < 0{\cdot}001$), reflecting the fact that the effect of a particular level of one vitamin varied according to the level of the other vitamin with which it was given. Thus, for example, the addition of 1000D to the diet containing the basal level of vitamin A significantly reduced the growth of the chicks, whereas it did not influence growth when added to the 1000A diet. It was not until the chicks were 2 weeks of age that a reduction in growth was noted in those groups

fed the highest level of vitamin A or D, which supports the view that the differences observed subsequently were treatment effects rather than effects due to position in the brooders. Depression in live-weight gain compared with controls was also observed in the experimental groups of chicks in Expt 2.

Table 1. *Expt 1: mean live weights (g) of chicks at 5 weeks of age given four levels of vitamin A and four levels of vitamin D*

(Means for ten chicks/treatment)

Level of vitamin A (× basal level)	Wt with level of vitamin D (× basal level) of: 1	10	100	1000	Mean wt
1	438	459	445	264	402
10	482	474	445	289	418
100	449	403	456	368	442
1000	366	239	276	278	275
Mean	419	416	401	300	—

SE of the difference between means in the body of the table = 24·2.
SE of the difference between vitamin A and vitamin D means = 12·1.

Table 2. *Expt 1: mean values for concentration (mg/100 ml) of plasma calcium for chicks 4, 5 and 6 weeks of age given four levels of vitamin A and four of vitamin D*

(Means for six chicks/treatment)

Level of vitamin A (× basal level)	Concentration with level of vitamin D (× basal level) of: 1	10	100	1000	Mean concentration
1	11·0	10·5	11·3	13·1	11·5
10	10·8	10·8	10·9	13·1	11·4
100	10·6	11·2	10·8	12·9	11·4
1000	9·6	10·0	10·3	10·7	10·2
Mean	10·5	10·7	10·8	12·5	—

SE of the difference between means in the body of the table = 0·57.
SE of the difference between vitamin A and vitamin D means = 0·29.

PCV. Results were obtained for chicks at 4, 5 and 6 weeks of age in Expt 1, and at 2, 4 and 5 weeks in Expt 2. In both experiments differences from control values were observed only in the birds fed 1000 times the basal level of vitamin A, in which a striking depression was observed from 4 weeks of age. Analysis of variance showed that this depression was significant ($P < 0{\cdot}001$). No depression was observed at 2 weeks of age but it is possible that it may have occurred by the time the chicks were 3 weeks old. (Unfortunately no results were obtained for this stage.) The statistical analysis showed that vitamin D did not influence PCV, and the mean values for all twenty-four chicks fed vitamin A at 1, 10, 100 and 1000 times the basal level in Expt 1 (birds 4, 5 and 6 weeks of age being grouped together) were, respectively, 28·8, 28·8, 27·9 and 21·8: the standard error of the difference between these means, obtained from the analysis of variance, was 0·60. Similar results were obtained in Expt 2.

Plasma Ca. [illegible]pt 1 dietary additions of 10 and 100 times the basal level of vitamin A or D or both did not influence the plasma Ca at any age. The highest level of vitamin A significantly reduced the plasma Ca below the control value for the three lowest levels of vitamin D, while the diets containing 1000 times the basal level of vitamin D had a marked hypercalcaemic effect except when 1000 times the basal level of vitamin A was also given (Table 2). Analysis of variance showed that the effects of both vitamins were significant ($P < 0.001$) and that the interaction between the vitamins was not significant. The mean level of plasma Ca in the chicks on the (1000A 1000D) treatment did not differ significantly from the control level. These results were confirmed in Expt 2, and it is clear that there was a marked antagonism between vitamin A and vitamin D when fed in toxic amounts in respect of their action on the level of plasma Ca.

Table 3. *Expt 1: mean values for concentration (mg/100 ml) of plasma inorganic phosphorus for chicks 4, 5 and 6 weeks of age given four levels of vitamin A and four of vitamin D*

(Means for six chicks/treatment)

Level of vitamin A (× basal level)	Concentration with level of vitamin D (× basal level) of: 1	10	100	1000	Mean concentration
1	7·6	7·5	8·0	3·8	6·7
10	7·6	8·2	7·9	4·6	7·1
100	8·2	8·4	8·5	5·4	7·6
1000	7·4	7·8	8·0	8·1	7·8
Mean	7·7	8·0	8·2	5·4	—

SE of the difference between means in the body of the table = 0·62.
SE of the difference between vitamin A and vitamin D means = 0·31.

Plasma inorganic P. Analysis of variance of the combined results for chicks 4, 5 and 6 weeks of age in Expt 1 (Table 3) showed that there were significant differences due to vitamin A ($P < 0.01$) and to vitamin D ($P < 0.001$) and that the interaction between the vitamins was also significant ($P < 0.001$). Thus the two highest levels of vitamin A significantly increased the plasma inorganic P and the highest level of vitamin D depressed it. Comparisons between subtreatments showed that the only groups of chicks in which significant changes from the control level occurred were those fed the highest concentration of vitamin D in combination with the three lowest levels of vitamin A, in which a reduction in the plasma P was observed. The mean concentration of inorganic P in the twelve groups of chicks at 4, 5 and 6 weeks of age fed the three lowest levels of vitamin D (a total of sixty-nine chicks) was 7·9 mg/100 ml and in the groups 1A–1000D, 10A–1000D, 100A–1000D and 1000A–1000D, the means were, respectively, 3·8, 4·6, 5·4 and 8·1 mg/100 ml. In these groups there was a roughly inverse relationship between the levels of plasma Ca and P, and the antagonism between the two vitamins was once again apparent.

Total plasma proteins. None of the dietary treatments influenced these values.

Plasma alkaline phosphatase. The level of activity of this enzyme was very variable and no consistent effects of any of the dietary treatments were observed.

Plasma acid phosphatase. Analysis of variance showed that the only treatments that had a significant influence on the activity of this enzyme were those in which one or both of the vitamins were fed at 1000 times the basal level. Excess vitamin A substantially increased the activity of this enzyme in the plasma and vitamin D had no significant effect at this level of vitamin A. Excess vitamin D added to a diet containing the basal level of vitamin A significantly depressed the activity of acid phosphatase below the control level ($P < 0.001$) and the activity increased as increasing amounts of vitamin A were added. These effects, which were not observed until the chicks were 4 weeks of age, are shown in Table 4, in which the mean activities of plasma acid phosphatase in chicks at 4, 5 and 6 weeks of age are given for the treatments common to both experiments.

Table 4. *Mean activities (i.u.) of plasma acid phosphatase in chicks 5 and 6 weeks of age in Expt 1 and 4 and 5 weeks of age in Expt 2*

(Means for nine or ten chicks/treatment)

Level of vitamin A (× basal level)	Activity with level of vitamin D (× basal level) of: 1	10	100	1000
1	28·1		—	16·9***
10	—		—	21·0‡
100	—	—	—	27·8‡
1000†	47·4***	48·4***	47·1***	44·9***

*** Significantly different ($P < 0.001$) from control (1A, 1D) means.
† No significant differences within these four treatments.
‡ Significantly different ($P < 0.001$) from 1000A means.

Statistical treatment was by analysis of variance after log transformation, using the pooled SE of the means for tests of significance.

Plasma arylsulphatase and β-glucuronidase. No effects of the dietary treatments were observed until the chicks were 4 weeks of age, and then only in those groups fed the highest level of vitamin A, in which a marked elevation of both enzymes was observed. Analysis of variance showed that there was no significant effect due to vitamin D. To show the influence of excess vitamin A, the mean activities of both enzymes for all chicks 4 and 5 weeks of age fed 1000 times the basal level of vitamin A (twenty-four samples) have been compared with those of chicks receiving the three lowest levels of vitamin A (twenty-one samples). These means, with their standard errors, for '1000A' and 'less than 1000A' respectively were: arylsulphatase, 0·83 ± 0·10 and 0·45 ± 0·04 i.u.; β-glucuronidase, 0·19 ± 0·01 and 0·09 ± 0·01 i.u. For both enzymes the differences were highly significant ($P < 0.001$).

Plasma chitobiase. The activity of this enzyme was remarkably constant with all treatments.

DISCUSSION

The biochemical responses to excessive dietary intakes of the two vitamins may be grouped into three categories: (1) those affected by both vitamins and in which there was a marked antagonism between their actions (plasma Ca, P and acid phosphatase), (2) those affected only by vitamin A (PCV, plasma arylsulphatase and β-glucuronidase),

lase), (3) negative responses (total plasma proteins, plasma alkaline phosphatase and chitobiase).

It appears therefore that the interactions between these vitamins are complex and not limited to a single mechanism.

An anaemia associated with a reduction in the red cell count is known to occur in rats suffering from hypervitaminosis A (Pouneau-Delille, 1943; Walker, Eylenburg & Moore, 1947). Baker *et al.* (1967), however, did not observe a consistent effect of hypervitaminosis A on PCV in chicks at 2–4 weeks of age, although they noted a fall at the age at which we first detected a marked depression in PCV (28 days). Papke (1937) suggested that the blood picture in rats suffering from hypervitaminosis A pointed to a hypochromic anaemia with hyperplastic bone marrow. The marrow of the chicks in this experiment was not examined, but Dr E. H. Hemstad, Royal Berkshire Hospital, Reading, kindly examined marrow smears from Japanese quail (*Coturnix*) fed a diet containing the same concentration of vitamin A as that present in the 1000A diet. These quail showed a reduction in PCV similar to that in the chicks suffering from hypervitaminosis A. Whereas control smears contained large amounts of fat and were dominated by late normoblasts and mature heterophils, those from the quail intoxicated with vitamin A showed only small amounts of fat and consisted mainly of early normoblasts and immature heterophils.

The reduced PCV in the chicks given the 1000A diets could have been the result of an increase in the plasma volume, but the reduction was too great to make this explanation appear likely and it seems more probable that it was due to a reduction in the number of circulating red cells. Dingle & Lucy (1962) have shown that erythrocytes suffer rapid haemolysis when incubated with retinol (10 μg/ml) in vitro at 37°, and it is suggested that excess vitamin A may have a similar effect in vivo. An increased fragility of the erythrocytes would be expected to reduce their life-span, to increase their rate of formation (indicated by hyperplasia of the marrow) and, if the rate of formation were unable to keep pace with the rate of destruction, to lead to a reduction in the number of circulating red cells and thus in the PCV. Vitamin D did not appear to influence this suggested course of events in these experiments.

The hypercalcaemic effect of excess vitamin D, one of its most characteristic actions, was shown in the chicks given the 1000D diet containing the three lowest levels of vitamin A. The hypocalcaemic effect of high levels of vitamin A, which we observed in the birds on the 1000A diets, has been reported recently in rats by Belanger & Clark (1967). The physiological interaction between the two vitamins when both were administered at the highest level, resulting in a restoration of the plasma Ca to the control level, was most striking and it was noted also by Belanger & Clark (1967); the mechanism of this interaction, however, is obscure.

A question that is provoked by the observation that diets high in vitamin A induce a hypocalcaemia in both rats and chicks is: 'Why does not the parathyroid restore the plasma Ca to normal in this condition?' Any answer to this question must inevitably be highly speculative, but it is possible that bone does not respond to parathyroid hormone in hypervitaminosis A, just as it fails to respond in vitamin D deficiency (Harrison, Harrison & Park, 1958).

The hypercalcaemia observed in animals fed toxic levels of vitamin D is thought to be due both to increased absorption of Ca from the gut and to an increase in bone resorption. In the rat it is accompanied by a decrease in the plasma level of inorganic P (Belanger & Clark, 1967), and this was also observed in the chicks on treatment 1A-1000D in the present experiment. The plasma inorganic P increased progressively when the concentration of vitamin A given together with the excess vitamin D increased and the mean level in the chicks on treatment 1000A-1000D did not differ significantly from the control (1A-1D) level (Table 3). Belanger & Clark (1967) observed a significant decrease in the level of plasma inorganic P in rats intoxicated with vitamin A, and a significant increase in those given excessive amounts of both vitamins, but neither of these effects was observed in the chicks in the present experiment.

In so far as the plasma levels of Ca and P reflect changes in bone metabolism it appears that excess vitamin D exerts a similar action in both rats and chicks, but that excess vitamin A has a different effect in the two species. Hypervitaminosis A is associated with bone fractures in rats but not in chicks and the biochemical differences reported here may be related to the different clinical findings.

The increase in the activity of three lysosomal acid hydrolases (acid phosphatase, β-glucuronidase and arylsulphatase) in the plasma of the chicks given the 1000A diets suggests that excess vitamin A causes the release of these enzymes from lysosomes in vivo just as it does in vitro (Dingle, 1961), but no information is available as to the precise tissue or tissues from which they were derived. It is surprising that there was no increase in the activity of chitobiase in the plasma of the 1000A chicks, as this enzyme has a similar distribution and is released under the same conditions as β-glucuronidase and arylsulphatase (Beaufay, Jacques, Baudhuin, Sellinger, Berthet & de Duve, 1964; Vaes, 1965; Bowers & de Duve, 1967). It is possible that it is readily inactivated in the general circulation or that it is rapidly excreted by the kidney. There is some support for the latter suggestion in the work of Morris (1967), who, in a study of the urinary excretion of enzymes in laying hens, found that approx. 1 l. plasma/24 h was cleared of chitobiase compared with 10 ml for β-glucuronidase and 400 ml for arylsulphatase.

Baker *et al.* (1967) reported a decrease in the level of plasma alkaline phosphatase in chicks treated with excess vitamin A, although there was no indication whether or not this decrease was statistically significant, whereas we were unable to observe any consistent treatment effects on the activity of this enzyme. Variations both within and between groups of chicks were extremely large.

It appears from these experiments that there is no general antagonism between excess vitamin A and excess vitamin D. There was, however, an antagonism in respect of the action of these vitamins on plasma Ca, P and acid phosphatase, i.e. on some of the substances involved in bone metabolism, and it seems probable therefore that the main source of this particular acid hydrolase was the skeleton. Both osteoclasts and mature osteocytes are rich in acid phosphatase and it may be suggested that vitamin A weakens the membranes of the lysosomes in these cells and that vitamin D tends to stabilize them.

nor by Taylor and Fine.[20] The urinary calcium, on the other hand, has been shown by Greenwald and Gross,[45] by Aub and his associates and by two of the present writers to be definitely increased by parathormone. Urechia and Popovicu,[17] as a result of their experiments upon dogs concluded that irradiated ergosterol was without value for the relief of parathyroid tetany. Morgan and Garrison[15] reported that vitamin D increased the susceptibility of dogs to overdosage with parathormone. Large doses of parathormone given to puppies caused only a slight rise in the serum calcium when the animals were upon a diet deficient in vitamin D, and symptoms of overdosage did not appear. These results, though indicating an interrelationship between vitamin D and the parathyroids, point to some association between the two other than the one which several of the observers cited above, and we ourselves, suggest. Jones, Rapoport and Hodes,[19] from their results, are also unable to subscribe to the view that the parathyroids are stimulated by vitamin D. A similar view is taken by Bauer and Marble.[20] The former authors failed to observe that irradiated ergosterol produced any symptoms resembling parathyroid overdosage. They have concluded that since hypercalcemia was caused by irradiated ergosterol given after a simple thyroparathyroidectomy, the parathyroid hormone could not be involved in the mechanism whereby this substance induced its effects upon calcium metabolism. The findings of these observers, as well as those of Morgan and Garrison, and Bauer and Marble, will be discussed in the latter part of this paper.

EXPERIMENTAL

Dogs were employed in the majority of our experiments, but the effects of irradiated ergosterol and parathyroid overdosage respectively have been studied also in cats, rats, rabbits, mice, guinea-pigs and chickens. The experiments were planned with three objects in view.

1. To determine the influence of irradiated ergosterol upon the course of so-called parathyroid tetany.

2. To investigate the action, if any, of this agent when administered in large doses to animals from which all parathyroid tissue, so far as was possible, had been removed.

3. To compare the effects upon normal animals of excessive doses of irradiated ergosterol with those resulting from parathyroid overdosage.

THE INFLUENCE OF IRRADIATED ERGOSTEROL UPON THE COURSE OF PARATHYROID TETANY

In these experiments the usual operation of thyroparathyroidectomy was performed. In the dog, on each side of the neck, at or a little below the level of the cricoid cartilage a separate thyroid gland is situated. Each of these with the parathyroids attached was removed. Not all the animals operated upon developed definite tetanic symptoms; in some, stiffness of the hind limbs alone was noticeable; in others, slight tremors or hyperexcitability of the muscles to tapping were the only signs. In all the animals some reduction in the serum calcium was observed, but in one there was a reduction of only 1 mg. per 100 c.c. It is generally assumed that in those animals which do not show definite tetanic manifestations the fall in the serum calcium to the level at which the symptoms appear is prevented by the liberation of a sufficient amount of hormone from accessory parathyroid tissue which the ordinary operation has not removed. This belief is supported by the well-known fact that if animals can be tided over the critical period after the operation by the administration of calcium they not infrequently survive though they receive little further treatment.

In several animals severe tetany developed. Muscular hypertonicity, clonic spasms, trismus or chattering of the teeth, tremors of the muscles, rapid breathing and salivation were prominent features. The calcium fell to the usual low level characteristic of the tetanic condition. In eight of these animals irradiated ergosterol was given by mouth when the disturbances were at their height. The animals were permitted to pass as far into the tetanic state as was possible without incurring the risk of their succumbing to the convulsions. If the jaws are tightly clamped together and salivation is profuse it is often very difficult to administer the ergosterol and ensure that it passes into the stomach and is not lost in the saliva drooling from the mouth. In only one of these eight animals did death occur as a result of the tetanic seizures. In this case the ergosterol had almost certainly been given too late, since the animal died of asphyxial spasm in three-quarters of an hour after the administration. The material had to be forced between the teeth, which could only be pried open a fraction of an inch. The salivation was very profuse. It is doubtful if the required amount of the material had reached the stomach, even had the animal lived long enough for this to have taken effect. In all cases treated with irradiated ergosterol there was, at the time the dose was given, complete prostration and utter inability to make any voluntary movement. Consciousness, however, was not completely lost.

In the first animal 1 c.c. of the irradiated ergosterol (10,000 D)* was given by mouth

* Kindly supplied to us by Mead Johnson & Co. We gratefully acknowledge their generosity.

the response was dramatic. Practically complete recovery occurred within one hour. At the end of this time the spasms and tremors of the muscles had entirely ceased. There was no salivation and the respirations were normal. The animal was able to walk about the cage and was apparently normal in every way. Some of the hypertonicity of the muscles could still be detected. After another hour this too had practically disappeared.

In another animal 1 c.c. of irradiated ergosterol (10,000 D) was given subcutaneously when the tetanic convulsions were violent and the animal as in the previous case lay prostrate upon the floor and completely incapacitated. An hour later there was no appreciable amelioration of the symptoms. One c.c. of irradiated ergosterol was then given by mouth. The symptoms were entirely relieved an hour and a half later. The relief of tetany in the other five animals was just as prompt and as striking when this amount of ergosterol was given orally. Definite improvement was noted within an hour and complete relief within at most two hours. After 24 hours a second dose of 1 c.c. was given in most instances, but no dose subsequent to this was administered. These animals remained perfectly free from tetany, ate their food, and were active, bright and normal in every way for a period of from one to two weeks. The muscles of the legs and jaws were, so far as could be judged, perfectly free from hyperexcitability. The actual length of time over which the symptoms might remain in abeyance as a result of these two doses has not so far been determined, since after a period considered to be sufficiently long for the purpose of demonstrating that the cure was complete the animals were employed for overdosage experiments. In the past five years one of us has performed a large number of parathyroidectomies and never has a *rapid, spontaneous* and *sustained* cure of tetany been observed. In the past any animals which developed symptoms comparable with those shown by the animals of this series invariably died within an hour or two if left untreated by injections of either parathormone or calcium. The question of the recovery being merely coincidental with the administration of the sterol may therefore be dismissed. The only possible explanation for the very prompt and complete cure which followed the administration of irradiated ergosterol is the obvious one,—that this substance in large doses is an anti-tetanic agent no less effective than parathormone.

The failure of previous observers to obtain similar results is we believe due to the fact that a sufficiently large dose of irradiated ergosterol had not been given for the demonstration of its parathyroid-like action. A typical protocol illustrating our results follows.

Dog 26, female, weight 7.5 kilos, recently lactating.

January 27, 1930.—Thyroparathyroidectomy, 4 p.m.

January 24th, 6 p.m.—Early signs of tetany. Stiffness of hind limbs.

11 p.m.—Rapid breathing, twitchings of muscles of face, shoulders and legs. Walks stiffly and with difficulty. Carried down from animal room to laboratory for observation.

11.30 p.m.—Respirations very rapid and noisy; they are difficult to count, but are over 160 per minute. Salivation, claw-like paws, twitchings and tremors of practically all muscles of body and especially of facial muscles. Extreme rigidity of limb muscles. Can walk in a staggering fashion with a straddled stiff movement of hind legs; hind legs drag along ground. Pupils fully dilated; do not react to light.

11.45 p.m.—Cannot rise or even support herself upon four legs, but can raise upper part of body on front legs. Lies on floor on her side with all legs stretched out and trembling violently. Teeth chattering, temporal muscles twitching actively. Attacks of asphyxia occur occasionally, apparently due to spasm of glottis. All signs of tetany extreme. Respirations are very rapid and noisy, and can be heard in another room. The temperature was not taken, but apparently high. Nose hot and dry.

11.50 p.m.—Complete prostration; cannot raise head from floor.

11.53 p.m.—1 c.c. irradiated ergosterol (10,000 D) given by mouth. Head held up until most of the material had trickled into throat. Animal swallowed.

12.10 a.m.—Respirations somewhat less violent and not so rapid; 140 per minute. Can raise front part of body. There is a slight, but, it is thought, definite improvement.

12.30 a.m.—Gets on to four feet and walks in a staggering stiff manner half across the room and then falls down. Gets up again and staggers to a corner where she lies down. Respirations about the same as at 12.10. No chattering of the teeth. Muscles less rigid. Spasms of glottis less frequent and of shorter duration.

12.50 a.m.—Breathing very much quieter, though still rapid. Respirations cannot be heard 20 feet away.

1.00 a.m.—At times breathing is comparatively slow and quiet. No muscular twitchings or chattering of teeth. Tapping muscles of face does not evoke any noticeable contractions.

1.15 a.m.—Respirations are quiet and at a general rate of 102, but short runs of rapid breathing occur at a rate of 120 or so every now and then. Dog appears quiet and comfortable. Lies upon her side and has lost that frightened, harassed look. She is resting, apparently exhausted after the seizure. Has no inclination to rise, but when placed upon her feet follows one across the room with a comparatively normal gait, though stiffness of the hind legs is still quite noticeable. Respirations just audible at a distance of 2 feet; rate 90 per minute.

1.30 a.m.—Apparently normal. Respirations perfectly quiet and not noticeably increased in rate. Nose cool and moist. Put upon a table 3 feet high, when

called jumps to floor with very slight disturbance of balance. Upon a second test she jumps to floor with perfect balance. Walks practically normally. Pricks up ears and takes interest in surrounding objects.

1.50 a.m.—Follows observer up two flights of stairs to animal room. Lopes up last flight 2 or 3 steps at a time. Put in cage and left for the night.

January 25th, 10.00 a.m.—Appears quite normal. Given no further treatment in order to determine whether or not the cure would be maintained after a single dose.

January 26th.—Normal.

It may be that much smaller doses than those which we employed would have had just as pronounced an influence upon the course of tetany. Though smaller amounts were not administered it is scarcely probable that we, by chance, chose the minimal curative dose. The question of dosage must be left for future investigation. The serum calcium was not determined in these animals after the ergosterol was given, since the drawing of blood would have confused us in arriving at any conclusion concerning the action of the administered substance. It is well known that definite though temporary relief of tetanic symptoms frequently follows bleeding. The ameliorating effect of blood letting is evidently due simply, as shown by Swingle and Wenner,[21] to the rise in serum calcium. The rise, however, occurs relatively gradually, as also does the abatement of symptoms. Though the serum calcium was not determined at the critical moment in our experiments, nevertheless our experience with normal animals has been that the calcium does not show a prompt rise after ergosterol administration, even in maximal doses. We have observed on the contrary a slight initial fall. In view of the known rôle played by calcium in the pathogenesis and cure of tetany this feature is very puzzling and requires elucidation. It is quite possible that when the supplies of available calcium are increased by irradiated ergosterol that the first call upon this suddenly mobilized calcium is made by the excitable tissues, and that it is only after these have become satisfied that the calcium rises in the blood.

THE INFLUENCE OF IRRADIATED ERGOSTEROL UPON THE COURSE AND SYMPTOMS OF OVERDOSAGE IN ANIMALS FROM WHICH ALL ACCESSORY PARATHYROID TISSUE SO FAR AS POSSIBLE HAD BEEN REMOVED

In this series of animals an extensive operation for the removal of parathyroid tissue was performed. All accessible sites which might harbour parathyroid tissue were carefully cleaned. In addition to the removal of the thyroid and the associated parathyroids a very free dissection of the neck tissues was made from the level of the hyoid bone and the angle of the jaw to the sternum. All the animals operated upon in this way developed tetany to a greater or less degree. *The tetany was remarkably refractory to the administration of irradiated ergosterol.* In animals which showed less urgent tetanic symptoms, several days of irradiated ergosterol treatment in 1 or 2 c.c. doses twice daily caused only slight improvement in the condition. Twitchings of muscles, chattering of the teeth, trismus and low serum calcium persisted for days. When the dosage was continued for two weeks or more the hyperexcitability gradually disappeared and the signs of overdosage ultimately made their appearance. All these animals developed the symptoms described below as characteristic of irradiated ergosterol or parathormone overdosage, and eventually succumbed. The survival time, however, was from 300 to 360 hours, as compared with 60 to 108 hours for normal adult animals of approximately the same weights. Judging from the duration of life after the commencement of ergosterol administration, dogs upon which the ordinary operation of thyroparathyroidectomy was performed showed a greater resistance than normal animals to the effects of overdosage, but less resistance than those upon which the complete operation had been performed. The former succumbed to overdosage in from 168 to 212 hours.

Animals upon which the complete operation was performed and which developed violent tetany were not relieved by maximal oral doses of irradiated ergosterol. Nor did any appreciable amelioration of the condition result, though in some cases several large doses were given over a period of from 3 to 7 hours before death occurred. The absence of any response to irradiated ergosterol in these subjects of severe tetany was in striking contrast to the rapid cure which followed the administration of the vitamin to animals deprived of the thyroparathyroid structures alone. The following is a specimen protocol of an animal which having had the neck fully dissected showed mild tetany and was treated with maximal doses of irradiated ergosterol twice daily.

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Dog 23.—Black bull terrier, male, 8 kilo. March 7, 1930: complete operation. March 9th, 11 p.m.; twitching muscles, chattering of teeth, jaws stiff, leg muscles rigid. One c.c. of irradiated ergosterol (10,000 D) given by mouth. No improvement in an hour. Dose repeated. March 10th; condition unchanged, tremors, stiffness of jaws, etc. One c.c. ergosterol given twice daily, 10 a.m. and 3 p.m. March 11th to 16th; tetanic symptoms still show little change; perhaps there is slight improvement. The animal has received 1 c.c. of ergosterol twice daily since March 10th. March 17th; muscles still stiff, but tremors are less, chattering of teeth has disappeared. March 18th: stiffness of leg muscles and hyperexcitability still present, though there is definite improvement. March 30th; vomiting, passage of blood in stools. March 21st; very weak, blood in stools. March 24th; death; usual signs of overdosage found in gastro-intestinal tract and lungs.

THE EFFECTS OF MAXIMAL DOSES OF IRRADIATED ERGOSTEROL UPON NORMAL ANIMALS AND A COMPARISON OF THESE EFFECTS WITH THOSE RESULTING FROM PARATHYROID OVERDOSAGE

Adult dogs.—After the first parathyroidectomized animal was relieved of its tetanic symptoms by irradiated ergosterol a normal dog (No. 28) was selected and large doses (1 c.c.) of the material were given twice daily. It came as a great surprise to us when the animal, weighing about 30 lbs. and apparently perfectly healthy at the time that the experiment was commenced died 84 hours later with *the symptoms of parathyroid overdosage,* and features identical with those of this condition were found post mortem. On the second day after the first dose was given this animal became depressed, weak and lethargic, and muscular tone was strikingly reduced. Diarrhœa and vomiting began later and upon the third day the stools became dark with blood and bloody fluid was vomited. Collapse and death occurred a few hours later—on the night of the third day. At autopsy the gastro-intestinal tract as far as the lower ileum was intensely hæmorrhagic. The interior of the stomach was a chocolate brown shade due to the extravasation of blood into the mucosa and submucosa, the colour lightening to a port-wine red in the small intestine. Areas of the gastric mucosa appeared necrotic. Upon microscopical examination the capillary vessels appeared widely dilated and masses of red cells were found between the gland tubules, in the submucous coat and in the muscularis mucosæ. The lungs also showed great numbers of leucocytes and red cells. Over large areas of the lung the alveoli were completely obliterated. This was due to the extravasation of blood into and the disintegration of the alveolar walls and not to the exudation of fluid or to the migration of blood cells into the air spaces. The serum calcium before death was elevated to 19 mg. per 100 c.c. The dose given to this dog (No. 28) was 0.15 c.c. per kilo per day. Considering the strength of the material (10,000 D) we employed, this is admittedly an enormous dose when compared with the therapeutic dose of viosterol (250 D). The latter has only 1/40 the concentration in vitamin D. The quantity of the preparation which we employed per kilo in this experiment is equivalent to 6.0 c.c., or about 180 drops of the therapeutic material. Death was caused however in 6 days (Dog 10) by daily doses as small as 0.09 c.c. of 10,000 D per kilo. In another adult female dog (39) weighing 16 kilograms, five doses of 1 c.c. each on the first and on the mornings and afternoons of the two successive days were given. The animal died on the fifth day. If the total dose of 5 c.c. be divided by the number of days elapsing from the beginning of the administration until death, the average daily dosage works out to 0.068 c.c. per kilo, or about 20 times the maximal therapeutic dose recommended for the infant. This was the smallest dose employed in adult dogs. In a subsequent paper it will be shown that the lethal dose for young dogs is considerably less than this.*

The effects of irradiated ergosterol have been studied upon some forty adult dogs. All of these, with two exceptions which are described below, showed symptoms during life similar to those just described. The post-mortem findings were also practically identical in all. The details from 6 experiments are given as examples in Table I.

A small proportion of animals appear to become tolerant to irradiated ergosterol in so far as the acute manifestations are concerned. If the administration is continued they ultimately die, but the disturbances are less evident, for they develop more slowly and are apparently chiefly of a metabolic nature. This apparent immunity to the intense effects of the sterol was

* Since the completion of these experiments Bills[22] has claimed that viosterol preparations which have been stated to possess an antirachitic potency 250 times greater than that of cod liver oil (250 D) are actually only 100 D. The material which we have employed (10,000 D or X according to Bills' designation) has therefore a potency probably 100 times rather than 40 times that of the therapeutic material.

TABLE I

THE ADMINISTRATION OF EXCESSIVE DOSES OF IRRADIATED ERGOSTEROL (10,000 D) TO NORMAL DOGS

Animal No. and Sex	Weight kilos.	Dosage c.c.					Dates			Survival time in hours	Serum calcium mg. per 100 c.c.	Post-mortem findings
		Total	Daily per animal	Daily per kilo.	Equivalent in 250 D per kilo.	x maximal* therapeutic dose per kilo.	Administration commenced	First symptoms	Death			
26 male	7.2	7	2	0.29	11.6	98	Mar. 7, 4 p.m.	Mar. 9	Early a.m. Mar. 10	84 approx.	18.3	Lungs, stomach and upper intestinal tract, hæmorrhagic. Large quantity of blood in stomach and bowel
28 female	14.0	7	2	0.15	6.0	50	Mar. 12, 10 a.m.	Mar. 13	Mar. 16 about 12 a.m.	84 approx.	19.5	Essentially the same as above. Gastro-intestinal hæmorrhage more intense, mucosa of stomach uniformly a deep chocolate colour resembling liver tissue.
30 female	16.1	5	0.8	0.068	2.4	20	Mar. 24, 4 p.m.	Mar. 26	Mar. 28 in p.m.	108 approx.	— —	Typical.
40 male	12.5	3	1	0.08	3.2	26	Mar. 27, 4 p.m.	Mar. 28	Mar. 29 p.m.	60 approx.	17.3	Usual signs, but exceptionally intense.
32 male	4.6	2	0.4	0.09	3.6	30	Mar. 20	Mar. 27	Mar. 29	228	18	Unusually severe reaction in lungs and gastro-intestinal tract
42 female	16.0	6	2	0.12	4.8	40	Apr. 3	— —	Apr. 6 p.m.	86 approx.	— —	Typical.

* The maximal therapeutic dose is taken as 0.6 c.c. (20 drops) per 5 kilo infant, i.e., 0.12 c.c. per kilo.

observed in two animals. Within three days after the administration of maximal daily doses had been commenced these animals showed the typical symptoms of overdosage. It was fully expected that death would result 24 or 48 hours later. On the contrary, recovery from the acute manifestations occurred and the animals lived for many days afterwards. At autopsy the usual gastro-intestinal signs were absent. One of these dogs (No. 8) did not die until 30 days after the commencement of the treatment, though this had been continued daily without intermission. There was the most extreme emaciation, the animal having lost 40 per cent of its original weight. Hypercalcæmia was present in the early stages, and was 140 per cent above normal during the last few days of life. For a short time during the mid-period the serum calcium was not greatly elevated. There was a gradual rise in the blood phosphate which reached a maximum of 26 mg. on the last day of life. After death the gastric mucosa showed no recent lesions, but large dark brown areas were observed which were quite evidently old blood extravasations. The discoloured epithelium bore witness to the effects of irradiated ergosterol upon the gastric wall during the first few days of the treatment and from which the animal had recovered. In the second animal (No. 5) which showed tolerance after a period of acute symptoms, the gastric mucosa appeared normal after death. This animal lived for 15 days. There were extreme emaciation and hypercalcæmia. A third dog (No. 17) showed a certain degree of tolerance to ergosterol action after the first three or four days of treatment. When the dose was doubled death occurred rapidly with the usual symptoms, and the typical signs were found at autopsy. It may be remarked here that animals also occasionally show a tolerance to parathormone—a phenomenon which has been remarked upon by Collip and by Anb and his associates.[15] We have also observed it upon several occasions.

The great emaciation and the metabolic disturbances of comparatively slow development described above in animals which have become tolerant to maximal doses, have been found by us to be a common occurrence when irradiated ergosterol has been given in smaller, though still excessive, dosage. The phenomena result-

...ther cases have been observed for most part in puppies, since in these we have been engaged in determining the minimal dose that would cause death.

A very constant feature of irradiated ergosterol overdosage in young pups has been the hemorrhagic state of the bone marrow. This may also occur in older dogs, but, so far, our attention has not been especially directed toward the investigation of this phenomenon in adult animals. In all the pups dying of irradiated ergosterol poisoning, in which the bones were examined at autopsy, this feature was observed. The bones of some 15 animals have been examined in this manner. The effect of overdosage with *parathormone* upon the medulla of the bones has been investigated in only two young animals. In these, however, the state of the marrow was identical with that found after overdosage with irradiated ergosterol. By the examination of the bones of amputated limbs, and by sacrificing healthy animals, we have acquainted ourselves with the appearance of the normal marrow of young pups. It is of a healthy flesh pink colour and quite unlike the dark red colour of clotted blood found in the animals killed by parathormone or irradiated ergosterol.

It has already been mentioned that the survival time in animals upon which a free dissection of the neck was performed was three or four times longer than the duration of life of normal animals when maximal doses were given in both cases. Nevertheless, the extension of life of these animals was not due to their possessing a natural tolerance similar to that of the animals described above, since in the end the usual symptoms appeared and the gastro-intestinal and pulmonary changes found post-mortem were just as intense as in the animals which died in the usual time. Animals in which a simple thyroparathyroidectomy was performed were also, as judged by the survival time, from two to three times more resistant to overdosage than normal animals. The shortest time which a normal animal survived was 58 hours. The average time was about 90 hours.

These effects of ergosterol—the relief of tetany, the hypercalcæmia and the autopsy appearances—showed so strong a resemblance to the action of the parathyroid hormone that it seemed highly probable that a fundamental association between the two substances existed. Experiments were devised to study more closely the effects of ergosterol and parathyroid overdosage respectively, with the view of discovering whether or not there were other features common to both agents.

THE EFFECTS OF PARATHYROID OVERDOSAGE AND THOSE OF ERGOSTEROL POISONING COMPARED

The general symptoms and the appearance found post-mortem.—Collip[23, 25] and Collip, Clarke and Scott[24] reported that an acid extract of the parathyroid glands of the ox when administered subcutaneously or intravenously to dogs induced hypercalcæmia within a few hours, and if the injections were continued death occurred within a few days. The first signs of overdosage were lassitude, refusal of food, vomiting, lethargy and muscular hypotonicity. Later, usually toward the end of the second or during the third day, emaciation was noticed, the stools became blood stained, and blood was usually vomited. The blood when drawn at this time appeared thick and viscous, and after it had clotted and had been centrifuged seemed greatly concentrated. Weakness soon became profound and collapse, quickly followed by death, occurred about the middle of the fourth day after the administration of the hormone had been commenced. At autopsy the gastric and intestinal mucosæ were found to be of a deep plum or chocolate colour, due to hæmorrhagic extravasation, and the stomach was usually half filled with dark blood. These remarkable effects of parathyroid overdosage were confirmed in practically every detail and reported shortly afterwards by Macleod and Taylor. The lungs in most instances, also, showed the most intense vascular engorgement, and large hæmorrhagic areas, often involving the greater part of one or two lobes, were found. The liver and spleen were also deeply congested. Upon microscopical examination masses of red cells were found throughout the connective tissue and parenchyma of these organs.

The symptoms and general course of their development and the effects upon the gastro-intestinal tract and lungs (Fig. 3, Plate I), liver and spleen after parathyroid overdosage are in all respects identical with those occurring in adult dogs after overdosage with irradiated ergosterol. In Plate I are shown for purposes

of comparison the gastro-intestinal tracts in the two conditions. Though minor variations occur between individuals in both instances, it is never possible, even for one who has seen many examples of each, to distinguish either by gross or microscopical examination the effects of parathyroid overdosage from those induced by irradiated ergosterol. The appearances after ergosterol poisoning have already been described in detail in dog 28. This description may be taken as exemplifying the effects induced in practically all the adult dogs given doses exceeding 20 times the maximal therapeutic dose of viosterol.

The blood changes.—The *serum calcium* was estimated by the Kramer-Tisdall[26] method, as modified by Clarke and Collip.[27] The outstanding effect upon the blood chemistry of the parathyroid hormone is the elevation of the serum calcium, whether this has been normal or has been depressed as a result of parathyroidectomy. The fact seems now to be well established that vitamin D, either in the form of cod liver oil or irradiated ergosterol, raises the calcium of the serum from the sub-normal to the normal level. It has already been mentioned that we have found that maximal doses of irradiated ergosterol relieve the tetany quite as rapidly as parathyroid extract, and raise the serum calcium from the tetany level to normal and ultimately above normal. This substance in large doses is also capable of inducing hypercalcæmia in normal animals. Jones, Rapoport and Hodes[19] reported that irradiated ergosterol raised the serum calcium to a higher level than it was possible to attain by means of parathormone, and concluded logically from this result that the vitamin does not exert its effects through stimulation of the parathyroid glands. However, one of the present writers has observed a serum calcium as high as 24 mg. per 100 c.c. as a result of parathormone administration. The highest serum calcium reported by Jones, Rapoport and Hodes[39] in their last paper is 20 mg. per 100 c.c., a value quite common after parathormone administration. They gave larger doses of ergosterol in experiments reported in their earlier paper. In these experiments the calcium in only one animal was raised beyond 21 mg. per 100 c.c. In this animal they state that the serum calcium was 47.3 mg. per 100 c.c.! This is a higher value than we have seen under any circumstances. Even by the injection of maximal doses of calcium chloride intravenously we have never been able to raise the calcium above 28 mg. per cent, and then only for a few minutes. It has also always been our experience that high calcium values, as a result of either parathyroid extract or ergosterol overdosage, are associated with urgent symptoms resulting in death. In the animal in which this extraordinarily high serum calcium is reported to have occurred, no symptoms beyond the refusal of food are mentioned, and the animal lived for nearly six weeks afterwards.

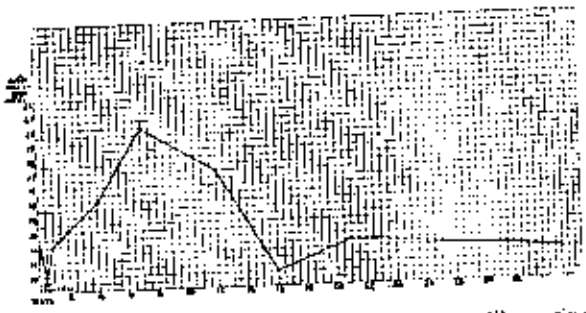

CHART I.—Serum calcium curve of a dog illustrating the effect of a large dose of irradiated ergosterol given intravenously in divided doses over a period of four hours. Note the early fall in the calcium level.

The form of the calcium curve after a single large dose of irradiated ergosterol differs in one important respect from that characteristic of parathyroid administration. The latent period, that is, the time elapsing between the moment the dose is given and the rise in the curve, is much longer after ergosterol. During this time the serum calcium is usually 2 mg. or more *below* the normal. This latter feature will be considered presently. In Chart I is shown the effect upon the serum calcium of a large amount of irradiated ergosterol given intravenously in divided doses over a period of 4 hours. It will be seen that no elevation of the serum calcium has occurred 12 hours later. Even after 60 hours there is only a moderate rise. The maximal height was not reached until the 7th day, when a serum calcium content of 20 mg. per 100 c.c. was observed. The animal received no ergosterol after the first series had been given in quick succession at the commencement of the experiment. On the 12th day the calcium curve was still well above normal. This slow response, the sustained height of the curve after a maximum had been reached,

PLATE 1

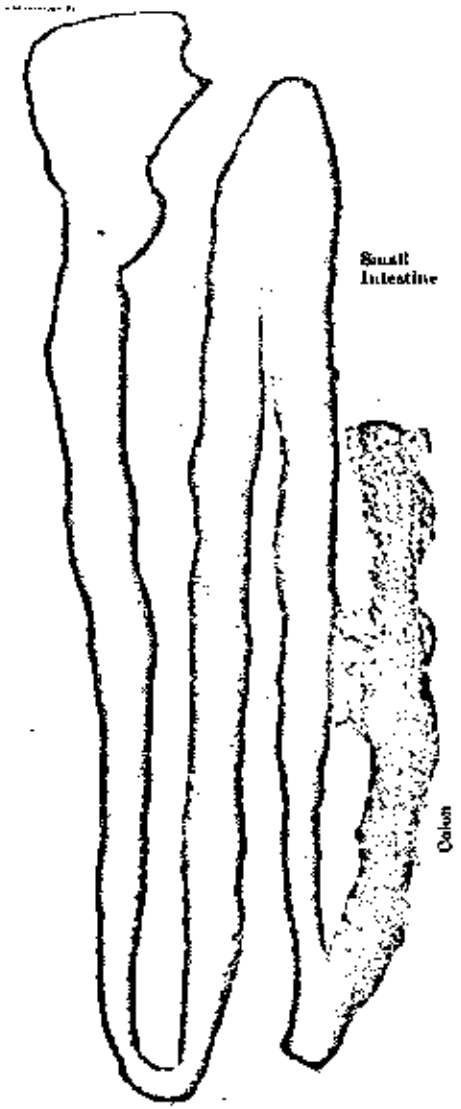

FIG. 1.—Gastro-intestinal tract of a dog (weight 6 kilos) which had received 1 c.c. irradiated ergosterol (10,000 D) daily for 5 days.

(Coloured photograph)

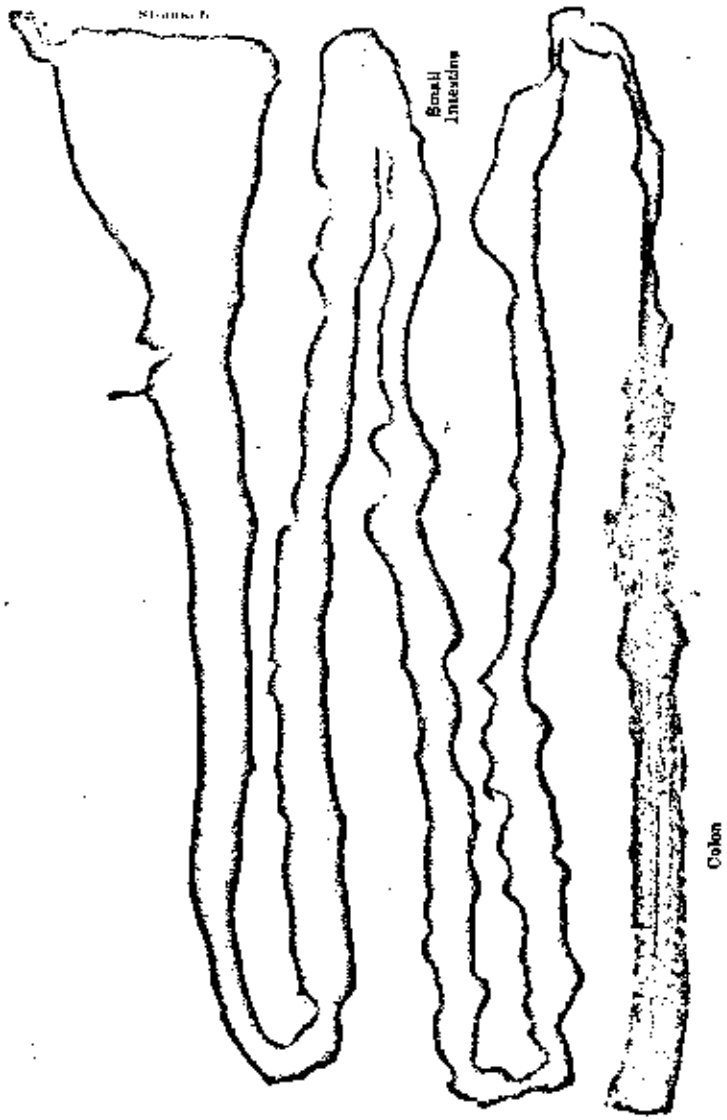

FIG. 2.—Gastro-intestinal tract of a dog (weight 12 kilos) which had received 40 units of parathyroid extract daily for 4 days.

(Coloured photograph)

FIG. 3.—Lung of a dog which had died as a result of overdosage with irradiated ergosterol.

(Coloured photograph)

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We wish to thank Mr J. Pearson for the [illegible]e of the chicks, Mr John Taylor for analytical assistance and Mr R. Morley Jones for statistical advice.

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VITAMIN-D ABSORPTION AFTER PARTIAL GASTRECTOMY

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PARTIAL gastrectomy is possibly the commonest cause of osteomalacia in Europe today. In one study of 60 patients with severe osteomalacia, for example, nearly a third had previously undergone partial gastrectomy (de Seze et al. 1962).

Patients with post-gastrectomy osteomalacia have subnormal levels of vitamin D in their plasma (Thompson, Lewis, and Booth 1964), and the osteomalacia may be cured by treatment with small doses of vitamin D (Morgan et al. 1965). These findings suggest that the condition is due either to inadequate intake in the diet or to intestinal malabsorption of the vitamin (Stammers and Williams 1963). Since the majority of patients with post-gastrectomy osteomalacia have steatorrhœa (Hall and Neale 1963, Deller et al. 1963), it has been suggested that malabsorption of vitamin D may be an important etiological factor, but hitherto no direct measurements of its absorption after partial gastrectomy have been reported.

TABLE I—DATA OF PATIENTS WITH POST-GASTRECTOMY OSTEOMALACIA

Patient no.	Age (yr.)	Yr. post op.	Serum Ca (mEq. per l.)	Serum P (mEq. per l.)	Serum Alkaline phosphatase (K.-A. units per 100 ml.)	Serum Albumin (g. per 100 ml.)	Bone X-rays	Bone biopsy
1	80	16	4·6	1·2	25	3·0	Rarefaction	Osteomalacia
2	76	14	4·2	1·6	43	2·8	Rarefaction	"
3	82	16	4·6	1·4	28	4·0	Secondary hyperparathyroidism	"
4	79	9	4·8	1·8	28	4·8	Pseudo-fractures	"
5	75	30	3·8	1·8	26	3·9	"	"
6	52	5	3·8	2·0	24	1·6	Rarefaction	"
Normal range			4·7–5·5	1·5–2·5	3–13	3·5–5·2		

It is now possible to study vitamin-D absorption in man, using tritium-labelled vitamin D_3 (Thompson, Lewis, and Booth 1966). This paper describes studies of its absorption in 6 patients with post-gastrectomy osteomalacia and, for comparison, in 4 post-gastrectomy patients without osteomalacia.

Methods

Vitamin-D absorption was measured using tritium-labelled vitamin D_3, as described by Thompson et al. (1966). The patients were given oral doses of 0·5–1·0 mg. of ^{3}H-vitamin-D_3, of specific activity 5–15 μC per mg. The vitamin was given by mouth in arachis oil immediately after a meal and was rinsed down with 250 ml. of milk.

Plasma samples were taken at intervals during the next 72 hours in some subjects, and faeces were collected for two successive 3-day periods in all patients. Radioactivity was measured in plasma and faeces, the net absorption of vitamin D being calculated from the faecal excretion of radioactivity. In 3 patients urinary radioactivity was also measured during the 24 hours after the oral dose; it ranged from 0 to 1·9% of the absorbed dose, which is similar to the excretion in control subjects (Thompson et al. 1966).

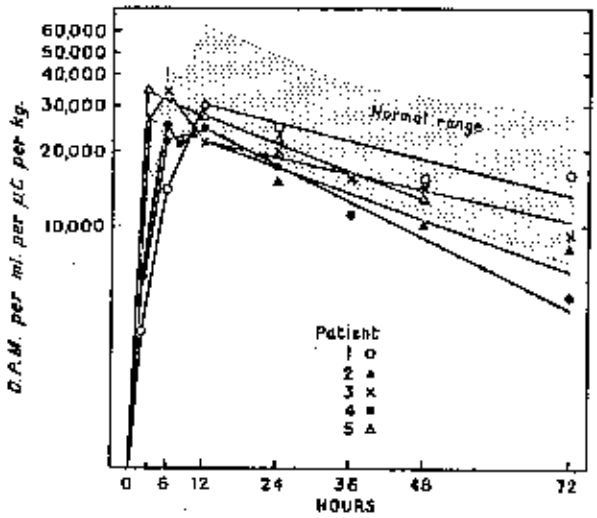
Faecal fat excretion was measured during the vitamin-D-absorption test by the method of Bowers et al. (1964); the patients received a diet containing approximately 70 g. of fat per day.

Other investigations included measurements of *serum calcium, inorganic phosphate*, and *alkaline phosphatase*, which were performed as described by Wootton (1964). The *serum-albumin* was measured by cellulose-acetate electrophoresis (Kohn 1960). *Radiological skeletal surveys* included X-rays of the chest, hands, pelvis, and thoracolumbar spine. *Iliac-crest bone biopsies* were performed by the method of Stammers and Williams (1963). Undecalcified sections were stained with von Kossa, and osteoid seams exceeding 15 μ in width were regarded as diagnostic of osteomalacia.

Patients Studied

Vitamin-D absorption was studied in 6 patients with post-gastrectomy osteomalacia (table I) and in 4 other post-gastrectomy patients without osteomalacia. The age of the patients with osteomalacia ranged from 52 to 82 years and they were studied at between 5 and 30 years after gastrectomy. All 6 patients had presented with biochemical evidence of osteomalacia, and this was confirmed by iliac-crest bone biopsy in 5 (patients 1–5, table I). There was evidence of skeletal rarefaction in all 6 patients; in addition, there were pseudo-fractures in 2 (patients 4 and 5), and in 1 (patient 3) there was evidence of secondary hyperparathyroidism. 4 patients (patients 1–3 and 5) had had Polya partial gastrectomy, 1 (patient 4) had undergone the Billroth-I operation, and in 1 other an initial Polya gastrectomy had been subsequently modified by a jejunal interposition operation. In this last patient, there was also evidence of a severe defect of pancreatic exocrine secretion (patient 6). All the patients with osteomalacia had been treated with vitamin D before their absorption of ^{3}H-vitamin-D_3 was studied.

The ages of the 4 patients without osteomalacia ranged from 45 to 73 years, and they were studied at between 3 months and 18 years postoperatively. 2 of the patients had undergone a Polya gastrectomy; the other 2 had had Billroth-I type gastrectomy.



Plasma radioactivity in 6 patients with post-gastrectomy osteomalacia after 0·5–1·0 mg. oral doses of ^{3}H-vitamin-D_3.
The shaded area represents the normal range.

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Results

Plasma Radioactivity after Oral ^{3}H-vitamin-D_3

The plasma radioactivity in 5 of the patients with post-gastrectomy osteomalacia (patients 1–5, table I) at various intervals after receiving oral ^{3}H-vitamin-D^3 is shown in the accompanying figure. Radioactivity has been expressed as disintegrations per minute (D.P.M.) per millilitre of plasma and corrected for dose and body-weight. All 5 patients had peak levels of radioactivity either within or only just below the normal range, usually by 3 to 6 hours after the oral dose.

Net Absorption of ^{3}H-vitamin-D_3

The net absorption of 0·5–1·0 mg. doses of ^{3}H-vitamin-D_3 was measured in each of the 10 patients. The results, together with the faecal fat excretions, are shown in table II. The observed range of absorption of similar

TABLE II—NET ABSORPTION OF 0·5–1·0 mg. DOSES OF ^{3}H-VITAMIN-D_3 IN POST-GASTRECTOMY PATIENTS

Patient no.	Type of operation	Faecal fat (g. per day)	^{3}H-vitamin-D_3 (% absorption)	
1	Polya	5·1	86·8	With osteomalacia
2	,,	9·5	52·8	
3	,,	11·5	61·5	
4	Billroth I	10·0	56·6	
5	Polya	8·4	54·6	
6*	,, with jejunal interposition	35·0	0·0	
7	Billroth I	2·7	89·4	Without osteomalacia
8	,,	15·6	87·9	
9	Polya	1·5	80·5	
10	,,	10·0	76·4	
Normal range		<6	62·4–91·3	

* Test of pancreatic function (Lundh 1962) showed complete absence of trypsin.

doses of ^{3}H-vitamin-D_3 in control subjects is 62·4 to 91·3% (Thompson et al. 1966).

In 1 of the patients with osteomalacia (patient 1), the absorption of ^{3}H-vitamin-D_3 was 86·8%, which is within the normal range; this patient did not have steatorrhœa. In 4 other patients with osteomalacia (patients 2–5) there was mild steatorrhœa, the faecal fat excretion ranging from 8·4 to 11·5 g. per day; and the absorption of ^{3}H-vitamin-D_3 was slightly impaired, ranging from 52·8 to 61·5%. The remaining patient with osteomalacia (patient 6) had a total defect of ^{3}H-vitamin-D_3 absorption, together with severe steatorrhœa; this was the patient with severe pancreatic dysfunction.

In contrast, all the post-gastrectomy patients without osteomalacia had a normal absorption of ^{3}H-vitamin-D_3, ranging from 76·4 to 89·4% (patients 7–10). 2 of these patients (patients 8 and 10) had moderate steatorrhœa, excreting 15·6 and 10·0 g. fat daily.

Discussion

These results show that the operation of partial gastrectomy alone does not cause malabsorption of vitamin D. All 4 of the post-gastrectomy patients who did not have osteomalacia absorbed ^{3}H-vitamin-D_3 normally, even though 2 of them had steatorrhœa. Furthermore, the results indicate that osteomalacia can occur after gastrectomy even when vitamin-D absorption is apparently normal. One of the patients studied had severe osteomalacia, yet absorbed ^{3}H-vitamin-D_3 normally; and it seems possible that in this patient an inadequate dietary intake of vitamin D was the major aetiological factor. In contrast, in 4 other patients with osteomalacia, all of whom had mild steatorrhœa, there was a slight defect of ^{3}H-vitamin-D_3 absorption. In these patients a combination of prolonged mild malabsorption of vitamin D with an inadequate dietary intake may possibly have caused the osteomalacia. The remaining patient with osteomalacia, whose pancreatic function was severely impaired, had a total defect of ^{3}H-vitamin-D_3 absorption, behaving similarly in this respect to patients with steatorrhœa due to chronic pancreatitis (Thompson et al. 1966).

The actual site of absorption of vitamin D in man is not known, although Schachter et al. (1964) have shown in rats that the capacity to absorb vitamin D is greatest in the mid-jejunum. There is no evidence to suggest that the duodenum is the main site of absorption, and malabsorption of vitamin D after Polya-type operations cannot be simply due to the duodenal bypass, as suggested by Morgan et al. (1965). The common association between osteomalacia and Polya gastrectomies can probably be attributed partly to the frequency with which this operation has been performed, and partly to its increased liability to cause steatorrhœa (Shingleton et al. 1957).

Dietary vitamin D, which may be partly protein-bound, would not necessarily be absorbed to the same extent as the much larger amounts of vitamin D used in these absorption studies. However, our results do suggest that vitamin-D deficiency may arise in two ways after partial gastrectomy—namely, by dietary lack and by malabsorption. Although either alone could lead to post-gastrectomy osteomalacia, it seems probable that a combination of these two factors is usually responsible.

Summary

The absorption of 0·5–1·0 mg. oral doses of tritium-labelled vitamin D_3 was studied in 10 post-gastrectomy patients, 6 of whom had osteomalacia.

The net absorption of ^{3}H-vitamin-D_3 was slightly subnormal in 4 of the 6 patients with osteomalacia, but in the 5th was nil, owing to an associated pancreatic defect; these 5 patients had steatorrhœa. The 6th patient with osteomalacia absorbed ^{3}H-vitamin-D_3 normally and did not have steatorrhœa. Plasma radioactivity was measured in 5 of these patients and was within or just below the normal range.

The net absorption of ^{3}H-vitamin-D_3 was normal in each of the 4 patients without osteomalacia; 2 of these patients had steatorrhœa.

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Vitamin D and Phospholipid Metabolism*

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Although it has been known for many years that the primary physiological action of vitamin D is to improve calcium absorption from the small intestine (1, 2), studies on calcium metabolism alone have thus far failed to elucidate the biochemical mechanism of action of vitamin D. By using everted intestinal sacs, Schachter and Rosen (3) have shown that vitamin D increases the transport of ^{45}Ca against a concentration gradient in the proximal portion of the small intestine. Harrison and Harrison have confirmed these findings (4) and have also reported that vitamin D increases the rate of diffusion of calcium across the entire length of the intestinal wall. In this laboratory, DeLuca *et al.* (5, 6) have demonstrated an action of vitamin D on the behavior and morphology of kidney mitochondria. More recently, Engstrom and DeLuca (7) have reported that vitamin D, *in vivo* or *in vitro*, stimulates the release of calcium from isolated rat kidney mitochondria. Studies in this laboratory on the subcellular distribution of vitamin D in rat kidney by biological assay (8) and by use of tritium labeled vitamins D_2 and D_3[1] have demonstrated that vitamin D is associated with all membrane fractions. These findings suggest that the action of vitamin D at the cellular level may be closely associated with a function of the cellular membranes.

Hokin and Hokin (9) have studied the secretion of proteins, polypeptides, and other hydrophilic material and have found that an increase in the secretion of these materials is accompanied by an increase in the incorporation of ^{32}P-orthophosphate into the phospholipids. Further work (10) on the synthesis of phosphatidic acid from diglyceride and adenosine triphosphate led to a possible scheme for the participation of phosphatidic acid in the transport of hydrophilic secretory materials across lipoid membranes. This scheme has more recently been modified to account for the number of sodium ions transported per ATP used (11). Several other investigators have proposed that phospholipids might function in the movement of cations and other hydrophilic material across cellular membranes (12, 13). Karnovsky and Wallach (14) have reported that $^{32}P_i$ incorporation into various phospholipids is increased during phagocytosis.

Studies indicating that (*a*) vitamin D is involved in the transport of calcium across biological membranes, (*b*) vitamin D is associated with and has an effect on lipoid membranes, and (*c*) phospholipids are involved in the secretion of hydrophilic material and have been implicated in the transport of cations across lipoid membranes stimulated an investigation of the effect of vitamin D on phospholipid metabolism.

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[1] A. W. Norman and H. F. DeLuca, in preparation.

EXPERIMENTAL PROCEDURE

Preparation of Rats—Male albino rats of the Sprague-Dawley strain, weighing 50 to 70 g, were housed in individual hanging wire cages and given food and distilled water *ad libitum*. The rats were fed a modified version of the adequate calcium and phosphorus diet of Steenbock and Herting (15), except that vitamin-free casein plus 0.2% L-cystine replaced egg white as the protein source. Fat-soluble vitamins A, E, and K were given orally in a cottonseed (Wesson) oil solution three times a week. In each experiment, except in the case of the response studies, one half of the animals received no vitamin D, while the other half received 75 i.u. of vitamin D_2 in cottonseed oil three times a week. In experiments concerned with the response to vitamin D, rats were killed 3 or 6 hours after an oral dose of 2000 i.u. of the vitamin.

Isotopes—$^{32}P_i$, carrier-free, was obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee.

Glycerol-1,3-^{14}C (specific activity, 1.0 mc per mmole) was purchased from Nuclear Research Chemicals, Inc., Orlando, Florida.

DL-Serine-3-^{14}C (specific activity, 6.0 mc per mmole) was obtained from Volk Radiochemical Company, Skokie, Illinois.

Incubations—After the rats had been on the diet for 21 to 25 days, two vitamin D-deficient and two vitamin D-fed rats were killed by a sharp blow on the head followed by decapitation. The upper one-half (approximately 30 cm) of the small intestine was quickly removed, slit open, and placed in ice cold 0.9% NaCl solution. The intestine was rinsed three times in cold NaCl solution, blotted on filter paper, and laid on a flat glass surface. The mucosa was removed by scraping with a microscope slide (16) and then weighed on a torsion balance. Mucosa (2 g) was placed in 20 ml of Krebs-Ringer-bicarbonate buffer (17), and the cells were suspended by drawing them up several times in a serological pipette. The suspensions were shaken for 5 minutes at 37° for thermal equilibration before initiation of the reaction by the addition of 160 μc of $^{32}P_i$ (yielding a specific activity of 5.2 μc per μmole of phosphorus) plus NaCl solution (isotonic NaCl-KCl, 25:1, total volume of 4 ml) or, in some cases, 4 μc of ^{14}C labeled glycerol or serine plus NaCl solution. The cells were incubated with shaking for 1 hour at 37° with 95% O_2-5% CO_2 flowing continuously as the gas phase. Samples of 3.2 ml were removed by pipette at various times and added to 10 ml of cold chloroform-methanol, 1:2, to stop the reaction.

In experiments with kidney and liver slices, the tissues were sliced with a hand microtome (17), and 250 mg of tissue were placed in 2.5 ml of Krebs-Ringer bicarbonate buffer in a 25-ml Erlenmeyer flask. Samples were initially incubated for 5 minutes at 37°, after which 10 μc of $^{32}P_i$ plus 0.9% NaCl solution (in a total volume of 0.5 ml) were added to each flask, and the

sample was incubated for the desired time. A gas phase of 95% O_2-5% CO_2 was maintained throughout the incubation. All other treatment was identical with that described for the intestinal mucosa.

Liver and kidney mitochondria were prepared by the standard method of Schneider (18). The mitochondrial pellet was washed once in isotonic sucrose and finally suspended in isotonic sucrose. The mitochondrial suspensions were incubated for 5 minutes as described by Garbus *et al.* (19). The reaction was terminated by the addition of chloroform-methanol, 1:2.

The fractionation of intestinal mucosa was carried out by a method devised in this laboratory.[2] The intestinal mucosa was homogenized without diluent in a conventional Potter-Elvehjem homogenizer fitted with a Teflon pestle, or was ground with sand in a chilled mortar for 1 minute, then made up to a 10% homogenate with isotonic mannitol. After preparation of the homogenates, the fractionation was carried out by the conventional method of Schneider (18).

Extraction of Lipids. In all experiments the lipids were extracted by the procedure of Bligh and Dyer (20). The samples, cells + incubation medium-chloroform-methanol (0.8:1:2), which forms a single phase, were homogenized in a Potter-Elvehjem homogenizer for 1 minute. One volume of chloroform was added, and then 1 volume of water, with the resulting mixture being homogenized after each addition. The final biphasic mixture was filtered through glass wool into a graduated centrifuge tube, and the phases were allowed to separate. An aliquot of the chloroform layer was removed and plated on an aluminum planchet to determine lipid-associated radioactivity. All radioactivity measurements were made in the Geiger region with a Nuclear-Chicago automatic counting device.

When ^{14}C-labeled glycerol was added to the incubation medium, total lipid soluble radioactivity was determined in the manner described above. For separation of the neutral and phospholipids, 2 ml of the lipid extract were removed and to this were added 2 ml of chloroform and 0.5 g of silicic acid (see below). This was shaken, the silicic acid was allowed to settle, and 1 ml of the chloroform was plated for radioactivity determination. The radioactivity remaining in the chloroform was taken to be associated with neutral lipids; the radioactivity of phospholipid was determined by difference.

Organic phosphate in the aqueous-methanol phase from the lipid extraction procedure was determined by the method of Martin and Doty (21). In this procedure the inorganic phosphate is complexed by molybdate and become soluble in a butanol-benzene phase, leaving only the organic phosphate in the aqueous phase. An aliquot of this aqueous phase was plated for radioactivity measurement.

Chromatography. Columns, 0.8 cm × 10 cm, were dry-packed with 4.0 g of Bio Rad (Calbiochem) silicic acid (mesh 325 mesh, specially prepared for the chromatography of lipids). The silicic acid had previously been placed in a vacuum desiccator for 48 hours, then activated at 135° for at least 24 hours prior to use. Chloroform (Analytical Reagent) was run through the column under slight (approximately 5 pounds) pressure until 50 ml of acid became clear (approximately 75 ml of chloroform). The column was allowed to stand overnight before the sample was applied. Preliminary experiments had indicated that better recovery of the radioactivity was obtained with an "aged" column.

The lipid extracts of two or three identical incubation samples were combined, evaporated to dryness under nitrogen, and redissolved in chloroform. A hyperbolic gradient (22) was utilized for separation of the phospholipids. The mixing chamber was initially filled with 300 ml of chloroform, and the holding chamber contained 60% methanol in chloroform. All ground glass joints were equipped with Teflon sleeves to prevent leaking. A flow rate of 1.0 ml per minute was maintained by a slight pressure. Fractions of 5 ml were collected. From each fraction, 1 ml was removed and analyzed for radioactivity; the remaining 4 ml were evaporated to dryness in a filtered air stream and total organic solids were determined by the method of Johnson (23). In some instances the fractions from the column were evaporated under a stream of nitrogen and rechromatographed on thin layer plates for further identification of the components. Three solvent systems were used: chloroform-methanol-water, 80:25:3 (24); chloroform-methanol-water, 100:25:3; and chloroform-methanol-7 N ammonia, 60:35:5 (25).

RESULTS

Fig. 1 illustrates the effect of vitamin D on the incorporation of $^{32}P_i$ into the lipids of intestinal mucosa. The stimulation by vitamin D was apparent even within the first 15 minutes of incubation, and was greater than 3-fold at the end of 1 hour. These results were quite reproducible as long as the animals used were of the same age. This incorporation of $^{32}P_i$ into the phospholipids is totally dependent on oxidative metabolism. The incorporation was completely abolished when 95% N_2-5% CO_2 was the gas phase, and the results were variable when the samples were not gassed with 95% O_2-5% CO_2 throughout the incubation period. The effects of several metabolic inhibitors on $^{32}P_i$ incorporation into the lipids were studied. The results are shown in Fig. 2. The incorporation was almost completely blocked in the presence of 10^{-4} M sodium cyanide, 7×10^{-5} M Dicumarol, 5×10^{-7} M antimycin A, and 10^{-5} M phlorizin, and was partially blocked by 10^{-7} M gramicidin and 10^{-6} M oligomycin. Iodoacetate (10^{-4} M) and 10^{-5} M *p*-chloromercuribenzoate[3] showed no inhibition. The incorporation was also insensitive to dinitrophenol at a concentration of 2×10^{-5} M.

In an effort to determine whether or not vitamin D was stimulating the general conversion of inorganic phosphate to organic

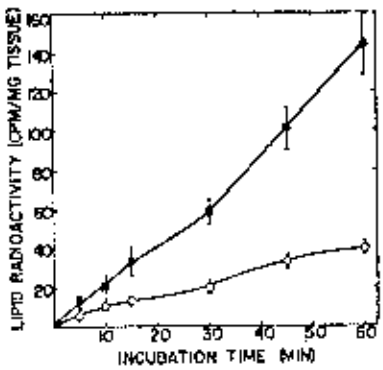

FIG. 1. Stimulation by vitamin D of incorporation of $^{32}P_i$ into the lipids of intestinal mucosa. ○—○, vitamin D deficient; ●—●, vitamin D fed. Each *point* is an average of six experiments ± standard deviation. Intestinal mucosa was incubated in Krebs bicarbonate buffer at 37° as described in text.

[2] A. Sudhgrove, personal communication.

[3] Aldrich Chemical Company, Milwaukee.

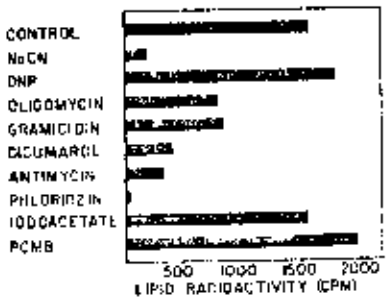

FIG. 2. The effect of metabolic inhibitors on the incorporation of $^{32}P_i$ into the lipids of intestinal mucosa from a vitamin D-deficient rat. Incubation was carried out in Krebs bicarbonate buffer at 37° for 30 minutes as described in the text. The samples were incubated for 10 minutes with the inhibitor before the addition of the $^{32}P_i$. The concentrations of inhibitors were: 10^{-4} M NaCN, 2×10^{-5} M dinitrophenol (*DNP*), 10^{-6} M oligomycin B, 10^{-7} M gramicidin S, 7×10^{-6} M Dicumarol, 5×10^{-7} M antimycin A, 10^{-3} M phlorizin, 10^{-4} M iodoacetate, and 10^{-3} M *p*-chloromercuribenzoate (*PCMB*).

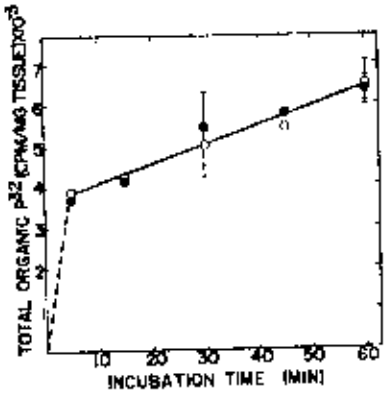

FIG. 3. Lack of an effect of vitamin D on $^{32}P_i$ incorporation into nonlipid organic phosphate. Intestinal mucosa was incubated in Krebs-bicarbonate buffer at 37° as described in text. Organic ^{32}P was determined by the method of Martin and Doty (21). ○—○, vitamin D-deficient; ●---●, vitamin D-fed. The 30- and 60-minute values are averages of five experiments ± standard deviation. *Other points* are averages of two experiments.

phosphate, water-soluble organic ^{32}P in the aqueous methanol phase from the lipid extraction procedure was measured by the method of Martin and Doty (21). The results, shown in Fig. 3, indicate that vitamin D did not affect the general incorporation of $^{32}P_i$ into organic compounds, and since ATP is a precursor of such phosphate compounds, there was no great effect of vitamin D on ATP labeling. However, it cannot be concluded that vitamin D does not affect some phosphate compounds other than the phospholipids, only that it does not have a general effect.

Because of the well established relationship between vitamin D and calcium metabolism, the effect of omitting calcium from the incubation medium was studied, and it was found that the omission of calcium has no significant effect on the labeling of the intestinal mucosa lipids from vitamin D deficient or vitamin D-fed rats.

The stimulation of $^{32}P_i$ incorporation due to vitamin D has been observed in rats fed high calcium rachitogenic diets as well as in those fed the nonrachitogenic diet routinely used. This demonstrates that the increase due to vitamin D is not merely a reflection of increased growth, since it has been previously observed that vitamin D depresses growth with diets of a high Ca:P ratio (15).

Further evidence that the stimulation due to vitamin D is not a result of increased growth is the increased lipid-^{32}P incorporation as early as 3 hours after administration of an oral dose of 2000 i.u. of vitamin D (Fig. 4). The radioactivity in the samples (*B* and *B'*) from rats that received vitamin D 3 hours before death was significantly greater than that in the samples from the vitamin D-deficient controls (*A* and *A'*) after 30 minutes ($p < 0.05$) and 60 minutes ($p < 0.01$) of incubation. The response 6 hours after the administration of vitamin D (*C* and *C'*) was obviously highly significant. In some experiments, the vitamin D-deficient controls were given 0.2 ml of pure Wesson oil at the same time that the others were given vitamin D; no difference was noted between these controls and those not given oil.

To ascertain whether or not specific components of the cells were affected by vitamin D, the distribution of the lipid-^{32}P in the subcellular fractions was studied. The whole cells were incubated for 60 minutes with $^{32}P_i$ and then fractionated. The results are given in Table I. The method for fractionation of intestinal mucosa cells was not totally satisfactory because of the difficulty in breaking the cells. Further, the difficulties were multiplied when attempts were made to fractionate cells which had been incubated previously. The stimulation by vitamin D of $^{32}P_i$ incorporation into the lipids was associated with all of the cell fractions, but in general the difference was greater in the mitochondrial and microsomal-supernatant fractions than in the nuclear-debris fraction.

The effect of vitamin D on the incorporation of $^{32}P_i$ into the lipids of kidney and liver slices was studied. The kidney slices (Fig. 5, *left*) showed a very large incorporation during the first 5 minutes of incubation. This initial phase was unaffected by vitamin D, but in the 30- and 60-minute samples the relative incorporation was increased by vitamin D. In liver slices (Fig. 5, *right*), a stimulation due to vitamin D was not observed.

Experiments conducted with ^{14}C-labeled phospholipid precursors showed that there was no effect of vitamin D on the

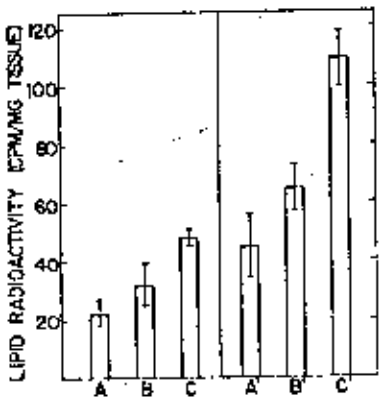

FIG. 4. Response to vitamin D of the incorporation of $^{32}P_i$ into intestinal mucosa phospholipids. Mucosa was incubated for 30 minutes (*A*, *B*, *C*) and 60 minutes (*A'*, *B'*, *C'*) in Krebs bicarbonate buffer at 37° as described in the text. The height of each *column* represents the mean of four to nine values. Standard deviations are shown as *short transverse lines*. *A*, *A'*, vitamin D-deficient controls; *B*, *B'*, given 2000 i.u. of vitamin D 3 hours before killing; *C*, *C'*, given 2000 i.u. 6 hours before killing. $B > A$ ($p < 0.05$); $B' > A'$ ($p < 0.01$).

incorporation of glycerol or serine into the phospholipids. The data are given in Table II.

Garbus *et al.* (19) reported a lipid in liver and kidney mitochondria which becomes labeled even more rapidly than phosphatidic acid and the polyglycerol phosphatides. They suggested that this might be a di- or triphosphoinositide. This was subsequently confirmed by Galliard and Hawthorne (26), who identified the compound as diphosphoinositide. The data given in Table III show that vitamin D had no effect on the rapid labeling of the mitochondrial lipids.

Chromatography of the lipid extracts by gradient elution from a silicic acid column was carried out as described under "Experimental Procedure." Seven peaks could be distinguished in the lipid extract of intestinal mucosa by this procedure. These are indicated by Roman numerals I to VII in Fig. 6. The peaks were identified by comparison with commercial preparations and by thin layer chromatography in at least two solvents. The "standards" for the thin layer chromatography were purified either by silicic acid column chromatography or by previous thin layer chromatography and elution of the main component. The commercial preparations were found to be quite impure

TABLE I

Distribution of lipid-^{32}P in subcellular fractions of intestinal mucosa from vitamin D deficient and vitamin D-fed rats

Intestinal mucosa was incubated in Krebs bicarbonate buffer at 37° for 1 hour as described in the text. In Experiments 1 to 3, the cells were broken by homogenizing without diluent in a Potter-Elvehjem homogenizer; in Experiments 4 to 6, by grinding with sand in a chilled mortar. The results are expressed as counts per minute per mg of mucosa.

Experiment	Whole cells		Nuclei + debris		Mitochondria		Microsomes + supernatant	
	−D	+D	−D	+D	−D	+D	−D	+D
1	34.6	176	41.2	103	7.9	25.6	18.1	58.0
2	75.5	312	27.8	85.6	6.8	36.4	32.5	144
3	64.0	314	16.7	35.9	7.9	30.4	25.3	97.8
4	31.0	72.0	13.5	12.7	2.3	11.6	4.9	16.1
5	82.8	160	41.2	35.0	5.4	10.0	9.2	12.1
6	52.9	220	38.5	83.7	3.1	14.7	7.4	27.6

TABLE II

Incorporation of glycerol-1,3-^{14}C and serine-3-^{14}C into phospholipids of intestinal mucosa

Intestinal mucosa was incubated in Krebs bicarbonate buffer at 37° as described in the text. The reaction mixture contained either 0.27 mM DL-serine-3-^{14}C (0.17 μc per ml, Volk Radiochemical Company) or 0.17 mM glycerol-1,3-^{14}C (0.17 μc per ml, Nuclear Research Chemicals). Results are expressed as counts per minute per mg of mucosa, and each number is an average of four experiments ± standard deviation.

Incubation time	^{14}C-Glycerol		^{14}C-Serine	
	−D	+D	−D	+D
min				
4			4.8 ± 1.3	4.3 ± 0.3
10	9.3 ± 2.0	8.0 ± 3.1	5.9 ± 1.5	6.2 ± 0.3
30	11.0 ± 3.2	9.2 ± 2.1	7.8 ± 2.3	7.8 ± 2.1
60	14.7 ± 3.9	11.2 ± 1.9	7.7 ± 1.0	8.9 ± 2.4

when first examined by thin layer chromatography. This heterogeneity has also been commented on by another investigator (27). The peaks from the silicic acid columns in most cases gave only a single spot on thin layer chromatography.

The two major components, Peaks IV and VII, have been identified as phosphatidylethanolamine and lecithin, respectively. The tail of VII was analyzed separately by thin layer chromatography to see whether it contained another component, but none was observed. Peak V has been identified as phosphatidylserine. Peak VI also contained some phosphatidylserine, but the major component appeared to be phosphatidylinositol. Ninhydrin analysis and organic solids run on corresponding samples showed that the profiles of the two analyses were identical through Peaks IV and V and that some ninhydrin-positive material was present in Peak VI, but it did not follow the organic solids curve. Peaks I, II, and III have not been identified. Peak II probably contained cardiolipin and also any phosphatidic acid which was present. Peaks I and II were never completely separated by the system used, and they occasionally appeared as a single peak. Peak I may not be a phospholipid. Clement and Dicostanzo (28) recently reported that some di- and monoglycerides of

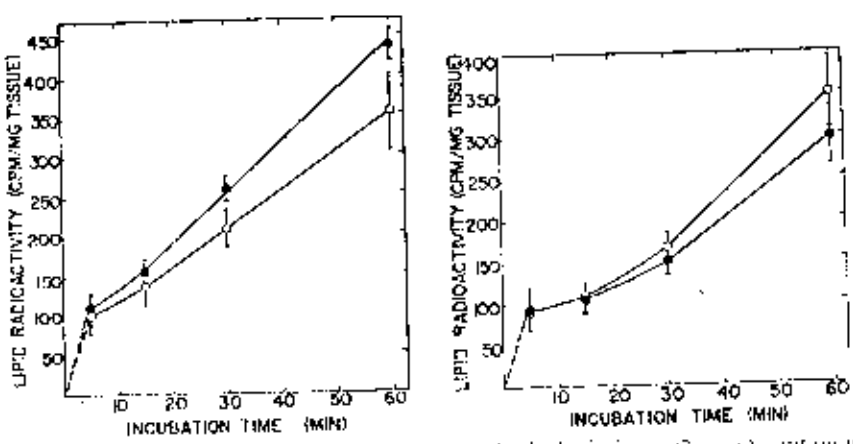

FIG. 5. Effect of vitamin D on the incorporation of ^{32}P, into lipids of kidney slices (*left*) and liver slices (*right*). Incubation was carried out in Krebs bicarbonate buffer at 37° as described in the text. Each *point* is an average of four to six experiments ± standard deviation. ○ - ○, vitamin D deficient; ● - ●, vitamin D fed. In the *left hand graph*, ● - fed, >○ deficient ($p < 0.01$) at 30 and 60 minutes. No significant differences were found at the 95% level in the experiments with liver slices (*right*).

intestinal mucosa were not removed from the silica acid column with chloroform, but were eluted with 2% methanol in chloroform.

Neither the total amount of phospholipid present in intestinal mucosa nor the amount of any particular phosphatide was affected by vitamin D. Table IV gives the percentage of the total phospholipid found in each of the peaks shown on the chromatogram (Fig. 6). The percentage found in Peaks V and VI is given as a single figure because of the variability and lack of separation of the two. This variation occurred because the amount of solids present in the fractions was in the lower range of the sensitivity of the determination. Phosphatidylethanolamine (IV) and lecithin (VII) constitute 65% of the phospholipid.

TABLE III

Rapid incorporation of $^{32}P_i$ into mitochondrial lipids from vitamin D-deficient and vitamin D-fed rats

Mitochondria from 0.5 g of tissue in 0.5 ml of isotonic sucrose were incubated for 5 minutes at 30° in medium containing 20 μmoles of Tris-HCl buffer, pH 7.4, 15 μmoles of potassium glutamate, 10 μmoles of $MgCl_2$, and 5 μc of ^{32}P, in a total volume of 1.0 ml. Each number is given as the average ± standard deviation (number of samples). All experimental values are corrected for a blank.

Tissue	No vitamin D	Plus vitamin D
	c.p.m.	*c.p.m.*
Liver	2775 ± 185 (8)	2490 ± 600 (8)
Kidney	3080 ± 1150 (6)	3310 ± 1350 (6)

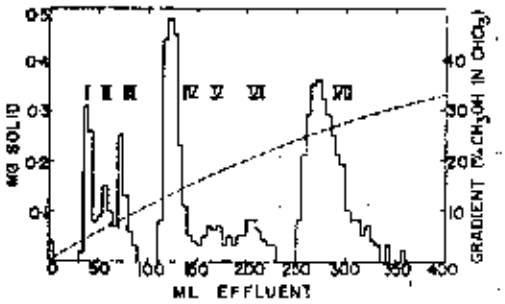

FIG. 6. Silicic acid column chromatography of phospholipid extract from 500 mg of intestinal mucosa. The conditions are described in the text. Identification of peaks: *I* (30 to 50 ml), unidentified, *II* (50 to 65 ml), cardiolipid, phosphatidic acid; *III* (65 to 90 ml), unidentified; *IV* (110 to 150 ml), phosphatidylethanolamine; *V* (150 to 180 ml), phosphatidylserine; *VI* (180 to 230 ml), phosphatidylinositol; *VII* (250 to 335 ml), lecithin.

TABLE IV

Percentage of total phospholipid contributed by each phosphatide from intestinal mucosa of vitamin D-deficient and vitamin D-fed rats

Peaks I to VII are as shown in Fig. 6. Each number is the average of at least 14 experiments ± standard deviation.

Peak	Vitamin D-deficient	Vitamin D-fed
I	8.3 ± 2.8	7.0 ± 2.5
II	6.4 ± 1.4	5.1 ± 1.3
III	8.0 ± 1.0	7.5 ± 1.3
IV	25.2 ± 1.5	26.7 ± 4.7
V + VI	13.2 ± 4.3	13.4 ± 3.0
VII	38.0 ± 1.3	39.4 ± 3.6

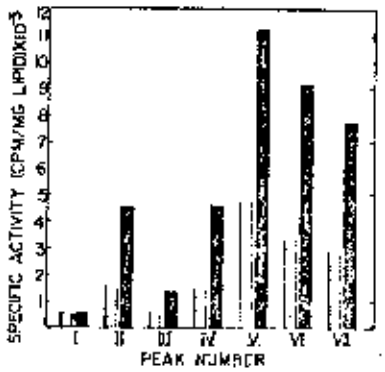

FIG. 7. Specific activity of phosphatide components I to VII (as shown in Fig. 6) from intestinal mucosa samples that were incubated for 60 minutes. *Solid black columns*, vitamin D-fed; *cross hatched columns*, vitamin D-deficient. The height of each column is the mean of four values.

Fig. 7 shows the specific activities (counts per minute per mg of lipid) × 10^{-3}) of the individual phosphatides from samples which had been incubated for 60 minutes. In every case except one (Peak I), vitamin D increased the specific activity approximately 3-fold. The small amount of radioactivity present in Peak I was thought to be contamination from Peak II. Thus it appeared that vitamin D, by increasing the incorporation of ^{32}P into each of the phospholipids, has a general effect on phospholipid metabolism rather than influencing a single compound.

The pattern of ^{32}P incorporation into the individual phosphatides differed widely: the incorporation into Peak II was rapid initially and leveled off; the specific activity of phosphatidylethanolamine increased linearly throughout the 1 hour of incubation; and lecithin was labeled very little during the first 20 minutes of incubation, but increased rapidly during the remaining 40 minutes. These patterns are similar to those observed by Karnovsky and Wallach (14).

DISCUSSION

The results show that vitamin D stimulates the incorporation of $^{32}P_i$ into the phospholipids of intestinal mucosa. The observance of a stimulation only 3 hours after the administration of 2,000 i.u. of vitamin D is of considerable importance. This is the earliest time at which a response to vitamin D has been observed when a dose of this magnitude has been given. The effect of vitamin D on calcium transport in everted intestinal sacs was detected 4 hours after administering 1,000 i.u. (9); however, Schachter has reported an increase in calcium transport only 1 hour after a 50,000-i.u. dose of vitamin D (29). Doses of this magnitude have not been studied with respect to phospholipid metabolism.

Attempts to stimulate the incorporation of ^{32}P into lipids by the addition of vitamin D *in vitro* were unsuccessful; however other workers have also failed to produce effects *in vitro* when whole cell systems were involved (6). Effects *in vitro* have been produced in mitochondrial systems used for studying vitamin D oxidation (11) and calcium translocation (10).

The incorporation of $^{32}P_i$ into the phospholipids is totally dependent on oxidative metabolism. Anaerobiosis completely prevented labeling of the lipids. This is in agreement with observations of other investigators (30, 31). The effects of 6

inhibitors agree closely with those found by Strickland (30). It is also of interest to note that at a concentration of 10^{-5} M, an inhibition of phospholipid labeling in brain slices by 2,4-dinitrophenol was observed by Strickland, while this concentration did stimulate oxidation. At concentrations of 10^{-4} and 10^{-3} M, dinitrophenol did inhibit the labeling of phospholipid in skin slices.

Several different compounds have been reported to stimulate $^{32}P_i$ incorporation into the phospholipids of various tissues: acetylcholine in salt gland and brain slices (9, 32), potassium in brain slices (32), estrogens in the uterus (33), and insulin in rat diaphragm (34). The stimulation in the small intestine due to vitamin D appears to be unique in the magnitude of the effect and the number of compounds affected. The only other general effect reported was that of estradiol in the uterus, and this appeared to be due to a large increase in the net synthesis of phospholipid.

Several experiments were tried in an attempt to examine the effect of vitamin D on phospholipids in subcellular systems, but these were not successful. Homogenates, mitochondria, and mitochondria plus mitochondrial supernatant of intestinal mucosa failed to incorporate $^{32}P_i$ in any of the systems tried. Incorporation into kidney and liver mitochondria did occur, but the major phospholipids did not become labeled. Other investigators (35, 36) have reported the inability of homogenates, mitochondrial, and microsomal systems to bring about an appreciable incorporation of $^{32}P_i$ into lecithin and phosphatidylethanolamine, yet considerable incorporation did occur in the polyglycerol phosphatides and phosphatidic acids. In any case, no difference due to vitamin D was observed in the incorporation into kidney and liver mitochondrial lipids.

Because the stimulation by vitamin D is observed in the intestine and kidney, but not in the liver, this phospholipid effect may be a primary action of vitamin D, possibly directly related to calcium transport. The lack of an effect due to calcium in the medium is not particularly disturbing to this postulation. The effect on the phospholipids may not require the presence of calcium, or, if calcium is required, sufficient calcium may have been present in the tissue.

SUMMARY

The addition of vitamin D to the diet of rats caused a 3-fold increase in the incorporation of ^{32}P-orthophosphate into the phospholipids of intestinal mucosa, while the incorporation into nonlipid organic phosphate was not altered by the vitamin. Smaller, but much smaller, stimulation of $^{32}P_i$ incorporation into the lipids of kidney slices, but not of liver slices, was observed. The incorporation of ^{32}C-labeled glycerol and serine into phospholipids of intestinal mucosa was not stimulated by vitamin D, nor was the total amount of phosphorus present in intestinal mucosa altered by vitamin D. A stimulation of the incorporation of ^{32}P into the lipids could be observed 3 hours after the administration of 2000 I.U. of vitamin D. The effect of the vitamin was not dependent upon the presence of calcium in the medium.

The incorporation of $^{32}P_i$ into the phospholipids was totally dependent on oxidative metabolism. Complete inhibition was caused by anaerobiosis, or by the addition of cyanide. Dicumarol, salmycin A, and phlorizin to the medium, and partial inhibition was caused by gramicidin and oligomycin. Dinitrophenol at a concentration of 2×10^{-5} M failed to inhibit.

Silicic acid column chromatography of the intestinal mucosa phospholipids indicated that vitamin D increased the specific activity of each of the phosphatides. Further identification of the phosphatides was accomplished by thin layer chromatography. The major phosphatides were found to be lecithin and phosphatidylethanolamine, comprising 39 and 26% of the total phospholipid, respectively.

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The Effect of Parathyroid Hormone and Vitamin D on Serum Calcium in Rats

By

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Abstract

Toverud, S. U. *The effect of parathyroid hormone and vitamin D on serum calcium in rats.* Acta physiol. scand. 1964. 62, 391—406. — Conflicting evidence exists regarding the hypothesis that vitamin D is essential for the calcium-mobilizing action of parathyroid hormone. A re-investigation was undertaken by feeding weanling vitamin D-deficient rats a vitamin D-free diet containing 0.7 % calcium and 0.6 % phosphorus (diet D_1) for 3 weeks. At day 1, when the mean serum calcium level was 7.1 mg/100 ml, the diet was replaced by 10 % glucose in saline. Different groups received either A) 100 I.U. vitamin D on day 2; B) injections of 200 and 100 units parathyroid extract (PTE) on days 4 and 5, respectively; C) a combination of treatments A and B; or D) saline injections. The serum calcium levels, determined 6 hours after the last PTE injection, permitted calculation of mean individual increase expressed in mg/100 ml: A) [illegible] 1.01, B) [illegible] 0.86, C) [illegible] 2.58, D) [illegible] 0.57.

Uncommon feeding with diet D_1 or one containing less protein resulted also in a significant response to PTE. Parathyroidectomy, but not sham operation, resulted in a substantial fall in serum calcium in rats fed diet D_1.

The conclusions are: 1) The calcium-mobilizing action of parathyroid hormone in the rat is either independent of, or requires only minute amounts of, vitamin D. 2) At the one dose level investigated, no evidence for interaction between the effects of vitamin D and parathyroid extract was found when intestinal calcium absorption was minimal.

Vitamin D is now regarded as one of the two major factors which maintain a normal plasma calcium level (McLean 1960), the other factor being the classic parathyroid hormone. This concept is partly based on experiments in vitamin D-deficient rats showing that vitamin D can bring about an increase in the serum calcium level which is partly independent of the effect of the vitamin on intestinal calcium absorption (Carlsson 1952, Nicolaysen and Eeg-Larsen 1956). Recently it has been claimed that vitamin D has the additional role in calcium homeostasis of permitting the parathyroid hormone to [illegible] its action upon the bone and possibly also other target organs (Rasmussen [illegible] Jefferstein 1962). This postulate is partly founded upon experiments by Harrison, Harrison and Park (1958), showing that injections of parathyroid extract into vitamin D-deficient young rats did not raise the serum calcium level unless the rats were pretreated with vitamin D. Similar results have been obtained by Marnay and Raoul (1959)

TABLE I. Composition of vitamin D-free diets[1]

	"High-protein"[2] (%)	"Low-protein"[2] (%)	Stock diet (%)
Dextrose	—	23.6	—
Cornstarch	—	42.5	—
Ground whole wheat, rye, oats and corn	—	—	60
Wheat germ	—	—	5
Wheat flour (70% extraction)	59.78	—	—
Casein, acid-precipitated	20	17	—
Egg albumin, crystalline	5	—	—
Dried skimmed milk	—	—	24
Arachis oil	10	7.5	5
Brewer's yeast	—	4.7	5
B-vitamin mixture[4]	0.1	—	—
Salt mixture[5]	—	4.7	—
Salt mixture (Ca- and P-free)[4]	2.0	—	—
Choline chloride	0.05	—	—
Calcium carbonate	1.75	—	5
Potassium diphosphate	1.32	—	—
Sodium chloride	—	—	5
Protein	31	18	16
Calcium	[6]0.70–0.73	0.61	0.52
Phosphorus	[6]0.54–0.59	0.67	0.54

[1] Containing no known sources of vitamin D.
[2] As described by Harrison et al. (1958).
[3] Supplements are described in the text.
[4] As described by Gran (1960a).
[5] As described by Wesson (1932).
[6] Range of 6 batches (6 samples of each).

and Marnay (1961). Most recently Rasmussen et al. (1963) reported results that were confirmatory with regard to low and moderate doses of parathyroid extract. However, treatment with massive doses of the purified hormone resulted in a response also in vitamin D-deficient rats. Other workers have demonstrated that vitamin D deficiency does not prevent the calcium-mobilizing action of moderate doses of parathyroid extract in adult rats (Toverud 1963), mice (Nichols, Schartum and Vaes 1963) and hens (Hertelendy and Taylor 1960).

The relationship between vitamin D and parathyroid hormone has also been studied on the level of cell mitochondria from rats. It has been demonstrated that parathyroid hormone stimulates a vitamin D-dependent release of calcium from kidney mitochondria (DeLuca, Engstrom and Rasmussen 1962) and a vitamin D-independent uptake of inorganic phosphate by liver mitochondria (Sallis, DeLuca and Rasmussen 1963).

Because of the discrepancy in results from experiments *in vivo* and because of the importance of this type of information for the understanding of the mechanism of action

of vitamin D and parathyroid hormone, it was desirable to re-investigate the separate and combined effects of the two agents on the serum calcium level in mature and young vitamin D-deficient rats. In addition, young rats suffering from vitamin D deficiency were subjected to parathyroidectomy or a sham operation.

A preliminary account has been given at the 6th International Congress of Nutrition, Edinburgh, Aug. 9—15, 1963 (Toverud 1964).

Materials and methods

Rats

All rats came from the same colony of interbred strains of hooded and albino rats which has been maintained at this institute for over 30 years without additions from other colonies. Diets containing minimal amounts of vitamin D have been given to the stock rats for the last 20 years. The rats were weaned when approximately 24 days old. After a pre-treatment period of 20 days or longer, rats of both sexes were divided into treatment groups by formal randomization. In the experiment with adult rats only females were used and the pre-treatment period started at the age of 6 months.

Diets

The composition of the diets is given in Table I. The mothers of the experimental rats were fed during pregnancy and lactation the stock diet supplemented with whole milk ad libitum and weekly rations of bread, boiled meat, and carrots or spinach. In general, skimmed milk was substituted for whole milk during the last half of the lactation period. From time of weaning and throughout the experimental period the rats were fed one of the two vitamin D-free purified diets, except in one experiment (37) in which this diet was replaced by a calcium-free regime consisting of a solution of 10 per cent glucose and 0.9 per cent NaCl in de-ionized water (later referred to as glucose-in-saline diet) during the period of administration of vitamin D and parathyroid extract. All rats received supplements of 150 I.U. vitamin A-acetate every two days except the rats in exp. 37 while they received the glucose-in-saline diet.

Criteria for vitamin D deficiency

The pregnant and lactating mothers of the experimental rats were fed the same diet as mothers of rats used regularly in this laboratory for vitamin D assay (2-point factorial dose radiographic curative assay as described by Bliss and György 1951). After consuming a rachitogenic (high calcium, low phosphorus) diet for 10—12 days from the time of weaning, these rats develop severe rickets, as evidenced by lack of roentgenologically visible epiphyseal calcification of the proximal end of the tibia. A substantial healing response is frequently obtained with as little as 0.4 units of vitamin D daily for 12 days and regularly with 0.8 units, indicating that the rats contain only traces of vitamin D prior to assay. Since the assay rats and the present rats had identical maternal and dietary backgrounds until time of weaning, since the subsequent diets for the present rats contained no known source of vitamin D, and since the two groups were equally exposed to daylight, it was assumed that the present rats had about the same degree of vitamin D deficiency as the rachitic assay rats.

Parathyroidectomy

Parathyroidectomy was carried out by means of hot wire cautery as described in detail by Hirsch, Gauthier and Munson (1963).

Bleeding

1—1.5 ml samples of blood were taken from the tail while the rats were lightly anesthetized with ether. In general, blood samples were taken immediately before and after parathyroidectomy or the periods of treatment with parathyroid extract or vitamin D, respectively. The time of the latter sample will be referred to as the second bleeding.

Parathyroid extract and vitamin D

The parathyroid extract was assayed by Eli Lilly Co. to contain 375 units per ml. The extract was stored at 7° C for 1.5 years prior to use and may not have retained its full potency. When parathyroid extract was given, the animals received by subcutaneous injection 200 and 100 units 24 and 6—8 hours, respectively, before the second bleeding, assuming full potency of the

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TABLE II. Precision of calcium determinations expressed as the standard deviation of a single determination (S. D., Eq. 1)

Number of samples	Serum calcium range (mg/100 ml)	S. D. (mg/100 ml)
7	2.5—4.1	0.09
20	4.3—6.7	0.06
25	7.2—10.4	0.05

extract. Comparable injections into control rats contained an equal volume of physiological saline solution. In the experiment with adult rats 0.75 ml parathyroid extract (280 units) was injected.

Vitamin D (D-dry-fed D_3 — 2000, Philips — Duphar) was administered in arachis oil by dropper pipette 3 days before bleeding. This and similar preparations are routinely standardized in this institute by the above-mentioned 2-point bioassay.

Calcium analyses

Calcium analyses were carried out in duplicate on 0.2 ml aliquots of serum from freshly collected blood according to the method of Munson et al. (1955) with the following modifications: a) Into the cuvette were pipetted 6 ml de-ionized water and 0.2 ml of an aqueous solution (1 mg/ml) of ammonium purpurate in addition to serum: 5 drops of 9 N NaCl and 1 drop of cetyl alcohol. b) With the cuvette placed in a Coleman Junior Spectrophotometer 0.1 ml increments of a solution of EDTA (0.18 g/l) were added.

The accuracy of the method was tested in a recovery experiment. The addition of 10 μg calcium to each of 13 serum samples, containing from 13.8 to 20.1 μg calcium, resulted in a mean recovery of 10.1 μg. The standard deviation of this series was 0.3 μg, corresponding to a concentration of 0.2 mg/100 ml. In Table II, the precision of the method is again indicated by the standard deviation, based on the differences between the 2 determinations of each sample (Eq. 1).

It is of interest to note that Wilk and King (1963), in comparing several calcium microprocedures, found only one procedure, that of Munson et al. (1955), to be precise[1] to 0.2 mg/100 ml calcium.

Phosphorus analyses

Phosphorus in the diets was determined by the method of Fiske and SubbaRow (1925) modified so as to ensure complete hydrolysis of any pyrophosphate present after digestion of the diet with nitric and perchloric acids.

Statistical treatment

1. Precision of calcium determinations. The precision was defined as the standard deviation (S.D.) of a single determination, calculated as follows:

$$\text{S.D.} = \sqrt{1/n \sum_{i=1}^{n} \sum_{j=1}^{2} (x_{ij} - \bar{x}_i)^2} \qquad \text{(Eq. 1)}$$

where subscript i refers to individual serum samples, subscript j refers to the first or second determination of the ith sample and n refers to the number of samples in each series: 7, 20 or 25.

2. Test for additivity of separate effects of the treatments in experiment 34: Analysis of variance, 2-way classification with interactions. *Assumptions:* A correction term may possibly be added to the sum of the separate effects. *Null-hypothesis:* The correction term (interaction between the main effects) is equal to zero. Since the number of observations differed from one cell to the other, the formulas for the case of unequal numbers of observations were used for calculating the F value (variance ratio) as described by Scheffé (1959).

[1] Precision was defined as the maximum deviation between the values for each sample when multiple determinations were carried out on several 0.2 ml serum samples (Wilk 1963).

TABLE III. Effect of vitamin D and/or parathyroid extract on the serum calcium[1] of parathyroidectomized, vitamin D-deficient, adult female rats

Mean ± standard error of 7 rats in each group. The 24-month-old rats had been fed for 18 months the vitamin D-free stock diet with weekly supplements of carrots or spinach. The doses of vitamin D (D) and parathyroid extract (PTE) were 250 I.U. and 270 units, respectively. Range of body weights: 195—256 g.

Days after PTX[2]	Serum calcium (mg/100 ml) Group I Level	Group I Rise[3]	Group II Level	Group II Rise	Group III Level	Group III Rise
6	4.46 ± 0.23		5.20 ± 0.26	—	4.92 ± 0.18	—
10		—	D	D	D	D
12	PTE	PTE	—	—	PTE	PTE
13	6.67	2.20 ± 0.23	7.16	1.96 ± 0.31	8.08	4.24 ± 0.24
20	5.52	—	6.33	—	6.41	—
20	PTE	PTE	PTE	PTE	—	—
21	6.80	1.28 ± 0.17	8.15	1.82 ± 0.19		
25	5.15		[4]6.52	—	—	—

[1] Analyzed in single 0.5 ml samples of serum according to the method of Munson et al. (1955) without the noted modifications.

[2] Parathyroidectomy.

[3] Individual rise above the previous level.

[4] 6 rats.

3. Test for the effect of sex on the response to parathyroid extract. A test for additivity of separate effects of parathyroid extract and sex was based on results from rats eating 2 different diets (exps. 37 and 41): Analysis of variance, 3-way classification with interactions, the 3 factors being parathyroid extract, sex and diet. *Assumptions:* A correction term may possibly be added to the sum of the 2 first separate effects. *Null-hypothesis:* The correction term (interaction between the 2 first separate effects) is equal to zero. The usual analysis of variance test for this purpose was used for calculating the F value (variance ratio) (Scheffé 1959).

4. t test. After the analysis of variance in the form of a 2-way classification was carried out (exp. 37), the standard deviation within cells was used as the basis for a t test (51 degrees of freedom) with the purpose of testing the significance of the mean individual change in serum calcium in cells (groups) I and II:

$$t = \bar{x}/(\text{S.D.}/\sqrt{n}) \qquad \text{(Eq. 2)}$$

where n is the number of observations in the cell, and S.D. (standard deviation within cells) is 0.68 (N = 55, d.f. = 51).

Results

Adult rats

In the first experiment, 24-month-old female rats were parathyroidectomized after having consumed the vitamin D-free stock diet for 18 months. Six days after the operation the serum calcium level was uniformly low, thus indicating complete removal of

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TABLE IV. Plan of the experiment on the effect of parathyroid extract (PTE) and/or vitamin D on the serum calcium of vitamin D-deficient young rats

On the day of the first bleeding six-week-old rats, having been fed the "high-protein" diet for 20 days, were given a solution of 10 per cent glucose and 0.9 per cent NaCl in deionized water instead of solid food and tap water.

Time before second bleeding	Group I Control	Group II PTE	Group III Vit. D	Group IV Vit. D + PTE
4 days	First bleeding	First bleeding	First bleeding	First bleeding
3 days	-		Vit. D[3]	Vit. D[3]
24 hrs	0.9% NaCl[2]	PTE 200 u.	—	PTE 200 u.
6 - 8 hrs	0.9% NaCl[2]	PTE 100 u.	—	PTE 100 u.
0 hrs	Second bleeding	Second bleeding	Second bleeding	Second bleeding

[1] 100 I.U., [2] 0.30 ml, [3] 0.25 ml.

the glands (see Table III). On the 10th day, rats in groups II and III received 250 I.U. vitamin D and 2 days later the rats in groups I and III received an injection of approximately 280 units parathyroid extract. Blood samples taken next day showed that there was a mean rise in the serum calcium of approximately 2 mg/100 ml in groups I and II and a rise twice as high in group III. A subsequent return towards the base (6th day) level was indicated by the 20th day values. These results strongly suggest that vitamin D-deficient, parathyroidectomized rats can respond to treatment with vitamin D or parathyroid extract or both by a measurable rise in the serum calcium level. However, the exact magnitude of the rise cannot be equated with response to treatment since results from an untreated control group are lacking. The increase in serum calcium in groups I and II one day after another injection of parathyroid extract and the return to the base (20th day) level within 5 days provides further evidence for the conclusion that vitamin D deficiency need not prevent parathyroid extract from raising the serum calcium level in adult female rats.

Response to parathyroid extract and/or vitamin D in young rats

In order to compare properly the effect of parathyroid extract obtained in the presence of vitamin D with that obtained in the absence of the vitamin, it was necessary to eliminate or at least minimize the powerful effect of vitamin D on intestinal calcium absorption by feeding all rats a calcium-free diet. Increased absorption could lead to increased deposition of bone salt available for mobilization. According to Munson, Hirsch and Tashjian (1963) a distinction between the calcium-mobilizing and the other effects of vitamin D is indicated.

The plan for exp. 37 is outlined in Table IV. Six-week-old rats, which had been fed the "high-protein" diet for approximately 3 weeks, were given the glucose-in-saline diet at the time of the first bleeding. Three days before the second bleeding the rats in groups III and IV received 100 I.U. vitamin D. 24 hours before the second bleeding

TABLE V. Effect of parathyroid extract (PTE) and/or vitamin D on the serum calcium of vitamin D-deficient young rats (experiment 37)

The treatment plan is given in Table IV. The mean and standard error (S. E.) of serum calcium is given as mg/100 ml

		Group I Control	Group II PTE	Group III Vit. D	Group IV Vit. D + PTE
First bleeding	Mean	7.42	6.89	7.41	6.83
	S. E.	± 0.18	± 0.26	± 0.26	± 0.21
	No.[1]	16	11	15	13
Second bleeding	Mean	6.85	7.87	8.45	9.32
	S. E.	± 0.20	± 0.21	± 0.12	± 0.17
	No.	16	15	15	16
Individual increase	Mean	− 0.57	+ 0.86	+ 1.04	+ 2.58
	S. E.	± 0.13	± 0.25	± 0.17	± 0.15
	No.	16	11	15	13

[1] Number of observations.

the rats in groups II and IV received injections of 200 units of parathyroid extract. The same rats received another injection of 100 units of the extract 6—8 hours before the second bleeding. The rats in group I received control injections of saline on the same two occasions.

From Table V it can be seen that the initial serum calcium levels (first bleeding) were clearly below normal, thus showing that vitamin D deficiency had been achieved. Both the mean final calcium level (second bleeding) and the mean individual increase for each group from first to second bleeding illustrate the effects of the 4 treatments, but only the individual increase will be used in the following discussion. A statistically significant ($P < 0.01$) decrease was observed for the control group. Undoubtedly this is because there was a negligible amount of calcium in the diet.

In group II the parathyroid extract treatment had resulted in an increase in serum calcium of almost 0.9 mg/100 ml, significant by t test at $P < 0.001$. This result agrees with the observations in the adult rat described above, but not with those of Harrison et al. (1955). The response to vitamin D in group III was approximately 1 mg/100 ml, which means that a calcium-free regime does not prevent a rise in serum calcium after vitamin D administration.

The combined vitamin D-parathyroid extract treatment in group IV resulted, as expected, in a much greater rise in serum calcium than did the other treatments. The serum calcium response in this group is very nearly equal to the algebraic sum of the corresponding values for the three first groups, thus suggesting that the effects of vitamin D and parathyroid extract, given together, are additive. In order to test the hypothesis of additivity an analysis of variance was carried out in the form of a two-way classification with interaction. The design of the test is indicated in Table VI, where the mean individual increase in serum calcium for each group (Table V) has been written in its

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TABLE VI. Design of test for additivity of main effects of parathyroid extract (PTE) and vitamin D (D) (experiment 37)

$\bar{x}$ refers to the mean individual increase in serum calcium (Table V).

	No PTE	PTE
No D	Group I $\bar{x} = -0.57$	Group II $\bar{x} = -0.86$
D	Group III $\bar{x}$ 1.04	Group IV $\bar{x} = +2.58$

TABLE VII. Body weight before and weight loss after feeding rats a 10 per cent glucose-in-saline solution (experiment 37)

The rats were re-weighed after 3 or 4 days' feeding. The figures are mean, standard error (S. E.) and number of observations (No.)

Sex		Body weight (g)	Period (days)	Weight loss (g) Group I	Group II	Group III	Group IV
♂ + ♀	Mean	—	3	14.7	13.5	—	—
♂ + ♀	Mean	—	4	19.2	14.5	13.3	15.1
	S. E	—		± 1.0	± 0.9	± 1.1	± 0.9
♂	Mean	105.9	4	21.5	14.5	14.1	16.3
	No.	38		8	6	7	7
	S. E.	± 1.9					
♀	Mean	89.4	4	16.6	14.4	12.6	13.7
	No.	26		7	5	8	6
	S. E.	± 1.5					

appropriate cell. The null-hypothesis to be tested was that the interaction between the separate effects of vitamin D and parathyroid extract is equal to zero (absence of interaction is equivalent to additivity). The standard deviation (S.D.) within cells (N = 55, d.f. = 51) is 0.69 mg/100 ml and the F value (variance ratio) is 0.06, with 1 and 51 degrees of freedom. Since the critical "F" value for the 5 per cent significance level is 4.03, and thus far greater, one cannot reject the null-hypothesis of the interaction being equal to zero. It must therefore be concluded that the present data give no evidence against the hypothesis that the effects of vitamin D and parathyroid extract on the serum calcium level are additive and it would appear that the two treatments do not potentiate each other.

a. Effect of weight loss

After the change in diet from solid food to glucose-in-saline solution the rats consistently lost weight (see Table VII). The mean losses for the first 3 days, before control and

TABLE VIII. Effect of parathyroid extract (PTE) on the serum calcium of vitamin D-deficient rats consuming different diets

Mean ± standard error. Age and weight were recorded 1, 2 or 3 days before the first injection. The rats received the experimental diets "high-protein" in experiment 38 and "low-protein" in experiment 41 from time of weaning and throughout the period of observation. The experimental rats were given injections of 200 and 100 units parathyroid extract (approximately 24 and 6 units, respectively) before bleeding. The control rats received comparable injections of physiological saline solution. Rats in experiment 41 were also bled 1 day before the first injection was given.

Exp. and group	Age (days)	Weight (g)	No. rats	Serum calcium (mg/100 ml) Before treatment	After treatment	Difference
38 PTE	45	102 ± 4	17	—	8.77 ± 0.27	—
38 Control	45	101 ± 3	18	—	7.39 ± 0.29	
41 PTE	47	81 ± 2	14	5.65 ± 0.19	7.35 ± 0.20	1.70 ± 0.15
41 Control	47	78 ± 2	13	5.80 ± 0.17	6.22 ± 0.16	0.42 ± 0.10

parathyroid extract injections were given, were essentially the same for groups I and II. Unfortunately, rats in Group III and IV were only weighed at the end of 4 days. It is seen that the high 4-day figure for Group I is almost entirely due to heavy weight loss in the males during the last day. The reason for this deviation is unknown, but the importance of it is doubtful since males and females did not differ much in the other groups. Although weight loss was an undesirable effect of the liquid diet, these figures indicate that it was a fairly constant factor. It was consequently assumed that the weight loss did not materially affect the relative response of the serum calcium level to the different treatments described above.

b. Effect of diet

Confirmation of the positive response to parathyroid extract in the absence of vitamin D was sought under different conditions in exp. 38. The rats were fed the "high-protein" diet throughout the experiment and they were bled only at the end of the extract treatment (see Table VIII). The age, weight, dietary pre-treatment and the experimental procedure were otherwise essentially the same as for groups I and II in exp. 37. Table VIII shows that there was a statistically significant ($P < 0.01$) difference of approximately 1.4 mg/100 ml in the serum calcium level between control and parathyroid extract-injected rats. Consequently, under these conditions as well, parathyroid extract brought about a rise in the serum calcium of vitamin D-deficient rats.

In an attempt to resolve the difference in response to parathyroid extract in rats between the present investigation and that of Harrison et al. (1956), the same diet ("low-protein") as that used by these workers was fed to rats from time of weaning in exp. 41. The mothers of these rats had been given skimmed milk instead of whole milk from the 7th day of lactation as an added precaution against the transfer of even minute amounts

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TABLE IX. Effect of parathyroidectomy (PTX) and sham operation (Sham) on the serum calcium of vitamin D-deficient young rats

Mean ± standard error. Age and weight were recorded 1, 2 or 3 days before time of the operation. The operated rats represent that fraction of a larger group (35 rats in both cases) which had the lowest serum calcium levels. The "high-protein" diet was fed to all rats throughout the experiment. The calcium content of the diet in experiment 38 was reduced to 0.52% 29 days before the operation.

Exp. and group	Age (days)	Weight (g)	No. rats	Serum calcium (mg/100 ml) Before operation: 29 days	2 days	After operation	Individual difference
39 Sham	48	106 ± 6	6	—	8.10 ± 0.21	[1]7.81 ± 0.35	0.38 ± 0.21
39 PTX	48	113 ± 7	6	—	8.05 ± 0.23	[1]5.57 ± 0.39	−2.47 ± 0.3[illegible]
38 PTX	76	151 ± 6	7	6.23 ± 0.20	6.65 ± 0.16	[2]3.83 ± 0.06	−2.82 ± 0.1[illegible]

[1] 5 hours after the operation.
[2] 7 hours after the operation.

of vitamin D through the mothers' milk. At the end of 3 weeks' pre-treatment the rats were bled, to deal with parathyroid extract or saline and bled again, as in exp. 37. Table VIII [illegible] that the initial serum calcium level for both groups was far lower than in the [illegible] experiments. The serum calcium rise after parathyroid treatment was highly significant ($P < 0.001$) and was more than 4 times as high as the rise after control treatment, the latter rise being of questionable significance. Whether or not the low initial serum calcium level is the result of a higher degree of vitamin D deficiency, these results show that parathyroid extract may be active also in the face of severe hypocalcemia.

c. Effect of sex

Since rats of both sexes were used in all experiments, it was possible to test statistically the influence of sex on the serum calcium response to parathyroid extract. The results from groups I and II (control and PTE) from exp. 37 and 41 were used in an analysis of variance of a 3-way classification with interactions. The null-hypothesis was: interaction between parathyroid extract and sex is equal to zero. (Additivity is equivalent to zero interaction.) The calculated F (variance ratio) value with 1 and 32 degrees of freedom was 0.20 while the critical value at the 5% level is 4.14. Consequently, the present data give no reason to believe that males and females respond differently to parathyroid extract.

Response to parathyroidectomy

In general, results obtained after injection of an impure extract of an endocrine gland require more cautious evaluation than observations made after the removal of the same gland. Additional experiments were therefore carried out with rats before and after parathyroidectomy. In both experiments (Table IX) the rats having the lowest serum calcium levels were selected so as to give added assurance of a high degree of vitamin D deficiency. In exp. 39, 7-week-old rats were bled both 2 days before and 5 hours after parathyroidectomy or a comparable sham operation. The results showed a highly significant ($P < 0.001$) decrease of almost 2.5 mg/100 ml in the serum calcium in the parathyroidectomized animals, while there was essentially no change in the control rats.

In exp. 38 all 7 rats were bled on 3 occasions, 29 and 2 days before and 7 hours after parathyroidectomy. The initial serum calcium levels were remarkably constant in spite of the calcium content of the diet having accidentally been decreased from approximately 0.7 to 0.5 per cent 29 days before the operation. The reason for the difference in the initial serum calcium levels between the 2 experiments is not known. As soon as 7 hours after the removal of the parathyroids very severe hypocalcemia was found, the decrease in serum calcium being almost 3 mg/100 ml. This decrease is significant by t test at $P < 0.001$. A very similar response was obtained in adult rats by G. et al. (1960 b). In view of the marked and rapid nature of the decrease in serum calcium and in view of the significant decrease observed in the control group in exp. 39, it seems highly probable that the decrease was indeed due to the removal of the parathyroid glands, even though sham operated controls were not included in the experiment. Since removal of the source of parathyroid hormone resulted in a prompt fall in serum calcium, the hormone previously secreted by the intact glands must have been responsible for maintaining the higher serum calcium level.

After this work was carried out, Hirsch, Gauthier and Munson (1963) published a report showing that the fall in serum calcium subsequent to parathyroidectomy by cautery is not entirely due to the removal of the parathyroid hormone source, but apparently also to the action of a hypocalcemic substance released from the thyroid gland. Among the parathyroidectomized rats in exp. 39, the occurrence of tetany in 5 rats and death of 3 rats the day after the operation indicates that there was a long-lasting serum calcium depression which probably could only be ascribed to removal of the calcium-mobilizing hormone. Thus, further evidence has been obtained for the hypothesis that parathyroid hormone is active also in rats with a high degree of vitamin D deficiency.

Discussion

Parathyroid action during vitamin D deficiency

Evidence for an effect of parathyroid hormone on the serum calcium level has been obtained in vitamin D-deficient rats by subjecting them to injections of parathyroid extract or parathyroidectomy in several different experiments. Since the rats were practically free from vitamin D, this evidence could be interpreted to mean that either the action of the hormone is completely or partially independent of vitamin D or the action requires only traces of the vitamin. If the latter were true, one would expect the present rats to be less vitamin D deficient than the rats used by investigators who failed to obtain a response from moderate doses of parathyroid extract. An appraisal of the criteria for vitamin D deficiency, used by different authors, will be instrumental in shedding light on the question of the relative vitamin D status of the present rats.

In one report (Harrison et al. 1948) the evidence for vitamin D deficiency was reduced concentrations of citrate in bone and serum, normal serum phosphorus levels and hypocalcemia. Of these, only the last was measured in the present investigation, and it was found to be of the same severity when the "low-protein" diet was used in both investigations (exp. 41, Table VIII). The dietary calcium and phosphorus contents in

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this experiment were, by analysis, 0.61 and 0.67 per cent respectively, as against 0.7 and 0.56 per cent respectively, as reported by Harrison et al. (1958). This difference in dietary composition is probably of negligible importance for the serum calcium level (Steenbock and Herting 1955). It thus seems reasonable to assume that, in these two cases, equal degrees of hypocalcemia indicate equal degrees of vitamin D deficiency. In addition, the present rats could be assumed to contain no more vitamin D than do rachitic rats prior to their being assayed for vitamin D by a curative bio-assay method (see Criteria for vitamin D deficiency). The only possible sources of vitamin D in the diet of the stock rats (the mothers of the present rats and of the bio-assay rats) are 3—4 g portions of cod's liver twice a month until time of delivery and whole milk ad libitum during the 2 first weeks of lactation only. Since all experiments were carried out during the winter months, the mothers' vitamin D supply from the milk (approximately 0.2 I.U. daily) was undoubtedly too low to provide the offspring with more than traces of the vitamin. The body content of vitamin D would be further reduced during the subsequent 3-week period of rapid growth.

Rasmussen et al. (1963) report that they employed young rats obtained commercially from "... colonies fed ordinary diet containing minimal quantities of vitamin D." The specific criterion for vitamin D deficiency was a lack of growth when the young rats consumed a vitamin D-free, purified diet. When the diet contained 0.47 per cent calcium and 0.3 per cent phosphorus, the serum calcium levels were 6.9 and 5.2 mg/100 ml, respectively, in two groups at the end of 3 weeks, thus indicating the existence of approximately the same degree of hypocalcemia as was found by Harrison et al. (1958) and in the present study (Table V and VIII).

It should be apparent from this discussion that the methods used by the present author satisfy the requirements emphasized by Rasmussen et al. (1963) for procuring vitamin D-deficient rats, namely maintaining rats for generations on diets containing minimal amounts of vitamin D and employing standardized purified diets.

The fact that Rasmussen et al. (1963) were able to raise the serum calcium in vitamin D-deficient rats by injecting massive doses (2,000 units) of parathyroid extract is, according to the present author, indicative of the idea that vitamin D is not essential for the calcium-mobilizing action of parathyroid hormone in rats. However, this idea is apparently not supported by other results obtained by Rasmussen et al. (1963). They showed that only 0.03 I.U. vitamin D daily for 6 days was sufficient to maintain the serum calcium at approximately 7.4 mg/100 ml until parathyroidectomy, within 4.5 hours, brought the level down to 4.6 mg/100 ml. In the vitamin D-deficient rats, on the other hand, the serum calcium level of 4.9 mg/100 ml was unaffected by the same operation.

Since differences between investigations in the degree of vitamin D deficiency probably were negligible, other explanations must be sought for the discrepancy between the present results and those of Rasmussen et al. (1963) and Harrison et al. (1958). The latter group of investigators used only male rats as against rats of both sexes in the present work. However, sex was not found to have any effect on the response to parathyroid extract in the present investigation. Finally, there was a difference in the strain of rats used, and since there was no opportunity for testing other strains in this laboratory, the possibility remains that rat strains differ with regard to degree of parathyroid dependence on vitamin D.

The present data are in agreement with those from previous work with adult, vitamin D-deficient rats in this laboratory (Toverud 1963). It was shown that the serum calcium

level 6 and 12 days after parathyroidectomy by cautery was approximately 5 mg/100 ml, while the pre-operative level was approximately 9 mg/100 ml. A clear return of the serum calcium to and beyond the pre-operative level was subsequently obtained with treatment with parathyroid extract.

Although the conclusion reached by Marmay and Raoul (1959) and Marmay (1961) is the same as that of Harrison et al. (1958), the results obtained by Marmay (1961) in one experiment (a serum calcium increase of 0.34 ± 0.05 mg/100 ml) may have indicated the presence of a small response to parathyroid extract in vitamin D-deficient parathyroidectomized rats.

Nichols, Schlatum and Vaes (1963) were able to illustrate parathyroid action with vitamin D-deficient mice in two ways. After injecting parathyroid extract, these authors found an increase in the serum levels of calcium and phosphorus as well as an increase in passive (thermodynamic) solubility of bone mineral, the latter observation stemming from experiments with untreated and heat-inactivated bone samples incubated *in vitro*.

The present results seem to warrant the general conclusion that in the special strain of rats maintained at this laboratory the calcium-mobilizing action of parathyroid hormone is either independent of the presence of vitamin D or dependent on such minute amounts of the vitamin as may be present in severely rachitic rats.

Effect of vitamin D on the serum calcium

The present results strongly indicate that vitamin D, administered in a moderate dose (100—120 I.U. per 100 g body weight), can stimulate a rise in the serum calcium of rats deprived of either the parathyroid glands or of the dietary supply of calcium. In the former case (Table III) the rise was probably due to the intestinal action of vitamin D as well as its calcium-mobilizing action.

Carlsson and Lindquist (1955) observed that in 60-day-old rats the intestinal effect of vitamin D was maximal after a single dose of only 10 I.U., while at least 100 I.U. were required for maximum effect on the serum calcium level. Both in their study and in that of Harrison et al. (1958) the increase in serum calcium 3—5 days after the administration of 100 I.U. vitamin D was 2—3 times greater than that observed in the present exp. 37 (Table V). The reason for this difference is not readily apparent, although differences in the state of the bone mineral may be important. In the case of the work of Harrison et al. (1958) it could be argued that the effect was partly due to the intestinal action of vitamin D, since the rats continued ingesting moderate amounts of calcium.

According to Nicolaysen (1957) the increased net absorption of calcium obtained by vitamin D treatment of vitamin D-deficient young rats varied between 1 and 2 mg daily whether the calcium intake was 2.4 mg or as low as 0.7 mg. However, for the lowest intakes net absorption was negative also after the vitamin D treatment. In the present exp. 37 the calcium intake from the glucose solution was presumably within this range or lower. At such low intakes treatment with vitamin D will mainly affect the re-absorption of digestive juice calcium. If the secretion of these juices was low because of the non-stimulating nature of the protein-free and liquid diet, and if the calcium concentration of the juices was low because of hypocalcemia (Giran 1960 a), the increase in net absorption due to vitamin D would probably have been even lower than Nicolaysen's finding of 1—2 mg. Since calcium absorption probably was minimal, if not negligible, it seems reasonable to assume that the serum calcium rise was entirely due to the action of vitamin D on bone or another non-intestinal end-organ. It should be

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remembered that the purpose of employing the glucose-in-saline diet was not to eliminate absorption of calcium entirely, but to prevent the deposition of significant amounts of bone mineral under the influence of vitamin D (see below: Parathyroid-vitamin D interaction).

The mechanism of the calcium-mobilizing effect of vitamin D remains obscure. However, Nichols et al. (1965) have made the interesting observation that vitamin D, administered to vitamin D-deficient rats 3 days before sacrifice, increased the solubility of bone samples incubated *in vitro*. The effect was partly due to increased passive solubility and was therefore similar to that of parathyroid hormone.

Parathyroid-vitamin D interaction

There are two possible, equally plausible explanations for the failure to find evidence for non-additivity (mutual potentiation) of the effects of parathyroid extract and vitamin D: 1) There was some degree of potentiation, but it was too small to be detected in the present experiment. 2) The effects were additive. It is entirely possible that a potentiating effect of vitamin D could have been demonstrated if the parathyroid extract had been given in a lower dose so that the final serum calcium level after the combined treatment would be lower than it was in this experiment. The reason for this suggestion is that when the serum calcium approaches the normal level, or passes it, as happened in several rats in Group IV (Table V), homeostatic mechanisms aimed at lowering the serum calcium would presumably come into play and impede the rise stimulated by the treatment.

A series of experiments with different combinations of doses and times of administration of the hormone and the vitamin would be needed to clarify the problem of their interaction.

The most likely mechanism by which vitamin D could render a dose of parathyroid extract more effective is probably that of stimulating the absorption and deposition of increased amounts of calcium of dietary origin. Long-term vitamin D treatment will undoubtedly increase the amount of stable bone salt available for mobilization by parathyroid hormone. If, in addition, the solubility of this bone salt is increased through an action of vitamin D (see above), one would expect parathyroid hormone to appear more effective in mobilizing calcium than it would in the vitamin D-deficient rat. But even a single dose of vitamin D may have a similar effect when given prior to the hormone, provided parathyroid hormone in the rapidly growing animal can mobilize bone deposited within the last 24 hours, as suggested by Bronner (1961). This type of interaction, the importance of which remains unknown, was at any rate avoided in the present experiment. The purpose of the glucose-in-saline diet was to ensure that the amount of calcium absorbed and deposited in the skeleton in response to the vitamin D treatment would be minute and negligible compared to the total skeletal content of calcium available for mobilization.

The possibility of an interaction of the effects of the two factors on the renal handling of calcium and its importance for the serum calcium level can neither be ruled out nor discussed in the light of present knowledge.

In conclusion it may be said that the present results are consistent with the hypothesis that the effects of parathyroid extract and vitamin D on the serum calcium are additive, but the results are inadequate to rule out the possibility of an interaction between the effects.

Parathyroid action in rachitic children

Neither Jonxis (1961) nor Steendijk (1964) were able to significantly raise the serum calcium level in severely rachitic children by injecting parathyroid extract. Only after prolonged treatment with vitamin D was a response obtained in one patient after administration of 700 units parathyroid extract (Eli Lilly Co.) over 3 days (Steendijk 1964). However, there is also evidence to the contrary, namely that the hormone is active in rickets: 1) The serum calcium level in primary vitamin D deficient rickets is usually normal or slightly subnormal. 2) In cases of rickets and osteomalacia (Lin et al. 1941) with slight hypocalcemia and marked hypophosphatemia, vitamin D treatment results in only a slow and delayed return of the serum calcium level, but a rapid return of phosphate to the normal level (Steendijk 1962). 3) Snider et al. (1962) have shown that a rachitic child could respond to vitamin D treatment, while on a calcium-free diet, by an immediate drop in urinary phosphate excretion and an increase in renal tubular phosphate reabsorption (in per cent of filtered phosphate), in spite of a steadily rising serum phosphate level. If one of the effects of vitamin D in this case had been the re-establishment of end-organ responsiveness to parathyroid hormone, the well-established phosphaturic effect of this hormone would have counteracted the observed urinary changes. Relative hyperphosphaturia and hypophosphatemia do not fit the concept of parathyroid hormone inactivity in the absence of vitamin D, and these phosphate abnormalities may actually be partly explained by assuming the existence of a hyperparathyroid state secondary to rickets (Rasmussen and Reifenstein 1962).

The discrepancy between these findings may possibly be explained by assuming that the small amount of bone salt present in the severely rachitic child and perhaps also the lower solubility (see above: Effect of vitamin D on the serum calcium) of the bone make it necessary to inject a very high dose of parathyroid extract to obtain a measurable effect on the serum calcium.

The author wishes to express his gratitude to Dr. Paul L. Munson for advice on the design of experiments, to Mr. Dan Wiberg for advice on statistical treatment, and to Miss L. M. Kristoffersen for valuable technical assistance. The author greatly appreciates the gift of parathyroid extract from the Eli Lilly Co. through the courtesy of Dr. Otto K. Behrens.

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25-Hydroxycholecalciferol: Stimulation of Bone Resorption in Tissue Culture

Abstract. *25-Hydroxycholecalciferol stimulates release of previously incorporated calcium-45 from fetal rat bones in doses of 0.9 to 27 units per milliliter. This effect cannot be produced by much larger doses of vitamin D_3. Comparison of stimulation of bone resorption by 25-hydroxycholecalciferol and parathyroid hormone reveals similarities with respect to time course, dose-response slope, and inhibition by calcitonin.*

Although there is substantial evidence that vitamin D stimulates bone resorption in vivo (*1*), attempts to demonstrate an effect in tissue culture have required large doses and have given inconsistent results (*2, 3*). To

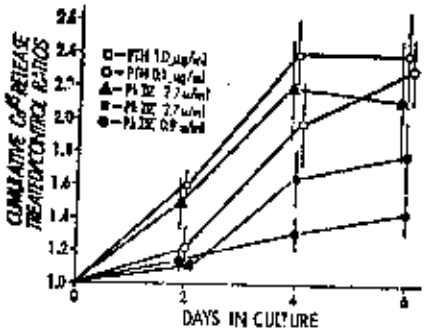

Fig. 1. Time course of the release of previously incorporated ^{45}Ca from fetal rat bones treated with either purified bovine parathyroid hormone (PTH) or 25-hydroxycholecalciferol (peak IV) in tissue culture. The shafts of the radius and ulna were dissected from 19-day fetal rats taken from mothers injected with 0.5 mc of ^{45}Ca on the previous day. The bones were cultured at 37°C in a chemically defined medium (*3*) in an atmosphere of 5 percent CO_2, 20 percent O_2, 75 percent N_2; 25-hydroxycholecalciferol was added in ethanol; test and control media were adjusted to contain 1 percent ethanol. Each point represents the mean ratio ± S.E. for cumulative ^{45}Ca release from four pairs of cultures.

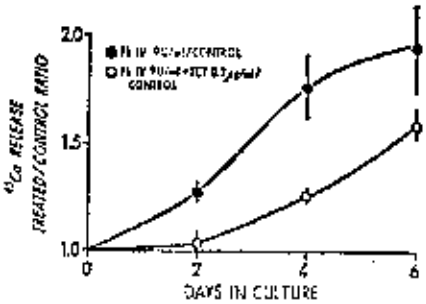

Fig. 2. The effect of purified rat thyroid calcitonin (TCT) on the stimulation of ^{45}Ca release from fetal rat bones by 25-hydroxycholecalciferol (peak IV) in tissue culture. Each point represents the mean ± S.E. for cumulative ^{45}Ca release from four pairs of cultures.

test whether vitamin D was less effective in vitro because it could not reach the appropriate site or because a transformation in vivo was required, a number of vitamin D preparations were tested in tissue cultures of long bone shafts of the fetal rat in a chemically defined medium (*4*). Resorption was measured as the release of previously incorporated ^{45}Ca from paired test and control bones (*5*). Crystalline vitamin D_3 (Eastman) dissolved in propylene glycol or ethanol, a water-soluble preparation (Vi-De-3 Hydrosol), and a water-dispersible preparation of vitamin D_2 (Mann Assay Laboratories) did not consistently enhance resorption during the first 3 days of culture. The crystalline vitamin D_3 preparations in doses of 300 to 400 units per milliliter caused variable increases in ^{45}Ca release during the 3rd to 6th day of culture; the others were without effect. These slow responses suggested that the effect might be the result of a transformation product of vitamin D. We therefore examined the effects of a physiologically active metabolite of vitamin D_3 found in serum and tissues, including bone (*6*). This material was originally called peak IV on the basis of its elution position on silicic acid chromatography. Purified samples of peak IV isolated from hog serum (*7*) stimulated bone resorption consistently during the first 3 days of culture. The active component of peak IV was then isolated in pure form and identified as 25-hydroxycholecalciferol (*7, 8*). This material had an effect on bone resorption qualitatively comparable to that of purified parathyroid hormone (PTH). Calcium-45 release was significantly increased by doses ranging from 0.9 to 27 units per milliliter (16 to 500 ng per milliliter or 4×10^{-8} to $1.2 \times 10^{-6} M$). The response to 25-hydroxycholecalciferol and purified PTH had the same time course (Fig. 1). The data for the first 2 days of treatment show parallel dose-response slopes for both agents. Low doses of 25-hydroxycholecalciferol and PTH produced significantly greater increases in ^{45}Ca release when added together than when added singly, suggesting a synergistic interaction of the two agents. Rat thyroid calcitonin (TCT) completely inhibited the stimulation of ^{45}Ca release by 25-hydroxycholecalciferol during the first 2 days of culture (Fig. 2). However, there was a subsequent escape from inhibition similar to that observed with PTH (*9*). Measurements of total bone calcium and decalcified dry weight confirmed the data on release of ^{45}Ca and indicated that losses of bone matrix and mineral were parallel during resorption stimulated by 25-hydroxycholecalciferol.

Our data indicate that low doses of an active metabolite of vitamin D_3 stimulate bone resorption in vitro. 25-Hydroxycholecalciferol is 1.4 times more active per unit weight than cholecalciferol in curing rickets in rats and chicks (*10*). It stimulates calcium transport in the intestine and bone resorption in vivo more rapidly than cholecalciferol does (*10*). The much greater effectiveness of the metabolite in tissue culture suggests that bone cannot convert the vitamin to an active form in this system or does so only slowly. With radioactive vitamin D_3, evidence which demonstrates that the liver is the major site of 25-hydroxycholecalciferol production has now been obtained (*11*). It is likely that bone resorption is stimulated in vivo only after vitamin D is transformed to 25-hydroxycholecalciferol and taken up by bone. The effective doses of 25-hydroxycholecalciferol used in our experiments are sufficiently low so that this material could account for stimulation of bone resorption after therapeutic doses of vitamin D in vivo and could explain earlier observations of different effects of serums from animals deficient in vitamin D and from animals treated with vitamin D (*2*). As a potent and direct stimulus for bone resorption, 25-hydroxycholecalciferol might provide a more reliable and rapid agent for the treatment of hypocalcemia than the various forms of vitamin D now used.

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Mode of Action of Vitamin D. The Effect of Vitamin D Deficiency in the Rat on Anaerobic Glycolysis and Pyruvate Oxidation by Epiphyseal Cartilage

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The role of vitamin D in calcification of bone still awaits elucidation; this is not to be wondered at since the mechanism of normal calcification is still far from clearly understood. The demonstration of alkaline phosphatase in bone and hypertrophic cartilage by Robison (1923) and Robison & Soames (1924) led to the suggestion that the enzyme promoted calcification by causing a local increase in concentration of inorganic phosphate. That this hypothesis did not explain fully the process of calcification was recognized by Robison, who postulated the presence of a second mechanism which was susceptible to the action of cyanide, fluoride and iodoacetate (Robison, MacLeod & Rosenheim, 1930; Robison & Rosenheim, 1931). Though Robison & Rosenheim (1934) did not make any suggestion about the nature of this second mechanism, in the light of the later knowledge of the action of the above-mentioned inhibitors in the intermediate metabolism of carbohydrates, it may be permissible to conclude that Robison's second mechanism must be very intimately connected with glycogen metabolism in hypertrophic cartilage.

Gutman & Gutman (1941) provided experimental evidence associating glycolysis with calcification occurring in calcifying cartilage. They demonstrated the presence of phosphorylase in significant concentration in the epiphyses of growing rats and rabbits and suggested that this enzyme could initiate conversion of the glycogen of hypertrophic cartilage cells into phosphoric ester substrates for cartilage phosphatase. Later Gutman, Warrick & Gutman (1942) observed that the addition of 0·000531 phloretin, which is a specific inhibitor for phosphorylase, completely blocked the calcification of rachitic cartilage in solutions containing calcium and inorganic phosphorus in concentrations of 8 and 5 mg. respectively/100 ml. of the medium. The addition of glucose 1-phosphate (G 1-P) restored the capacity of the cartilage to calcify even in the presence of phloretin. The authors concluded, therefore, that phosphorylase was necessary in the calcification mechanism. Further work with the use of inhibitors like cyanide, iodoacetate and fluoride suggested that the calcification of cartilage involved phosphorylative glycogenolysis at least to the point of phosphopyruvate formation.

Additional evidence in support of the important role of glycogenolysis in initiating calcification was forthcoming from the work of Albaum, Hirschfeld & Sobel (1952*a*) who observed that adenosine triphosphate (ATP), which is essential in these enzymic processes, was present in epiphyseal cartilage of rat tibiae at the same level as was normally found in liver and kidney (9·2–17·2 μg./rat, i.e. two tibiae). Albaum, Hirschfeld & Sobel (1952*b*) further demonstrated the presence of most of the glycogenolytic enzymes in epiphyseal cartilage and several of the phosphorylated intermediates by a chromatographic technique.

Although the role of glycogenolysis in calcification had thus been clearly demonstrated, current opinion appears to view this process only from the standpoint of its being able to provide phosphoric esters, which under the influence of alkaline phosphatase would provide the requisite concentration of inorganic phosphorus for the deposition of the bone salt. This concept, which lays emphasis on the

concentrations of calcium and inorganic phosphorus alone, does not fully explain the observed facts.

Dikshit & Patwardhan (1952) have shown that the epiphyseal cartilage of rachitic rat after treatment with vitamin D can undergo calcification *in vitro* in media in which the cartilage of untreated rats fails to calcify. These authors suggested that, on administration of vitamin D, some changes as yet ill understood take place in the cartilage which impart to the latter the capacity to calcify. A histochemical examination of rachitic epiphyseal cartilage from rats before and after treatment with vitamin D has recently been made by Ramalingaswami, Soundararajan, Dikshit, Tulpule & Patwardhan (1954). As a result of these studies, the authors concluded that failure of calcification in vitamin D deficiency probably was due to a disturbance or arrest of the metabolism of glycogen in the rachitic cartilage. Experimental evidence that such disturbance does *in fact* take place in induced rickets in rats is described in the present communication.

EXPERIMENTAL

The anaerobic breakdown of glycogen and hexose phosphates and the aerobic oxidation of pyruvate by the epiphyseal cartilage were studied by the Warburg manometric technique with the use of standard Warburg apparatus using flasks of 15 ml. capacity with centre well and one side chamber. The anaerobic respiration of cartilage was studied in the atmosphere of nitrogen and for aerobic oxidation of pyruvate, O_2 was used as the gas phase. All observations were made at 37°.

Preliminary trials with slices and homogenates of the epiphyseal cartilage of normal young rats showed that homogenates gave results which were not reproducible probably owing to inadequate homogenization of the cartilage. Hence all experiments described in this paper have been carried out with cartilage slices. Utter, Wood & Reiner (1945) had observed that the addition of ATP, diphosphopyridine nucleotide (DPN), Mg^{2+} and nicotinamide to buffered substrate was necessary to evoke maximum glycolytic activity of cell-free extracts and homogenates prepared from the central nervous system. Preliminary experiments showed that the same cofactors favourably influenced the glycolytic activity of cartilage and hence, they were incorporated in the reaction medium in concentrations given below. In experiments with pyruvate, cytochrome *c*, with ATP and DPN, was found to improve the rate of oxidation. The reaction mixtures for glycolysis and aerobic oxidation experiments were made up as follows:

(*a*) *Anaerobic experiment: gas phase, nitrogen*

		ml.
(i)	Na-K phosphate buffer, pH 7·3 (0·03 M)	1·0
(ii)	$NaHCO_3$ (0·36 M)	0·2
(iii)	$MgSO_4$ (0·24 M)	0·1
(iv)	ATP (0·0021 M)	0·1
(v)	DPN (0·0055 M)	0·1
(vi)	Nicotinamide (0·6 M)	0·1
(vii)	Fructose 1:6-diphosphate or Glycogen or G 1-P or G 6-P (0·0075 M)	0·2

The total volume was made up to 3·2 ml. with distilled water. Temperature equilibration was obtained by shaking the reaction mixtures for 10 min. before the cartilage slices were tipped into the chamber from the side arm. Readings were taken every 5 min. for half an hour. Enzyme activity was expressed as Q_{N_2} = µl. CO_2 liberated/mg. dry wt./hr.

(*b*) *Aerobic experiment: gas phase, oxygen*

		ml.
(i)	Krebs–Ringer bicarbonate buffer without $CaCl_2$ (pH 7·4)	1·0
(ii)	ATP (0·0021 M)	0·1
(iii)	DPN (0·0055 M)	0·1
(iv)	Cytochrome *c* (3×10^{-5} M)	0·1
(v)	Sodium pyruvate (0·05 M)	0·7

The total volume was made up to 3·0 ml. with the 0·1 water; 0·2 ml. of 10% (w/v) aqueous KOH was placed in the centre well. The cartilage slices were added to the side chamber from the beginning. Equilibration time was 10 min. Readings were taken every 20 min. for 1 hr.

The enzyme activity is expressed as Q_{O_2} = µl. of O_2 consumed/100 mg. dry tissue/hr. The results of pyruvate oxidation with the epiphyseal cartilage of normal 4- to 5-week-old rats are given in the last column of Table 2.

Experimental animals. 180 young and healthy albino rats weighing about 45 g. and between 24 and 28 days of age were caged individually and kept in a room where no direct or reflected sunlight could penetrate. All the animals were fed rachitogenic diet (Dikshit & Patwardhan, 1947) with Ca:P as 10:1. The control rats received 25 I.U. of vitamin D_2/day. At intervals of 5, 10, 15 and 20 days a fixed number of rats in each group was killed, tibial cartilages were dissected out, cut longitudinally in thin slices and immediately used for manometric observations. In rachitic animals the glycolytic activity was estimated on cartilage slices of individual rats since sufficient amount of cartilage could be obtained from each animal, but in case of vitamin D treated controls the cartilage from two animals had to be pooled for each trial. On the other hand, owing to very low oxidative capacity of the cartilage, slices from two animals in the rachitic group had to be pooled for experiments with pyruvate.

RESULTS AND DISCUSSION

Bywaters (1936*a*, *b*), Dickens & Weil-Malherbe (1936), Hill (1940) and Laskin, Sarnat & Bain (1952) have studied the respiratory activity in cartilage with the use of manometric technique. Their results pointed to the fact that the glycogenolytic process in the cartilage was predominantly anaerobic. Even so, the level of glycolytic activity of the epiphyseal cartilage of the tibia observed in the present investigation (Table 1) was much higher than that reported by the above mentioned workers. This might be due to the fact that ATP, DPN and Mg^{2+} were added by us to the reaction medium in suitable concentrations and that glycogen and phosphorylated hexoses were used as substrates. Hill (1940) and Laskin *et al.* (1952) observed a fourfold increase in the oxygen uptake of rabbit and horse articular cartilage when methylene blue was added to the reaction mixture. They also produced evidence to

show that the formation of pyruvate takes place in the metabolic process.

The results in Table 1 indicate that in the initial stages of rachitogenesis, the glycolytic activity of rachitic cartilage with all the substrates tried was considerably higher than that exhibited by the cartilage from vitamin D treated rats. However, as rickets became well established, a fall in glycolytic activity to the level of vitamin D treated controls was witnessed on the 15th day. There were fairly large variations in the values of Q_{CO_2} obtained at different stages of rachitogenesis in comparable animals. On the whole, however, the observed differences between the anaerobic glycolytic activity of cartilage in the rachitic and vitamin D treated animals were not found to be statistically significant. If anything, the values of Q_{CO_2} with G 1-P and G 6-P as substrates were slightly higher after 15 days in rachitic cartilage than in that of vitamin D treated animals.

One can tentatively conclude from these experiments that the mechanism of anaerobic breakdown of glycogen in cartilage is not disturbed in vitamin D deficiency.

The results of experiments dealing with pyruvate oxidation are summarized in Table 2.

As mentioned earlier, q_{O_2} (pyruvate) was very much less than the glycolytic activity displayed by rachitic cartilage. There was a difference in the early stages of rachitogenesis between the endogenous respiration of the cartilage and the rate at which the added pyruvic acid was oxidized. Statistical analysis of the results shows that this difference was more marked in vitamin D treated rats than in deficient animals ($P < 0{\cdot}001$), as observed on the fifth day after placing the rats on rachitogenic diets. As the rats continued on the diet and with the establishment of rickets the capacity to oxidize pyruvic acid suffered a decrease in rachitic cartilage and after 20 days on the rachitogenic diet, the rachitic cartilage seemed to have lost almost the capacity to oxidize pyruvic acid. On the other hand, in rats treated with vitamin D, pyruvate oxidation remained mostly unimpaired, as would be evident from a comparison of these results with those on cartilage of normal young rats given in Table 2. These results, therefore, clearly indicate that in vitamin D deficiency the rachitic epiphyseal cartilage of rats is unable to oxidize pyruvic acid, thus confirming the suspicions expressed in the earlier paper by Ramalinguswami *et al.* (1954).

Pyruvate oxidation trials were carried out with liver and kidney homogenates prepared according to the method of Green, Loomis & Auerbach (1948) from rachitic and vitamin D protected rats. The results showed that the capacity of these organs to oxidize pyruvate was not impaired in vitamin D deficiency. It appears possible, therefore, that the effect of vitamin D deficiency described in this paper is restricted to bone.

The antirachitic action of citrate demonstrated by Shohl (1937) and the essential similarity of histochemical changes in rachitic cartilage induced under the influence of vitamin D on the one hand and citrate on the other as observed by Ramalinguswami *et al.* (1954) had indicated that in vitamin D deficiency one was more likely to find an arrest or disturbance in the oxidative phase of glycogen metabolism than in the earlier and anaerobic stages of glycogen breakdown. The experiments described here have fulfilled these expectations. It is not yet clear, however, how the orderly progress of glycogen metabolism would be essential to calcification. Two tentative suggestions can be made at this stage. It is possible that the supply of citrate itself is inadequate in rickets owing to a progressive inability of the cartilage to deal with pyruvate. That citrate is of importance to bone can be concluded from the observation of Dickens (1941) that 90 % of citrate in the animal body is to be found in the skeleton. The same author has shown that in rickets citrate in bone is reduced. Further, the relationship between the state of vitamin D nutrition and citrate metabolism has been well brought out by the work of Harrison & Harrison (1952), who found that in

Table 1. *Glycolytic activity of rat epiphyseal cartilage*

		μl. CO_2/mg. dry weight, with substrate (averages only are given)			
		Glycogen	Glucose 1-phosphate	Glucose 6-phosphate	Fructose 1:6-diphosphate
Five days on rachitogenic diet	Without vitamin D (16 rats)	15·1	16·0	13·8	24·1
	With vitamin D in diet (10 rats)	6·3	7·8	7·2	7·1
Fifteen days on rachitogenic diet	Without vitamin D (10 rats)	7·8	6·8	7·6	7·0
	With vitamin D in diet (12 rats)	4·6*	3·9	3·5	6·7

Table 2. *Oxidation of pyruvate by epiphyseal cartilage of rat tibiae in rachitic and vitamin D protected animals*

	Rats on rachitogenic diets			Rats on rachitogenic diets and vitamin D			Normal rats on rahon diets (8)*
	Five days (32)*	Ten days (12)*	Twenty days (24)*	Five days (32)*	Ten days (12)*	Twenty days (20)*	
	S.E.		S.E.	S.E.		S.E.	S.E.
(a) μ_{O_2} with substrate in μl./100 mg. dry tissue/hr.	191·2 ± 10·57	187·4	132·1 ± 15·53	252·5 ± 13·85	288·4	257·7 ± 15·72	284·2 ± 14·41
(b) μ_{O_2} without substrate in μl./100 mg. dry tissue/hr.	137·3 ± 10·68	161·3	138·1 ± 10·41	139·2 ± 8·30	154·7	156·7 ± 9·72	175·5 ± 7·80
μ_{O_2} (pyruvate) (a − b)	53·9	26·1	−6·0	123·3	133·7	101·0	165·7
Student's t†	2·7358	—	0·3187	7·6409	—	6·8860	16·4470
P	<0·02	—	0·5	<0·001	—	<0·001	<0·001

The significance of differences in μ_{O_2} pyruvate within and between groups on 5th and 20th days is tabulated below:

(1) Rachitic 5th and 20th days	$t = 4·6244$ (26)†	P, <0·001
(2) Vitamin D 5th and 20th days	$t = 0·9290$ (24)†	Not significant P between 0·3 and 0·4
(3) Rachitic and vitamin D on 5th day	$t = 4·4318$ (36)†	P, <0·001

* Figures in parenthesis indicate the number of animals.
† Degrees of freedom.

rachitic infants the concentration of citrate in serum was significantly lower than in normal infants, and that it was raised on administration of vitamin D. Dixon & Perkins (1952) have shown the existence of enzyme systems, viz. citrogenase, aconitase and isocitric dehydrogenase in bones. It may not be unreasonable to conclude that the formation of citrate is inhibited in rickets, a conclusion of which experimental confirmation is now being sought.

On the other hand, other possible roles which pyruvate oxidation may play in calcification will have to be examined. Much of the energy needed for calcification must be derived from the progressive oxidation of pyruvic acid. Then again, our earlier observations (Ramalingaswami *et al.* 1954) suggested a possible defect in the formation of cartilage matrix in rickets which seems to undergo a qualitative change after treatment with vitamin D. It is possible that the formation of the appropriate matrix may be closely related to pyruvate metabolism in cartilage.

SUMMARY

1. Anaerobic breakdown of glycogen and phosphorylated hexoses and the oxidation of pyruvate by epiphyseal cartilage of the tibiae of rats kept on rachitogenic diets with and without vitamin D has been studied by the Warburg manometric technique.

2. In rachitic rats the glycolytic activity of the epiphyseal cartilage was almost the same as that observed in vitamin D protected rats in comparable experiments.

3. In well established rickets the epiphyseal cartilage of the rat loses the capacity to oxidize pyruvate.

4. The significance of the above findings in the mechanism of calcification has been discussed.

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GRADE "A" PASTEURIZED MILK ORDINANCE

1965 Recommendations
of the United States
Public Health Service

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
Bureau of Disease Prevention and Environmental Control
National Center for Urban and Industrial Health
Environmental Sanitation Program

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Part II

GRADE A PASTEURIZED MILK ORDINANCE WITH ADMINISTRATIVE PROCEDURES—1965 RECOMMENDATIONS OF THE UNITED STATES PUBLIC HEALTH SERVICE

An ordinance defining "milk" and certain "milk products," "milk producer," "pasteurization," etc.; prohibiting the sale of adulterated and misbranded milk and milk products; requiring permits for the sale of milk and milk products; regulating the inspection of dairy farms and milk plants, and the examination, labeling, pasteurization, distribution and sale of milk and milk products; providing for the construction of future dairy farms and milk plants, the enforcement of this Ordinance, and the fixing of penalties.

Be it ordained by the _____ of _____ [a,1] as follows:

SECTION I. DEFINITIONS

The following definitions shall apply in the interpretation and the enforcement of this *Ordinance:*

***A. Milk.**—Milk is hereby defined to be the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows, which contains not less than 8¼ percent milk solids-not-fat and not less than 3¼ percent milkfat. (Milkfat or butterfat is the fat of milk.)*

***A–1. Goat Milk.**—Goat milk is the lacteal secretion, practically free from colostrum, obtained by the complete milking of healthy goats. The word "milk" shall be interpreted to include goat milk.*

***B. Cream.**—Cream is the sweet, fatty liquid separated from milk, with or without the addition of milk or skim milk, which contains not less than 18 percent milkfat.*

***B–1. Light Cream, Coffee Cream, or Table Cream.**—Light cream, coffee cream, or table cream is cream which contains not less than 18 percent but less than 30 percent milkfat.*

***B–2. Whipping Cream.**—Whipping cream is cream which contains not less than 30 percent milkfat.*

[a] Numbered footnotes are assembled on p. 91.

***B–3. Light Whipping Cream.**—Light whipping cream is cream that contains not less than 30 percent but less than 36 percent milkfat.*

***B–4. Heavy Cream or Heavy Whipping Cream.**—Heavy cream or heavy whipping cream is cream which contains not less than 36 percent milkfat.*

***B–5. Whipped Cream.**—Whipped cream is whipping cream into which air or gas has been incorporated.*[2]

***B–6. Whipped Light Cream, Coffee Cream, or Table Cream.**—Whipped light cream, coffee cream, or table cream is light cream, coffee cream, or table cream into which air or gas has been incorporated.*[2]

***B–7. Sour Cream or Cultured Sour Cream.**—Sour cream or cultured sour cream is a fluid or semifluid cream resulting from the souring, by lactic acid producing bacteria or similiar culture, of pasteurized cream, which contains not less than 0.20 percent acidity expressed as lactic acid.*[2]

***C. Half-and-Half.**—Half-and-half is a product consisting of a mixture of milk and cream which contains not less than 10.5 percent milkfat.*[2]

***C–1. Sour Half-and-Half or Cultured Half-and-Half.**—Sour half-and-half or cultured half-and-half is fluid or semifluid half-and-half derived from the souring, by lactic acid producing bacteria or similar culture, of pasteurized half-and-half, which contains not less than 0.20 percent acidity expressed as lactic acid.*[2]

***D. Reconstituted or Recombined Milk and Milk Products.**—Reconstituted or recombined milk and/or milk products shall mean milk or milk products defined in this section which result from the recombining of milk constituents with potable water.*[2,3]

***E. Concentrated Milk.**—Concentrated milk is a fluid product, unsterilized and unsweetened, resulting from the removal of a considerable portion of the water from milk, which, when combined with potable water, results in a product conforming with the standards for milkfat and solids-not-fat of milk as defined above.*[2]

***E–1. Concentrated Milk Products.**—Concentrated milk products shall be taken to mean and to include homogenized concentrated milk, vitamin D concentrated milk, concentrated skim milk, fortified concentrated skim milk, concentrated lowfat milk, fortified concentrated lowfat milk, concentrated flavored milk, concentrated flavored milk products, and similar concentrated products made from concentrated milk or concentrated skim milk, and which, when combined with potable water in accordance with instructions printed on the container conform with the definitions of the corresponding milk products in this section.*[2]

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E–2. Frozen Milk Concentrate.—Frozen milk concentrate is a frozen milk product with a composition of milkfat and milk solids not fat in such proportions that when a given volume of concentrate is mixed with a given volume of water the reconstituted product conforms to the milkfat and milk solids not fat requirements of whole milk. In the manufacturing process, water may be used to adjust the primary concentrate to the final desired concentration. The adjusted primary concentrate is pasteurized, packaged, and immediately frozen. This product is stored, transported, and sold in the frozen state.[2]

F. Skim Milk or Skimmed Milk.—Skim milk or skimmed milk is milk from which sufficient milkfat has been removed to reduce its milkfat content to less than 0.50 percent.[2]

G. Lowfat Milk.—Lowfat milk is milk from which a sufficient portion of milkfat has been removed to reduce its milkfat content to not less than 0.50 percent and not more than 2.0 percent.[2]

H. Vitamin D Milk and Milk Products.—Vitamin D milk and milk products are milk and milk products, the vitamin D content of which has been increased by an approved method to at least 400 U.S.P. units per quart.[2]

I. Fortified Milk and Milk Products.—Fortified milk and milk products are milk and milk products other than vitamin D milk and milk products, the vitamin and/or mineral content of which have been increased by a method and in an amount approved by the health authority.[2]

J. Homogenized Milk.—Homogenized milk is milk which has been treated to insure breakup of the fat globules to such an extent that, after 48 hours of quiescent storage at 45° F., no visible cream separation occurs on the milk, and the fat percentage of the top 100 milliliters of milk in a quart, or of proportionate volumes in containers of other sizes, does not differ by more than 10 percent from the fat percentage of the remaining milk as determined after thorough mixing. The word "milk" shall be interpreted to include homogenized milk.[2]

K. Flavored Milk or Milk Products.—Flavored milk or milk products shall mean milk and milk products as defined in this Ordinance ***to which have been added a flavor and/or sweetener.***[2]

K–1. Eggnog Flavored Milk.—Eggnog flavored milk is a milk product consisting of a mixture of at least 3.25 percent butterfat, at least 0.5 percent egg yolk solids, sweetener, and flavoring. Emulsifier and a maximum of 0.5 percent stabilizer may be added.

K–2. Eggnog.—Eggnog is a milk product consisting of a mixture of milk or milk product of at least 6.0 percent butterfat, at least 1.0 percent egg yolk solids, sweetener, and flavoring. Emulsifier and not over 0.5 percent stabilizer may be added.

L. Buttermilk.—Buttermilk is a fluid product resulting from the manufacture of butter from milk or cream. It contains not less than 8¼ percent of milk solids-not-fat.[1]

L–1. Cultured Buttermilk.—Cultured buttermilk is a fluid product resulting from the souring, by lactic acid producing bacteria or similar culture, of pasteurized skim milk or pasteurized lowfat milk.[1]

M. Cultured Milk or Cultured Whole Milk Buttermilk.—Cultured milk or cultured whole milk buttermilk is a fluid product resulting from the souring, by lactic acid producing bacteria or similar culture, of pasteurized milk.[2]

N. Acidified Milk and Milk Products.—Acidified milk and milk products are milk and milk products obtained by the addition of food grade acids to pasteurized cream, half-and-half, milk, lowfat milk, or skim milk, resulting in a product acidity of not less than 0.20 percent expressed as lactic acid.[2]

O. Milk Products.—Milk products include cream, light cream, coffee cream, table cream, whipping cream, light whipping cream, heavy cream, heavy whipping cream, whipped cream, whipped light cream, whipped coffee cream, whipped table cream, sour cream, cultured sour cream, half-and-half, sour half-and-half, cultured half-and-half, reconstituted or recombined milk and milk products, concentrated milk, concentrated milk products, skim milk, skimmed milk, lowfat milk, fortified milk and milk products, vitamin D milk and milk products, homogenized milk, flavored milk or milk products, eggnog, eggnog flavored milk, buttermilk, cultured buttermilk, cultured milk, cultured whole milk buttermilk, and acidified milk and milk products.[2,3,1]

This definition is not intended to included such products as sterilized milk and milk products hermetically sealed in a container and so processed, either before or after sealing, as to prevent microbial spoilage, or evaporated milk, condensed milk, butter, ice cream and other frozen desserts, dry milk products (except as defined herein), or cheese except when they are combined with other substances to produce any pasteurized milk or milk product defined herein.

P. Grade A Dry Milk Products.—Grade A dry milk products are milk products which have been produced for use in Grade A pasteurized milk products and which have been manufactured under the provisions of Grade A Dry Milk Products—Recommended Sanitation Ordinance and Code for Dry Milk Products Used in Grade A Pasteurized Milk Products.

Q. Optional Ingredients.—Optional ingredients shall mean and include Grade A dry milk products, concentrated milk, concentrated milk products, flavors, sweeteners, stabilizers, emulsifiers, acidifiers, vitamins, minerals, and similar ingredients.

R. Adulterated Milk and Milk Products.—Any milk or milk product shall be deemed to be adulterated (1) if it bears or contains

any poisonous or deleterious substance in a quantity which may render it injurious to health; (2) if it bears or contains any added poisonous or deleterious substance for which no safe tolerance has been established by State or Federal regulation, or in excess of such tolerance if one has been established; (3) if it consists, in whole or in part, of any substance unfit for human consumption; (4) if it has been produced, processed, prepared, packed, or held under insanitary conditions; (5) if its container is composed, in whole or in part, of any poisonous or deleterious substance which may render the contents injurious to health; or (6) if any substance has been added thereto or mixed or packed therewith so as to increase its bulk or weight, or reduce its quality or strength, or make it appear better or of greater value than it is.

***R-1. Misbranded Milk and Milk Products.**—Milk and milk products are misbranded (1) when their container(s) bear or accompany any false or misleading written, printed or graphic matter; (2) when such milk and milk products do not conform to their definitions as contained in this* Ordinance; *and (3) when such products are not labeled in accordance with Section 4 of this* Ordinance.

***S. Pasteurization.**—The terms "pasteurization," "pasteurized," and similar terms shall mean the process of heating every particle of milk or milk product to at least 145° F., and holding it continuously at or above this temperature for at least 30 minutes, or to at least 161° F., and holding it continuously at or above this temperature for at least 15 seconds, in equipment which is properly operated and approved by the health authority:* Provided, *That milk products which have a higher milkfat content than milk and/or contain added sweeteners shall be heated to at least 150° F., and held continuously at or above this temperature for at least 30 minutes, or to at least 166° F., and held continuously at or above this temperature for at least 15 seconds,* Provided further, *That nothing in this definition shall be construed as barring any other pasteurization process which has been recognized by the United States Public Health Service to be equally efficient and which is approved by the State health authority.*

***T. Sanitization.**—Sanitization is the application of any effective method or substance to a clean surface for the destruction of pathogens, and of other organisms as far as is practicable. Such treatment shall not adversely affect the equipment, the milk or milk product or the health of consumers, and shall be acceptable to the health authority.*

***U. Milk Producer.**—A milk producer is any person who operates a dairy farm and provides, sells, or offers milk for sale to a milk plant, receiving station, or transfer station.*

***V. Milk Hauler.**—A milk hauler is any person who transports raw milk and/or raw milk products to or from a milk plant, receiving or transfer station.*

***W. Milk Distributor.**—A milk distributor is any person who offers for sale or sells to another any milk or milk products.*

***X. Health Authority.**—The health authority shall mean the ---------- of the ----------[1] or his authorized representative. The term, "Health Authority," wherever it appears in this* Ordinance, *shall mean the appropriate agency having jurisdiction and control over the matters embraced within this* Ordinance.

***Y. Dairy Farm.**—A dairy farm is any place or premises where one or more cows or goats are kept, and from which a part or all of the milk or milk product(s) is provided, sold, or offered for sale to a milk plant, transfer station, or receiving station.*

***Z. Milk Plant and/or Receiving Station.**—A milk plant and/or receiving station is any place, premises, or establishment where milk or milk products are collected, handled, processed, stored, pasteurized, bottled, or prepared for distribution.*

***Z-1. Transfer Station.**—A transfer station is any place, premises, or establishment where milk or milk products are transferred directly from one transport tank to another.*

***AA. Official Laboratory.**—An official laboratory is a biological, chemical, or physical laboratory which is under the direct supervision of the State or a local health authority.*

***BB. Officially Designated Laboratory.**—An officially designated laboratory is a commercial laboratory authorized to do official work by the supervising agency, or a milk industry laboratory officially designated by the supervising agency for the examination of producer samples of Grade A raw milk for pasteurization.*

***CC. Person.**—The word "person" shall mean any individual, plant operator, partnership, corporation, company, firm, trustee, or association.*

***DD. And/or.**—Where the term "and/or" is used, "and" shall apply where appropriate, otherwise "or" shall apply.*

SECTION 2. ADULTERATED OR MISBRANDED MILK OR MILK PRODUCTS

No person shall, within the municipality of ---------- of ----------,[1] or its police jurisdiction, produce, provide, sell, offer, or expose for sale, or have in possession with intent to sell any milk or milk product which is adulterated or misbranded: Provided, *That in an emergency, the sale of pasteurized milk and milk products which have not been graded, or the grade of which is unknown, may be authorized by the health authority, in which case such products shall be labeled "ungraded."*

Any adulterated or misbranded milk or milk product may be impounded by the health authority and disposed of in accordance with applicable laws or regulations.

ADMINISTRATIVE PROCEDURES

This section of the *Ordinance* shall be used in impounding the products of, or preferring charges against, persons who adulterate or misbrand their milk or milk products or label them with any grade designation not authorized by the health authority under the terms of this *Ordinance*, or who sell or deliver ungraded milk or milk products except as may be permitted under this section in an emergency. An emergency is defined as a general and acute shortage in the milkshed, not simply one distributor's shortage.

SECTION 3. PERMITS

It shall be unlawful for any person who does not possess a permit from the health authority of the _____ of _____[1] to bring into, send into, or receive into the _____ of _____[1] or its police jurisdiction, for sale, or to sell, or offer for sale therein, or to have in storage any milk or milk products defined in this Ordinance: Provided, *That, grocery stores, restaurants, soda fountains, and similar establishments where milk or milk products are served or sold at retail, but not processed, may be exempt from the requirements of this section.*

Only a person who complies with the requirements of this Ordinance *shall be entitled to receive and retain such a permit. Permits shall not be transferable with respect to persons and/or locations.*

The health authority shall suspend such permit, whenever he has reason to believe that a public health hazard exists; or whenever the permit holder has violated any of the requirements of this Ordinance; *or whenever the permit holder has interfered with the health authority in the performance of his duties;* Provided, *That the health authority shall, in all cases except where the milk or milk product involved creates, or appears to create, an imminent hazard to the public health; or in any case of a willful refusal to permit authorized inspection, serve upon the holder a written notice of intent to suspend permit, which notice shall specify with particularity the violation(s) in question and afford the holder such reasonable opportunity to correct such violation(s) as may be agreed to by the parties, or in the absence of agreement, fixed by the health authority, before making any order of suspension effective. A suspension of permit shall remain in effect until the violation has been corrected to the satisfaction of the health authority.*

Upon written application of any person whose permit has been suspended, or upon application within 48 hours of any person who has been served with a notice of intention to suspend, and in the latter case before suspension, the health authority shall within 72 hours proceed to a hearing to ascertain the facts of such violation or interference and upon evidence presented at such hearing shall affirm, modify, or rescind the suspension or intention to suspend.

Upon repeated violation(s), the health authority may revoke such permit following reasonable notice to the permit holder and an opportunity for a hearing. This section is not intended to preclude the institution of court action as provided in Sections 5 and 6.

ADMINISTRATIVE PROCEDURES

Issuance of Permits.—Every milk producer, milk distributor, milk hauler, and each milk plant, receiving station, and transfer station operator shall hold a valid permit. Milk producers who transport milk or milk products only from their own dairy farms and employees of a milk distributor or milk plant operator who possesses a valid permit, shall not be required to possess a hauler's permit. Grocery stores, restaurants, soda fountains, and similar establishments where milk or milk products are served or sold at retail but not processed, may be exempt from the requirements of this section.

Suspension of Permit.—When any requirement(s) of this *Ordinance* is violated, the permit holder is subject to the suspension of his permit.

The health authority may forgo suspension of the permit, provided the product or products in violation are not sold or offered for sale.

Hearings.—If a State or municipal administrative procedure act which provides procedures for administrative hearings and judicial review of administrative determinations is available, the act shall be made applicable by reference to the hearings provided for in the *Ordinance.* If such administrative procedure act is not available, appropriate procedures, including provision for notice, hearing officer, and his authority, record of hearing, rules or evidence, and court review shall be established by appropriate authority.

Reinstatement of Permits.—Any producer, distributor, hauler, or plant operator whose permit has been suspended may make written application for the reinstatement of his permit.

When the permit suspension has been due to a violation of any of the bacterial, coliform, or cooling-temperature standards, the health authority within 1 week after the receipt of a written application for reinstatement of permit, shall issue a temporary permit after determining by an inspection of the facilities and operating methods that

the conditions responsible for the violation have been corrected. Samples shall then be taken at the rate of not more than two per week on separate days within a 3-week period, and the health authority shall reinstate the permit upon compliance with the appropriate standard as determined in accordance with Section 6 of this *Ordinance*.

Whenever the permit suspension has been due to a violation of a requirement other than the bacteriological, coliform, or cooling-temperature standards, the application shall contain a written statement to the effect that the violation(s) has been corrected. Within 1 week of the receipt of such an application, the health authority shall make an inspection of the applicant's establishment, and as many additional inspections thereafter as are deemed necessary, to determine that the applicant's establishment is complying with the requirements. When the findings justify, the permit shall be reinstated.

SECTION 4. LABELING

All bottles, containers, and packages enclosing milk or milk products defined in Section 1 of this Ordinance *shall be conspicuously labeled or marked with (1) the name of the contents as given in the definition in this* Ordinance; (2) the word "*reconstituted*" *or* "*recombined*" *if the product is made by reconstitution or recombination;*[a] *(3) the grade of contents; (4) the word "pasteurized" if the contents are pasteurized and the identity of the plant where pasteurized; (5) the word "raw" if the contents are raw and the name or other identity of the producer; (6) the designation "vitamin D" and the number of U.S.P. units per quart in the case of vitamin D milk or milk products; (7) the volume or proportion of water to be added for recombining in the case of concentrated milk or milk products; (8) the words "nonfat milk solids added" and the percentage added if such solids have been added, except that this requirement shall not apply to reconstituted or recombined milk or milk products; (9) the words "artificially sweetened" in the name if non-nutritive and/or artificial sweeteners are used; and (10) the common name of stabilizers, distillates, and ingredients;* Provided, *That (a) only the identity of the milk producer shall be required on cans delivered to a milk plant which receives only Grade A raw milk for pasteurization, and which immediately dumps, washes, and returns the cans to the milk producer; (b) the identity of both milk producer and the grade shall be required on cans delivered to a milk plant which receives both Grade A raw milk for pasteurization and ungraded raw milk, and which immediately dumps, washes, and returns the cans to the milk producer; (c) in the case of concentrated milk products, the specific name of the product shall be substituted for the generic term "concentrated milk products" e.g., "homogenized concentrated milk," "concentrated skim milk," "concentrated chocolate milk," "concentrated chocolate flavored lowfat milk"; (d) in the case of flavored milk or flavored reconstituted milk, the name of the principal flavor shall be substituted for the word "flavored",*[b] *and (e) in the case of cultured milk and milk products, the special type culture used may be substituted for the word "cultured," e.g., "acidophilus buttermilk," "bulgarian buttermilk," and "yogurt."*

All vehicles and transport tanks containing milk or milk products shall be legibly marked with the name and address of the milk plant or hauler in possession of the contents.

Tanks transporting raw milk and milk products to a milk plant from sources of supply not under the routine supervision of the health authority are required to be marked with the name and address of the milk plant or hauler and shall be sealed; in addition, for each such shipment, a shipping statement shall be prepared containing at least the following information:

(1) Shipper's name, address, and permit number.
(2) Permit number of hauler, if not employee of shipper.
(3) Point of origin of shipment.
(4) Tanker identity number.
(5) Name of product.
(6) Weight of product.
(7) Grade of product.
(8) Temperature of product.
(9) Date of shipment.
(10) Name of supervising health authority at the point of origin.
(11) Whether the contents are raw, pasteurized, or otherwise heat treated.

Such statement shall be prepared in triplicate and shall be kept on file by the shipper, the consignee, and the carrier for a period of 6 months for the information of the health authority.

The labeling information which is required on all bottles, containers or packages of milk or milk products shall be in letters of an acceptable size, kind, and color satisfactory to the health authority and shall contain no marks or words which are misleading.

ADMINISTRATIVE PROCEDURES

Emergency Supplies—Labeling.—When the sale of ungraded milk or milk products is authorized during emergencies, under the terms of Section 2, the label must bear the designation "ungraded." When

[a] The Federal Food and Drug Administration considers the term "chocolate milk" misleading unless the flavoring ingredient used is "chocolate," "sweet chocolate," or "milk chocolate" complying with the appropriate standards of identity for these products. Milk and milk products flavored with "cocoa" may be labeled "chocolate flavored."

such labeling is not available, the health authority shall take immediate steps to inform the public that the particular supply is ungraded, and that the supply will be properly labeled as soon as the distributor can obtain the required labels.

Identity Labeling.—"Identity," as used in this section, is defined as the name and address of the milk plant at which the pasteurization takes place. It is recommended that the voluntary national uniform coding system for identification of pasteurization plants at which milk and milk products are packaged, be adopted in order to provide a uniform system of codes throughout the country.

In cases where several pasteurization plants are operated by one firm, the common firm name may be utilized on milk bottles or containers: *Provided*, That the location of the plant at which the contents were pasteurized is also shown, either directly or by a code. This requirement is necessary in order to enable the health authority to identify the source of the pasteurized milk. The street address of the pasteurizing plant need not be shown when only one plant of a given name is located within the municipality.

The identity labeling requirement may be interpreted as permitting plants and persons to purchase and distribute, under their own label, milk and milk products processed and packaged at another plant, provided, that the label reads, "Processed at ---------- (name and address)," or that the processing and packaging plant is identified by a proper code.

Misleading Labels.—The health authority shall not permit the use of any misleading marks, words, or endorsements upon the label. He may permit the use of registered trade designs or similar terms on the bottle cap or label when, in his opinion, they are not misleading and are not so used as to obscure the labeling required by the *Ordinance*. The use of super grade designations shall not be permitted. Grade designations such as "Grade AA Pasteurized," "Selected Grade A Pasteurized," "Special Grade A Pasteurized," "Premium," etc., give the consumer the impression that such a grade is significantly safer than Grade A pasteurized. Such an implication is false, because the *Ordinance* requirements for Grade A pasteurized milk, when properly enforced, will insure that this grade of milk will be as safe as milk can practicably be made.

Note.—Milk or milk products shall be labeled in accordance with the provisions of Section 4 of this *Ordinance* and, when shipped interstate, shall fulfill the applicable requirements of the Federal Food, Drug, and Cosmetic Act, as amended.

SECTION 5. INSPECTION OF DAIRY FARMS AND MILK PLANTS

Each dairy farm, milk plant, receiving station, and transfer station whose milk or milk products are intended for consumption within ----------[1] or its police jurisdiction shall be inspected by the health authority prior to the issuance of a permit. Following the issuance of a permit, each dairy farm and transfer station shall be inspected at lease once every 6 months and each milk plant and receiving station shall be inspected at least once every 3 months. Should the violation of any requirement set forth in Section 7 be found to exist on an inspection, a second inspection shall be required after the time deemed necessary to remedy the violation, but not before 3 days; this second inspection shall be used to determine compliance with the requirements of Section 7. Any violation of the same requirement of Section 7 on such second inspection shall call for permit suspension in accordance with Section 3 and/or court action.

One copy of the inspection report shall be handed to the operator, or other responsible person, or be posted in a conspicuous place on an inside wall of the establishment. Said inspection report shall not be defaced and shall be made available to the health authority upon request. An identical copy of the inspection report shall be filed with the records of the health authority.

Every milk producer, hauler, distributor, or plant operator shall, upon request of the health authority, permit access of officially designated persons to all parts of his establishment or facilities to determine compliance with the provisions of this Ordinance. *A distributor or plant operator shall furnish the health authority, upon request, for official use only, a true statement of the actual quantities of milk and milk products of each grade purchased and sold, and a list of all sources of such milk and milk products, records of inspections, tests, and pasteurization time and temperature records.*

It shall be unlawful for any person who in an official capacity obtains any information under the provisions of this Ordinance *which is entitled to protection as a trade secret (including information as to quantity, quality, source or disposition of milk or milk products, or results of inspections or tests thereof) to use such information to his own advantage or to reveal it to any unauthorized person.*

ADMINISTRATIVE PROCEDURES

Inspection Frequency.—One producer inspection every 6 months or one plant inspection every 3 months is not a desirable frequency; it is instead a legal minimum. Dairy farms and milk plants expe-

riencing difficulty meeting requirements should be visited more frequently. Inspections of dairy farms shall be made at milking time as often as possible, and of milk plants at different times of the day, in order to ascertain if the processes of equipment assembly, sanitizing, pasteurization, cleaning, and other procedures comply with the requirements of this *Ordinance*.

Enforcement Procedure.—This section provides that a dairy farm or milk plant shall be subject to suspension of permit, and/or court action, if two successive inspections disclose violation of the same requirement.

Experience has demonstrated that strict enforcement of the *Ordinance* leads to a better and friendlier relationship between the health authority and the milk industry than does a policy of enforcement which seeks to excuse violations and to defer penalty therefor. The sanitarian's criterion of satisfactory compliance should be neither too lenient nor unreasonably stringent. When a violation is discovered, the sanitarian should point out to the milk producer or plant operator the requirement that has been violated, discuss a method for correction, and set a time for correcting the violated requirement.

The penalties of suspension or revocation of permit, and/or court action, are provided to prevent continued violation of the provisions of this *Ordinance* but are worded to protect the dairy industry against unreasonable or arbitrary action. When a condition is found which constitutes an imminent health hazard, prompt action is necessary to protect the public health; therefore, the health authority is authorized, in Section 3, to suspend the permit immediately. However, except for such emergencies, no penalty is imposed on the producer or distributor upon the first violation of any of the sanitation requirements listed in Section 7. A producer or distributor found violating any requirement must be notified in writing and given a reasonable time to correct the violation(s) before a second inspection is made. The requirement of giving written notice shall be deemed to have been satisfied by the handing to the operator or by the posting of an inspection report, as required by this section. After receipt of a notice of violation, but before the allotted time has elapsed, the producer or distributor shall have an opportunity to appeal the sanitarian's interpretation to the health authority or for an extension of the time allowed for correction.

Certified Industry Inspection.—The health authority may certify industry personnel to carry out cooperatively the provisions of this *Ordinance* with respect to the supervision of dairy farms. Reports of all inspections conducted by such personnel to determine compliance with the provisions of this *Ordinance* shall be forwarded to the health authority. All punitive actions and all inspections for the issuance

or reinstatement of permits shall be performed by the health authority. Industry personnel shall be certified annually by the health authority in accordance with the provisions of Appendix B, p. 99.

Inspection Reports.—A copy of the inspection report shall be filed by the health authority and retained for at least 12 months. The results shall be entered on appropriate ledger forms. The use of a computer or other information retrieval system may be used. Examples of field inspection forms are included in Appendix L, p. 167.

SECTION 6. THE EXAMINATION OF MILK AND MILK PRODUCTS

During any consecutive 6 months, at least four samples of raw milk for pasteurization shall be taken from each producer and four samples of raw milk for pasteurization shall be taken from each milk plant after receipt of the milk by the milk plant and prior to pasteurization. In addition, during any consecutive 6 months, at least four samples of pasteurized milk and at least four samples of each milk product defined in this Ordinance *shall be taken from every milk plant. Samples of milk and milk products shall be taken while in possession of the producer or distributor at any time prior to final delivery. Samples of milk and milk products from dairy retail stores, food service establishments, grocery stores, and other places where milk and milk products are sold shall be examined periodically as determined by the health authority; and the results of such examination shall be used to determine compliance with Sections 2, 4, 10. Proprietors of such establishments shall furnish the health authority, upon his request, with the names of all distributors from whom milk or milk products are obtained.*

Required bacterial counts and cooling temperature checks shall be performed on raw milk for pasteurization. In addition, antibiotic tests on each producer's milk or on commingled raw milk shall be conducted at least four times during any consecutive 6 months. When commingled milk is tested, all producers shall be represented in the sample. All individual sources of milk shall be tested when test results on the commingled milk are positive. Required bacterial counts, coliform determinations, phosphatase, and cooling temperature checks shall be performed on pasteurized milk and milk products.

Whenever two of the last four consecutive bacteria counts, coliform determinations, or cooling temperatures, taken on separate days, exceed the limit of the standard for the milk and/or milk product, the health authority shall send a written notice thereof to the person concerned. This notice shall be in effect so long as two of the last four consecutive samples exceed the limit of the standard. An additional sample shall be taken within 14 days of the sending of such notice, but not before

the lapse of 3 days. Immediate suspension of permit in accordance with Section 4 and/or court action shall be instituted whenever the standard is violated by three of the last five bacteria counts, coliform determinations, or cooling temperatures.

Whenever a phosphatase test is positive, the cause shall be determined. Where the cause is improper pasteurization, it shall be corrected; and any milk or milk product involved shall not be offered for sale.

Samples shall be analyzed at an official or appropriate officially designated laboratory. All sampling procedures and required laboratory examinations shall be in substantial compliance with the ..... Edition of Standard Methods for the Examination of Dairy Products *of the American Public Health Association, and the ..... Edition of* Official Methods of Analyses *of the Association of Official Analytical Chemists. (Insert edition number current at time of adoption.) Such procedures and examinations shall be evaluated in accordance with the methods of evaluating milk laboratories recommended by the United States Public Health Service. Examinations and tests shall be conducted to detect adulterants, including pesticides, as the health authority shall require. Assays of vitamin D milk or milk products and/or fortified milk and milk products shall be made at least annually in a laboratory acceptable to the health authority.*

ADMINISTRATIVE PROCEDURES

Enforcement Procedures.—All violations of bacteria, coliform, and cooling temperature standards shall be followed promptly by inspection to determine and correct the cause. (See App. E, Examples of 3-out-of-5 Compliance Enforcement Procedures.)

Laboratory Techniques.—Procedures for the collection and holding of samples; the selection and preparation of apparatus, media and reagents; and the analytical procedures, incubation, reading, and reporting of results, shall be in substantial compliance with *Standard Methods for the Examination of Dairy Products* and the *Official Methods of Analyses.* The procedures shall be those specified therein for:

(1) Standard plate count at 32° C.
(2) Simplified methods for viable counts of raw milk at 32° C.
(3) Coliform test with solid media at 32° C.
(4) Disc assay methods for antibiotics
(5) APHA or AOAC phosphatase tests

The phosphatase test is an index of the efficiency of the pasteurization process. In the event the laboratory phosphatase test is positive, the cause shall be determined immediately. Where the cause is improper pasteurization, it shall be corrected. When a laboratory phosphatase test is positive, or if any doubt should arise as to the compliance of the equipment, standards or methods outlined in Section 7, item 16p., the health authority should immediately conduct field phosphatase tests at the plant (App. G, p. 133).

The direct microscopic count is useful as a screening test to detect suspicious tanker loads of milk for subsequent official examinations, and to determine the possible presence of abnormal milk.

Sampling Procedures.—When samples of raw milk for pasteurization are taken at a milk plant prior to pasteurization, they shall be drawn following adequate agitation from randomly selected storage tanks.

When bacterial counts and temperature determinations are made of several samples of the same milk or milk products collected from the same supply or processor, on the same day, these values are averaged arithmetically, and the results recorded as the count or temperature determinations of the milk or milk product for that day. All counts and temperatures should be recorded on the milk-ledger form PHS 1784 (or a similar form) for dairy farms, and form PHS 1782 (or a similar form) for milk plants as soon as reported by the laboratory.

A computer or other information retrieval system may be used.

See Appendix G, page 134, for a reference to antibiotics in milk and the conditions under which a positive phosphatase reaction may be encountered in properly pasteurized milk or cream.

Note.—The industry should be encouraged by the health authority to achieve day-to-day compliance with the foregoing standards by performing tests on each producer's milk, including platform tests for odors, temperature, and sediment. Bacterial counts should be conducted following laboratory pasteurization as a check for thermoduric organisms. Examinations for the presence of psychrophilic bacteria are also recommended. Periodic screening tests for presence of added water, antibiotics, and pesticide residues should be performed on producer milk. Plants should reject milk of abnormal odor and high temperature as well as milk that is found to be unsatisfactory by the sediment test. Followup inspections on the dairy farm should be made by the plant fieldman to determine the cause and to institute corrective measures whenever milk is rejected by the milk plant.

SECTION 7. STANDARDS FOR MILK AND MILK PRODUCTS

All Grade A raw milk for pasteurization and all Grade A pasteurized milk and milk products shall be produced, processed, and pasteurized to conform with the following chemical, bacteriological, and temperature standards, and the sanitation requirements of this section.

No process or manipulation other than pasteurization, processing methods integral therewith, and appropriate refrigeration shall be applied to milk and milk products for the purpose of removing or deactivating microorganisms.

may be enjoined from continuing such violations. Each day upon which such a violation occurs shall constitute a separate violation.

SECTION 17. REPEAL AND DATE OF EFFECT

All ordinances and parts of ordinances in conflict with this Ordinance *shall be repealed 12 months after the adoption of this* Ordinance, *at which time this* Ordinance *shall be in full force and effect, as provided by law.*

SECTION 18. UNCONSTITUTIONALITY CLAUSE

Should any section, paragraph, sentence, clause, or phrase, of this Ordinance *be declared unconstitutional or invalid for any reason, the remainder of this* Ordinance *shall not be affected thereby.*

FOOTNOTES

In the interest of clarity and to provide easy access to their information, all numbered footnotes have been removed from the body of this publication and are assembled in this section. A numerical reference in the text will always relate to its like-numbered footnote in this section.

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[1] Substitute proper legal jurisdiction here and in all similar places throughout the *Ordinance*.

[2] Optional ingredients as defined in Section 1, Definition Q, may be used in this product.

[3] Where State law does not permit the sale of reconstituted or recombined milk and/or milk products, Definition D and other corresponding references should be omitted.

[4] Communities desiring to regulate cottage cheese and creamed cottage cheese under the terms of this *Ordinance* should insert the following definitions:

—— *Cottage Cheese:* Cottage cheese is the soft uncured cheese obtained by adding lactic-acid-producing bacteria, with or without enzymatic action, to pasteurized skim milk, pasteurized lowfat milk, or pasteurized reconstituted skim milk.[3] It shall contain not more than 80 percent moisture. Cottage cheese may be seasoned with salt.

—— *Creamed Cottage Cheese:* Creamed cottage cheese is prepared by mixing cottage cheese with a pasteurized creaming mixture consisting of pasteurized cream and milk, dry milk products, concentrated skim milk, skim milk, or lowfat milk, to which salt, lactic acid, and flavor producing bacteria, rennet, lactic acid, citric acid, phosphoric acid or stabilizer may be added. The quantity of milkfat added in the creaming mixture shall be not less than 4 percent by weight of the finished creamed cottage cheese. Dry milk products or concentrated skim milk may be added, provided the amount of added solids does not exceed 3 percent of the weight of the creaming mixture. Creamed cottage cheese shall contain not more than 80 percent moisture.

[5] A certified copy may be secured from the Department of Health, Education, and Welfare, Public Health Service, Washington, D.C.

What you should know about GRADE A milk

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
Public Health Service
Division of Environmental Engineering and Food Protection
Milk and Food Branch
Washington, D.C. 20201

For sale by the Superintendent of Documents, U.S. Government Printing Office
Washington, D.C. 20402 - Price 15 cents

I. Introduction

Of all things in life which affect human welfare, none is more important than food. Properly selected food provides mankind with the mental and physical energy which has been the mainspring of all civilization. It repairs the structural damage which the wear and tear of life inflicts upon our bodies, and it helps make us resistant to disease. Disraeli, when he was head of the English government, once said, "The health of the people is really the foundation upon which all their happiness and all their powers as a State depend."

The importance of good nutrition has become of primary interest to governments throughout the world. The World Health Organization, formed in 1946, is authorized to ". . . promote, in cooperation with other specialized agencies where necessary, the improvement of nutrition," and to "develop, establish and promote international standards with respect to food."

Of the great variety of foods available today, none is more important than milk. Research carried out in all parts of the world tell the same story. Improved appearance and improvement in physical and mental performance have invariably followed the addition of milk to the diet. Let us then find out why milk makes this difference.

There are three important questions about milk which should be of concern to all persons. They are:

1. What is the nutrient value of milk and how much should we include in the daily diet for maximum health?

2. What is being done to safeguard milk to prevent the dissemination of disease through milk and milk products?

3. What is being done about cholesterol, chemical residues and radiological fallout?

II. Nutrient Value of Milk

Milk is the one food specifically prepared by nature for the young of mammals. Nearly every one will agree that a substance specifically prepared by nature for no other purpose than for food is more likely to contain elements necessary to sustain life and justly deserves the title, "the most nearly perfect food."

It is obvious that one of the most important attributes which a food should possess is that it be a good source of energy, since

Nutrie in Cow's Milk

[illegible]	AMOUNT IN 1 QUART	AMOUNT IN 1 GLASS (8 OZ.)*
Food Energy, Calories	670	168
Protein, gm	34	8.5
Calcium, gm	1.15	0.29
Phosphorous, gm	0.88	0.22
Iron, mg	0.33	0.08
Vitamin A, I. U.	1500	375
Thiamine, mg	0.33	0.08
Riboflavin, mg	1.65	0.41
Niacin Equivalents, mg	8.75	2.16
Ascorbic Acid, mg	14	3.50
Vitamin D, I. U.**	400	100

* Fluid whole milk **Applies only to vitamin D milk

every living thing needs a fresh supply every day. Milk is such a food and, furthermore, is a form of energy, low in cost in relation to all other foods.

Milk is also a good muscle builder. It is rich in protein of the highest quality. Under most conditions, milk protein is the most completely digested and easily assimilated of all food proteins.

Proteins are necessary for growth and body maintenance; without them, neither animal nor vegetable life is possible, because protein furnishes nitrogen which is an essential component of all plant and animal cells.

When proteins are eaten, they are broken down during digestion into substances called amino acids. There are eleven known essential amino acids which must be provided by the food we eat. The proteins of milk, casein, lactalbumin, and lactoglobulin, contain all of these essential amino acids.

The amount of high quality protein in one quart of milk supplies one-third to one-half of the daily requirement of protein for the adolescent and one-half the daily requirement for the adult. These essential amino acids are necessary in the ing of enzymes and hormones. They play a vital role as re ators in the digestion and oxidation processes of the body and are effective in promoting wound healing and restoring blood volume to normal.

Milk is a far more concentrated food from the standpoint of solids than most of us imagine. We think of milk as a liquid not much above the consistency of water; but it contains 13 percent of solids by weight, which is more than is contained in onions, beets, carrots, squash, pineapple, turnips, oysters, cabbage, radishes, cauliflower, spinach, tomatoes, asparagus, celery, lettuce, or cucumbers. When we buy one pint or one pound of milk, therefore, we buy more actual dry solid food than when we buy one pound of any of the above mentioned foods.

The sugar in milk is chiefly lactose. Each cup of milk contains approximately one tablespoon of this sugar which is not as sweet as other sugars. Lactose contributes a pleasing flavor to milk and like all carbohydrates is a source of energy. Lactose has a beneficial effect on the digestive tract, enabling the more complete absorption of calcium. In milk, nature has thus provided materials for building purposes and for energy in a form which assures their ready absorption and utilization.

The minerals in milk include calcium, phosphorous, potassium, sulfur, iron, copper, sodium, iodine, chlorine, magnesium, manganese, and other trace minerals. Each body cell contains small amounts of mineral elements which function in the physical and chemical changes in the body. These changes are vital to health and growth. Minerals aid in normal functioning of the heart muscles, make possible the quick response to stimuli, aid digestion, determine the process of oxidation and secretion, and aid in the development and reproduction of body cells.

Milk contains calcium and phosphorous in ideal proportions for the building of bones and teeth. Milk is the best source of calcium of all foods. It assists in the control of the heartbeat, aids digestion, is necessary for coagulation of blood, functions in the contraction of muscles, and effects the quickness with which we react to stimuli.

The fat of milk contains the fatty acids essential for a healthy skin. Milk is one of our best sources of vitamin A. One of the outstanding diet specialists of the world has stated that, "Of the three vitamins A, B, and C in milk, vitamin A is the factor of greatest practical importance to nutrition and health, because so many of our staple foods are poor in vitamin A, and because diets poor in this vitamin cause such widespread weakening of the body and increases its susceptability to so many infectious diseases."

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Mi an excellent source of vitamin A. This vitamin is essential to growth, essential for the health of the skin, hair, nails, and mucous membrane, helps prevent night blindness, and is necessary to maintain normal vision. It is essential in the maintenance of tooth enamel and the health of gums, respiratory tract, and digestive tract.

There is only a small amount of vitamin D naturally in milk. However, milk is the only food approved by the Council on Foods and Nutrition of the American Medical Association for fortification by adding vitamin D. The "Vitamin D Milk" sold on the market today contans 400 units of added vitamin D per quart, which is the recommended daily need of an adult. Vitamin D is essential in the prevention of rickets, aids in the absorption of calcium and phosphorous through the intestinal tract, promotes height growth or increases the length of the skeleton, and is essential for the placing of calcium in the blood stream.

Milk is the outstanding food source of riboflavin, or vitamin G. Riboflavin promotes growth, helps maintain health and vigor, is essential to cell oxidation processes, promotes resistance to infection, and promotes healthy lips, skin, and eyes.

Thiamine and niacin are found in significant amounts in milk. These two vitamins, like riboflavin, function in the oxidation in cells and the oxidation of carbohydrates in particular. Thiamine promotes growth, is important in reproduction and lactation, and prevents beriberi. Niacin is also necessary in the maintenance of healthy tissues and is effective in the treatment of pellagra.

Vitamin C is one of the more important vitamins of the diet. It is necessary for the formation of the cementing substance between the cells, and it promotes resistance to infection. Vitamin C is essential to the internal structure of the teeth and gums, and helps to regulate oxidation and reduction in cells. Milk contains only small amounts of this vitamin, so the diet must be supplemented with fresh fruits and vegetables.

Finally, milk is one of the most digestible of all foods due in great part to its fluid form.

It may be asked why milk is called "the most nearly perfect food" rather than "the perfect food." This is due to the fact that, while it is the most nearly perfect food, it is not an entirely dependable source of all vitamins nor does it contain sufficient iron. For this reason and because variety in the diet stimulates the appetite, we should not attempt to live on milk alone. The diet of children should include at least a quart of milk daily, supplemented with a selection of other foods, including orange juice and green vegetables. All adult diets should include at least a pint of milk daily in one form or another.

III. Prevention of Disease Transmission Through Milk and Milk Products

Milk is not only an excellent food for man but is also an ideal medium for the growth of bacteria. Consequently, milk must have constant protection from the healthy cow to the dining table.

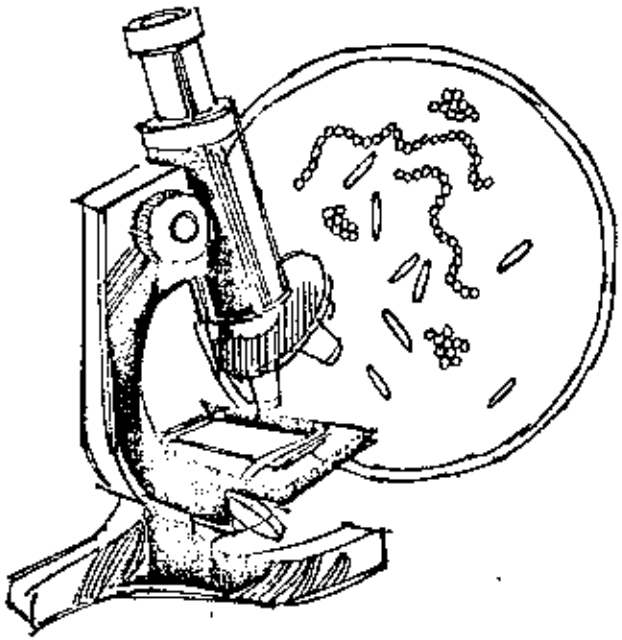
From the time milk leaves the udder of the cow, unless adequate safeguards are maintained, it may receive bacteria and other microorganisms from the surroundings. Additional organisms may come from the hands of the milker, in the handling of pails and other equipment used in milk processing, storage, and transportation. These are in most cases harmless.

Pathogenic (disease) organisms may enter the milk either from man or the cow and may retain their virulence long enough to produce illness in persons drinking raw milk.

Among the diseases that may be transmitted by milk are tuberculosis, typhoid fever, scarlet fever, diptheria, septic sore throat, Q- fever, undulant fever, and food poisoning. Although

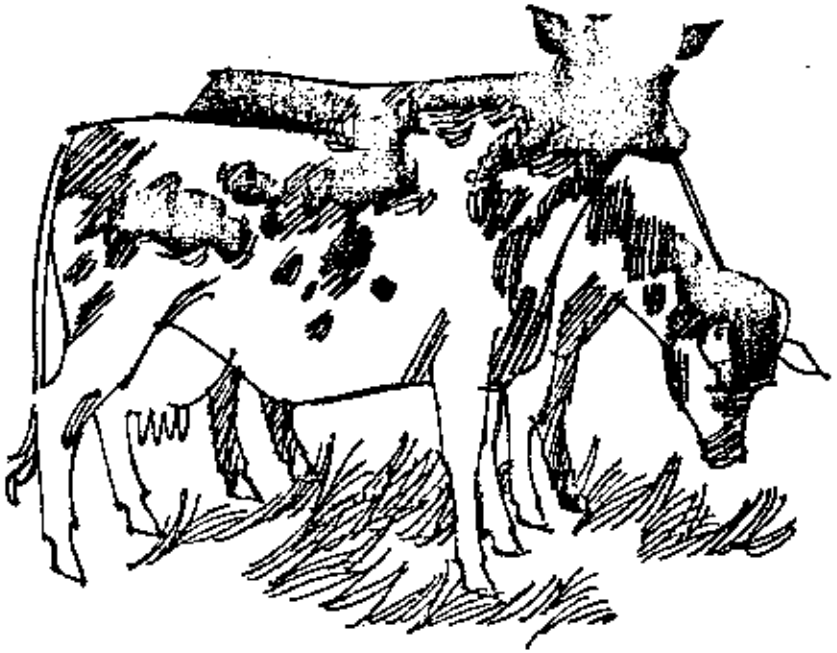
few epid s have been reported to the Public Health Service in the past years, prior to that time, 30 to 50 outbreaks were reported each year. The occasional outbreaks that still occur, however, serve to illustrate that it is the control of milkborne disease rather than the elimination of the disease itself that has been achieved.

Suppose you were buying raw milk from a dairyman. What could he do, other than pasteurization, to make sure that you or any of his other customers do not contact disease by drinking his milk?



In the case of tuberculosis, for example, about the only thing he could do would be to have his cows tested for tuberculosis and slaughter those that were infected. Suppose this dairyman had a herd of 100 splendid, purebred cattle, that he had them tested, found three or four to be infected with tuberculosis, had these three or four slaughtered and then continued in business. Would you and the other customers be protected from contracting bovine tuberculosis? Could you be sure that you would never regret having permitted your children to drink raw milk from this dairy?

Of course, the cows that had been slaughtered would no longer be a menace. But suppose that a year later your dairyman tested again and found another cow with tuberculosis. During this period, your child could have received, through this milk



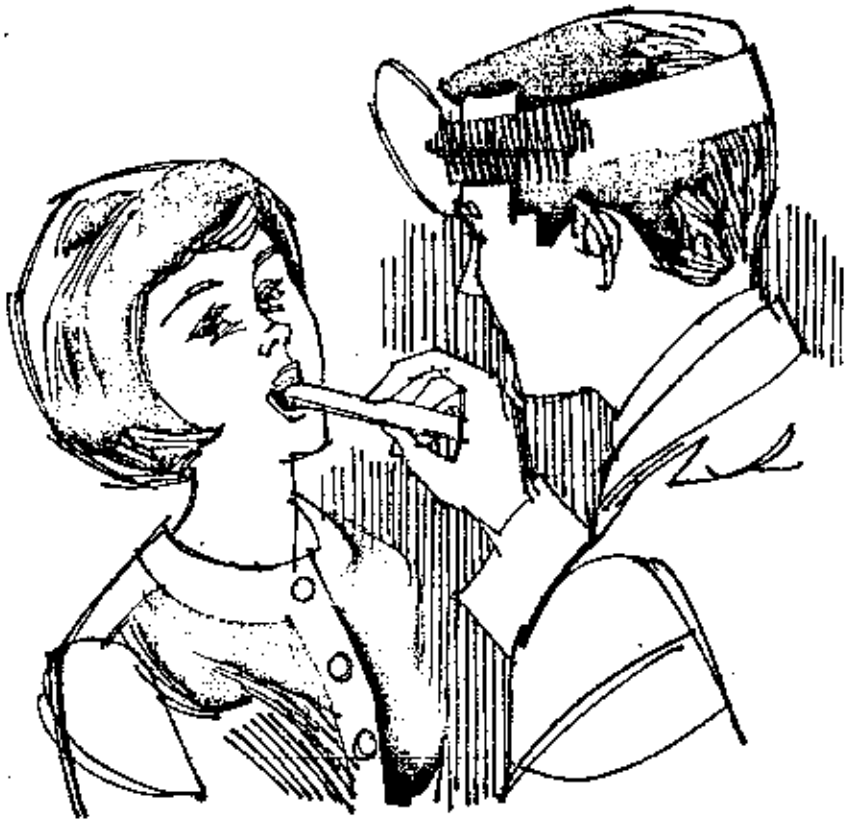
supply, the germs of tuberculosis, an infection which might not disclose itself for considerable time, until perhaps you had forgotten that you had ever bought milk from that dairyman.

Do not think that this cannot happen. It can and does happen even at certified raw milk dairies, and slaughtering the infected cows does not undo the damage that has already been done.

Now let us pass on to typhoid fever. If you were buying raw milk from a dairy, what would be the most effective way, short of pasteurization, for the dairyman to make sure the milk you were buying would not be carrying typhoid fever organisms?

Of course, if one of the milkers or other helpers contracted typhoid fever, he would at once be quarantined or sent to a hospital; and, if the dairyman acted promptly and carefully, there would probably be very little danger. But unfortunately, that is not usually the way epidemics of typhoid are caused by milk.

When milk becomes contaminated with typhoid fever organisms, it is usually not a sick person who is at fault, but, instead, a perfectly well individual who has had typhoid fever perhaps years ago and who possibly did not know that it was typhoid. Nevertheless, he has, as a result, become what is known as a typhoid carrier. Such a person is an apparently well individual. He doesn't look or feel sick but, unfortunately, he still carries typhoid fever organisms, either in his gall bladder or elsewhere, from which they are discharged in his feces or urine; and thus



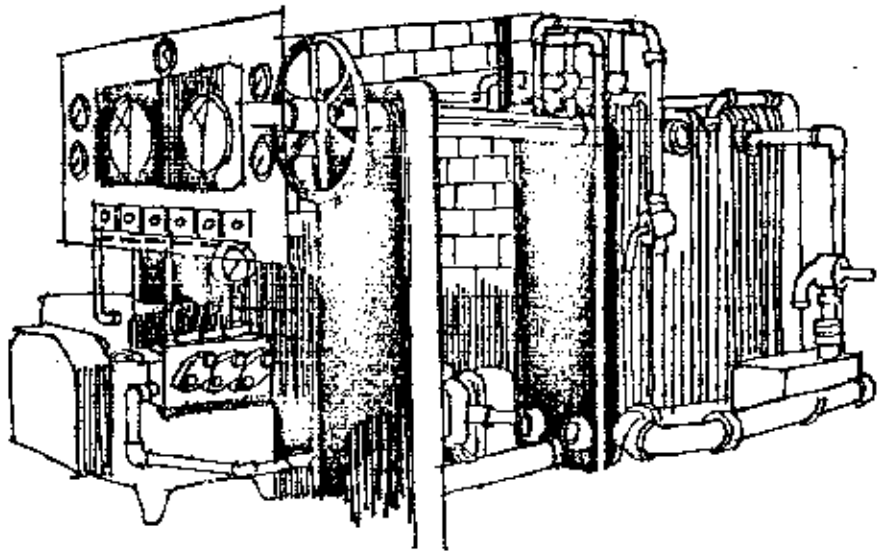
accidentally now and then find their way to his hands or clothing and eventually to the dairy equipment and the milk supply.

Of course, the typhoid carrier is not aware of his condition. If he were, he would, in most cases, be honest enough not to imperil the lives of others by working in a dairy. But this is the dangerous thing about it. The typhoid carrier is usually ignorant of the fact that he is a carrier of the disease.

Knowing these facts, what could the dairyman do? Of course, he could have everyone at the dairy send samples of their feces and urine to the health department laboratory so that it could be determined whether they contained typhoid organisms.

Suppose the laboratory reported that none of these specimens contained the organisms of typhoid fever, would this assure the dairyman that none of his employees is a typhoid carrier; and that you, as his customer, would never contract typhoid fever from drinking his milk?

Unfortunately, the answer is no. Many typhoid carriers do not discharge the typhoid organisms every day; and on, the day the specimens were collected and sent to the laboratory, the carrier, if there was one, may or may not have been discharging the organisms.



There is, therefore, no way of making absolutely sure that raw milk will never contain typhoid fever organisms; and, if you knew as much about the danger as the health officer does, you would live in constant fear of contracting typhoid fever if you consumed raw milk.

How about a third example—septic sore throat or strep throat? If you were drinking raw milk, what could your dairyman do to assure you that you could never contract this disease from his milk supply?

Frankly, he could not give you this assurance. A milker may think he has an ordinary cold, when really it is a strep throat. He may then infect the milk supply directly; or may infect the cow's udder during the milking process; and the milk from that cow may later be teeming with the organisms of the disease.

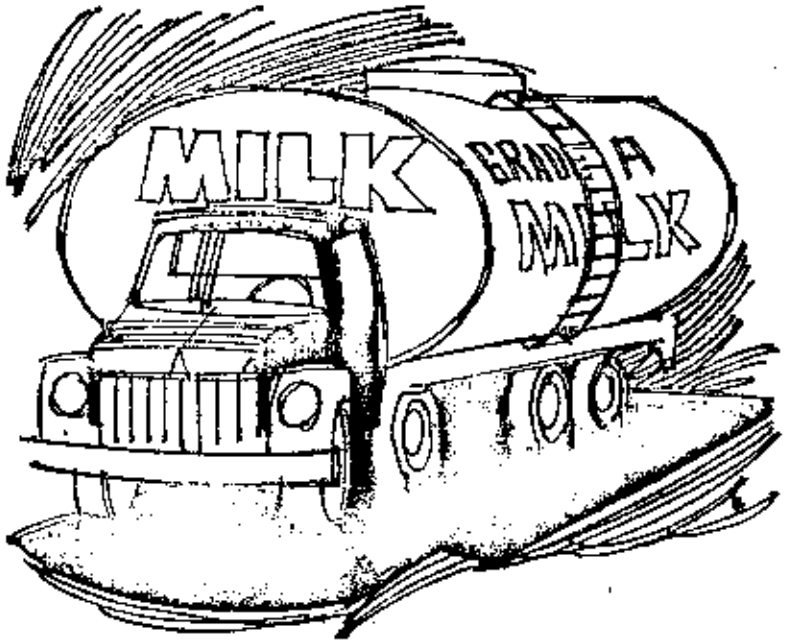
Suppose we were to examine every milker's throat every day and every cow's udder every day, even then we would not have done away with the danger; because, by the time the report came back from the laboratory, the milk would have been consumed. Of course, a daily examination would be out of the question, if for no other reason than the expense entailed.

From these few examples, it seems impossible to escape the conclusion that there is no method known to guarantee milk to be safe, except by pasteurization or boiling.

Should we rely on boiling? This is what is done in many parts of Europe and South America; and, as a result, they have practically no milkborn disease. But with these people, boiling milk is a necessity and a daily habit. In most of the areas in question, refrigeration is not available and milk is boiled to slow its souring.

In this ntry, we have two factors to consider; first, that most famil ve electric refrigeration and can keep milk sweet; and, second, most people do not like the taste of boiled milk. Even if the health authorities simply asked all people to boil their milk, it is very doubtful if they could depend upon sufficient numbers doing it to prevent epidemics.

The only other method then, to assure the safety of milk, is pasteurization, unless the milk is adulterated by the use of chemicals, which would not be acceptable in this country. That is why health authorities today agree that all milk should be pasteurized.



The term "pasteurization" refers to the process of heating every particle of milk to at least 145°F. and holding it continuously at or above this temperature for at least 30 minutes (vat or batch pasteurization), or to at least 161°F. and holding it continuously at or above this temperature for at least 15 seconds (high-temperature short-time pasteurization). In each case this must be done in equipment which is properly operated and approved by the health authorities. Pasteurization destroys all organisms capable of transmitting disease through milk.

Health authorities and physicians agree that pasteurization has little, if any, effect on the food value of milk. The nutrients in milk are unaffected by pasteurization except the ascorbic acid (vitamin C) and thiamine (vitamin B). Ascorbic acid content is reduced, but the level of this vitamin in milk is not of particular

importance, since a varied American diet inclu ther sources rich in ascorbic acid. Pasteurization reduces th. e somewhat, but the milk still supplies a significant amount in the daily diet.

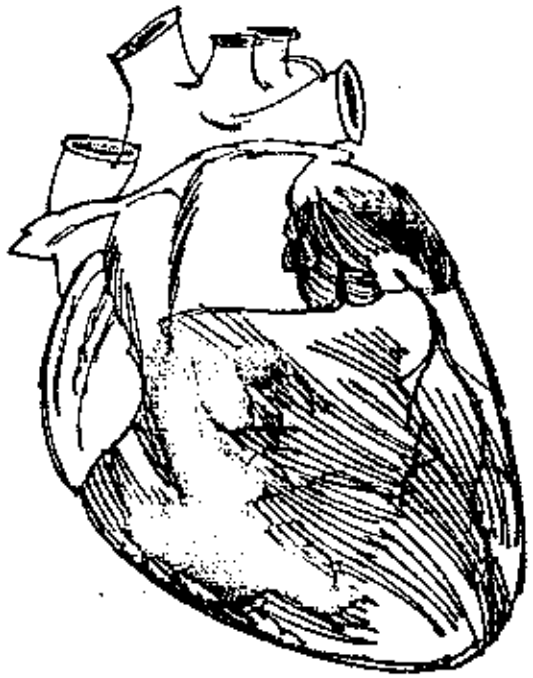
A number of years ago, the Public Health Service conducted an intensive study on about 3,700 children to determine whether those who drank pasteurized milk actually thrived as well as those who drank raw milk.

The results showed that the average weight of children receiving raw milk was 33.2 pounds, whereas, the weight of those fed pasteurized milk was 33.6 pounds. The average height of those receiving raw milk was 37.4 inches, those fed pasteurized milk was 37.5 inches. From the parents' reports, it was found that children who drank raw milk suffered with communicable diseases more frequently than did those who drank pasteurized milk. The final conclusion of the study was that, taking into account the average supplementary American child diet, children who are fed pasteurized milk thrive as well as children who are fed raw milk, and contract certain communicable diseases less frequently.

Of course, we do not rely on pasteurization as a cure-all and neglect all precautions on the farm or during transportation from the farm to the plant. The pasteurization process is operated by human beings and is as foolproof as modern science and know-how can make it. We insist that the milk we drink be not only properly pasteurized, but also that it be carefully produced on the farm so that we will have the maximum practicable protection from the cow to the consumer.

In every State and in most municipalities an active milk sanitation program is being carried on. To promote uniformity and effective control, the Public Health Service has developed a Milk Ordnance recommended for adoption by State and local health agencies. Currently, it is the basis of milk sanitation laws or regulations of 39 States. Its provisions have been voluntarily adopted by over 2,000 municipalities and counties—a population coverage of almost 117 million people.

Starting at the time of World War II, the demand for milk in the United States grew by leaps and bounds. Much of the milk brought into many communities from distant sources proved to be of inferior quality. Milk sanitation control officers became alarmed over the possibility of epidemics. Health organizations, including the American Public Health Association, the Association of State and Territorial Health Officers, and the Conference of State Sanitary Engineers asked for Federal action. The Surgeon General of the Public Health Service convened a National Conference on Interstate Milk Shipments to study the problem. An outcome of the conference, which was held in 1950, was agreement by



State and Federal representatives and the dairy industry on principles designed to govern a national milk certification program.

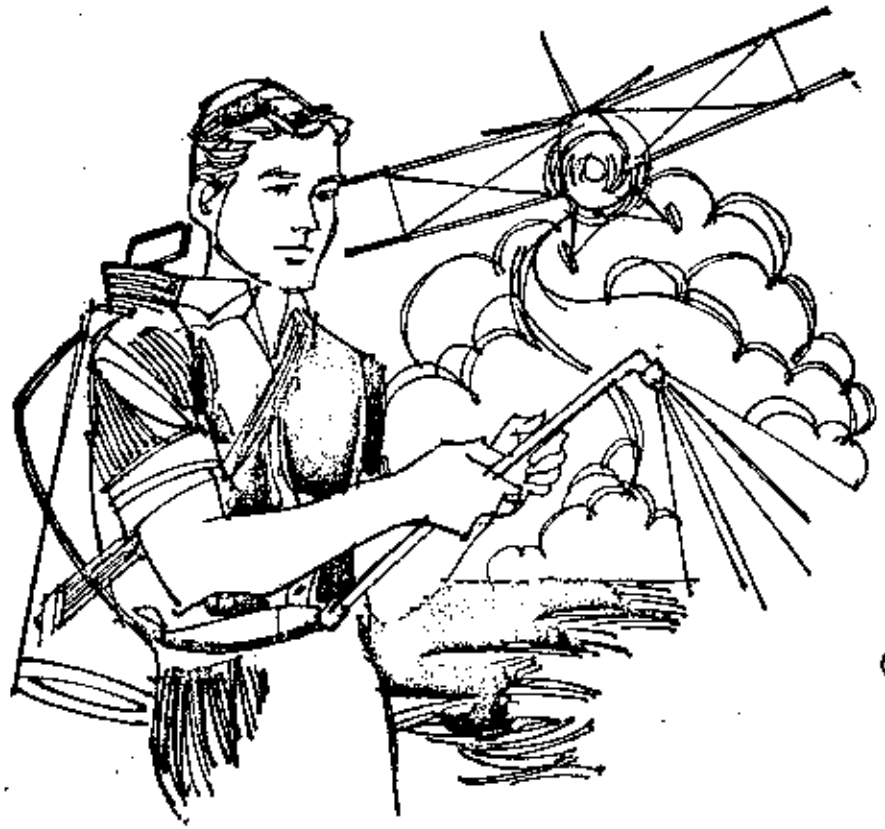
The program functions on a voluntary, cooperative basis to benefit the milk consumer. Existing State and local milk control agencies are utilized for inspection of raw and pasteurized milk and milk products at the source. Procedures and rating methods developed by the Public Health Service are used to determine compliance with the Grade "A" Pasteurized Milk Ordinance, which is used as the basic milk sanitation standard.

Ratings of producer farms, receiving stations, and processing plants are made. After the ratings are certified to the Public Health Service by the States, the ratings are published and distributed quarterly by the Department of Health, Education, and Welfare. This publication is utilized by many milk control authorities in obtaining milk supplies that are produced, handled, and inspected in accordance with established high sanitary standards.

In 1951, the first year of operation, the interstate milk shipper publication listed the names and ratings of 160 shippers located in 17 States, drawing milk from an estimated 30,000 grade A dairy farms. By January 1966, the number of States cooperating had risen to 47 and the number of certified shippers was 1,526 receiving milk from an estimated 136,000 grade A dairy farms. During 1965, over 12 billion pounds of milk were shipped in interstate commerce under this program.

Substantial benefits to the milk consumer ha ulted from this program. As more and more high quality milk has become available, questionable and often inferior and unsafe milk has been largely eliminated. Reliable information about the sanitary quality of milk from other areas has become readily available. The need for costly duplication of inspection has been eliminated, so that tax dollars have been saved. Since increased confidence for the work of other milk sanitation agencies has resulted, cooperation has extended to other environmental health areas. The program has also had considerable impact on intrastate shipment of milk, since the philosophy and principle of the interstate plan have been applicable to shipments of milk within State borders.

The Milk Shipper Certification Program has also stimulated a high degree of uniformity in the application of sanitary standards. At the request of States, the Public Health Service has certified the rating procedures of 151 State milk sanitation rating officers in 49 States. The Service has also assisted States in the training of milk sanitarians in the uniform interpretation and application of the Grade "A" Pasteurized Milk Ordinance recommended by the Public Health Service.



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IV. What eing Done About Cholesterol, Chemical Residues, and Radio-Active Fallout?

It is not difficult to understand the concern of the American people over heart and blood vessel diseases, since they claim over 900,000 lives yearly.

The most common cardiovascular disease is arteriosclerosis or hardening of the arteries. In this disease, a fatty material accumulates in the walls of the arteries. This material may build up to a point where the artery is closed off or the blood flow is slowed down to a point where the blood clots and closes off the artery. If the coronary artery which feeds the heart muscle is closed, a stroke results.

At present, no one knows what causes this fatty material to build up in the arteries. There are a number of theories as to the cause of arteriosclerosis, including the stress and strain of modern living, hormones, heredity, lack of exercise, overweight, and the use of animal fats in the daily diet.

Cholesterol appears in many animal fats and organ tissues and is also manufactured by the body from other foods. Although it is involved in the heart disease problem because cholesterol is one of the substances found in the fatty material that builds up in the walls of the blood vessels, it is also recognized as essential to good health.

Recent studies suggest that high levels of triglycerides, another form of fat in the blood, are another important indicator of potential arteriosclerosis. High levels of triglycerides in the blood may be caused by consumption of either fats or carbohydrates (sugars and starches).

Because there is an assumption that a relationship exists between high blood fat levels and the development of arteriosclerosis, the American Heart Association has outlined experimental diets for reducing blood fat levels.

The Public Health Service has taken the stand that dietary changes may be warranted in subjects with elevated blood lipids, evidence of existing heart disease, or evidence of high coronary heart disease susceptability. But such diets should be prescribed and supervised by the subject's physician. Available evidence is insufficient to warrant any recommendation that the general public modify its dietary habits; and there is no reliable evidence that changing one's diet or reducing blood lipid levels by whatever means will reduce heart-disease risk.

The danger in self prescription of high unsa d fat diets lies in the failure to recognize the true nutrient content of different foods. Animal protein sources such as dairy, poultry, and meat products must, therefore, be used to supply a high protein calorie restricted diet even though products contain saturated fats.

It should be kept in mind that only one milk nutrient—the fat—has been implicated in cardiovascular disease. Milk, even with its fat content removed, is still one of the finest sources of important food nutrients available in one food.

Recently, a new problem which has arisen is that of unintentional additives to fluid milk. Residues from pesticides, antibiotics and other chemicals may be excreted through the udders of the cow and result in adulteration of the milk. Some of these materials could cause toxic or allergic reactions in humans, and others have proven to be carcinogenic (cancer producing) in laboratory animal studies. (Constant monitoring and testing by both industry and government protect the consumer from significant levels of these dangerous materials. A real public health hazard does not, at this time, exist for humans due to the ingestion of milk or milk products.)

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The Fe[illegible] Food and Drug Administration has set a "no tolerance" le[illegible] on the residues of both pesticides and antibiotics in milk. This means that any milk or milk product containing such a residue is condemned and the producer is prohibited from further sales until the residues can no longer be detected. Recently developed procedures have made possible the detection of DDT in milk at levels of 0.02 parts in a million. Other pesticides may be detected in similar minute quantities.

A chief source of pesticide residues in milk is from the feed which the cow consumes. In order to control crop damage due to insects, the dairyman may use only those pesticides which have been approved by the U.S. Department of Agriculture. When used as directed these are considered relatively safe. However, misuse or accidental application of non-approved pesticides may result in contamination of the feed crops or pasture land.

Antibiotics, which are found by monitoring milk, are the result of treating cattle for disease. Milk regulations prohibit the sale of milk for such time as the material is being excreted in the milk. The careless operator is penalized by having his entire milk supply condemned when antibiotic residues are detected. This may be avoided by having animals treated by a competent veterinarian and by using the medicinal agents as directed by the manufacturers label.

Public concern has been expressed in the past few years because milk may contain certain radioactive materials, including strontium 89, strontium 90, cesium 137, and iodine 131. Some radioactivity has always been present in our environment and in food. Radioactive potassium, radium, and other trace materials occur in varying concentration in different foods and in different areas. In recent years, there has been added radioactivity because of man-made radioactive materials. Fission products resulting from the fissioning of uranium and other materials have appeared in foods including milk.

When large quantities of radioactive materials are deposited in the atmosphere as a result of testing of atomic weapons or an accident in a reactor power plant, this material is carried by air currents and eventually deposited as fallout on the earth. These particles can enter man and animals through air inhaled or through food and water. In general, foods including milk are the principal source. Cattle forage crops become contaminated with fallout. The contaminated forage is consumed by dairy cattle and a part of the radioactive material is excreted in the milk.

Since 1956, the Public Health Service in cooperation with State and local health agencies has conducted a system of surveillance for radioactive materials in the environment. Milk has been given particular emphasis in this surveillance s[illegible] so that a current knowledge exists as to the levels of var[illegible] radioactive materials in milk. Research efforts of the past several years have provided methods of reducing the amount of certain radioactive materials in milk, should such measures ever be indicated.

The Federal Radiation Council has made recommendations regarding potential hazards of radioactivity and measures to be taken to reduce such hazards. The present levels of radioactivity in milk are not sufficient to warrant public concern. The surveillance systems provide constant and current knowledge of radioactivity levels in the environment with particular emphasis on milk. Should protective measures ever be indicated, several means exist to reduce radioactivity exposure from milk without altering individual milk consumption habits.

Summary

Milk with its contribution to the nutritional needs of humans has justified its title as "nature's most nearly perfect food." It owes its importance in the diet to the excellent quality of its protein, to the completeness of its assortment of mineral elements and the excellent proportions in which they occur, to the high content of calcium which makes milk almost indispensable for ideal storage of this element during growth, to the presence of vitamins A, B, and G in quantities which make generous use of milk day by day a practical guarantee against deficiency of any of the three, and the calcium phosphorous ratio so importnat for the building of strong bones and teeth.

Grade A pasteurized milk must come from healthy animals and be produced, pasteurized, and handled under conditions of strict sanitary control by State and local milk sanitation officials. It is paradoxical that milk is both important to the maintenance of our health, and at the same time possesses the potential to serve as a carrier of such diseases as tuberculosis, brucellosis, Q-fever, and septic sore throat. Effective milk sanitation controls in every State today protect consumers against such diseases. Continued vigilance through adherence to these controls is, however, essential. One program which is actively improving milk quality and providing local milk sanitation authorities throughout the Nation with dependable information about the sanitary quality of milk received from beyond their local jurisdiction is the Cooperative State-Public Health Service Program for certification of Interstate Milk Shippers.

Pesticides, antibiotics, and radioactive fallout could create a problem. However, cooperative plans by industry, and local, State,

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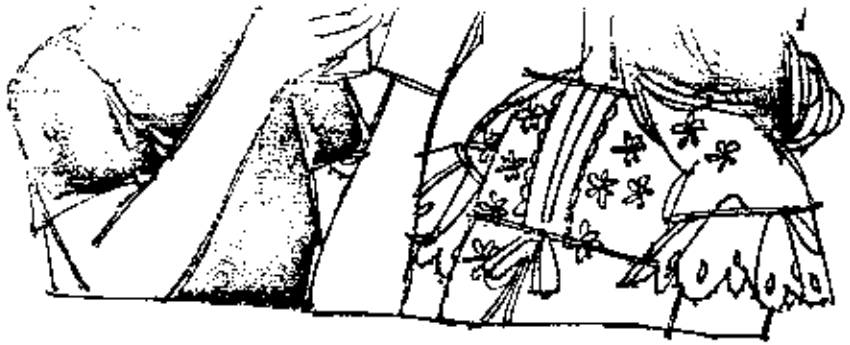
nd [illegible]ral authorities have been worked out so as to safegu[illegible] [illegible]rotect the milk consuming public from these harmful [illegible]s.

Today, consumers may be assured that pasteurized grade A milk and milk products that they purchase have been properly safeguarded to prevent illness when produced, processed, and packaged in accordance with the *Grade "A" Pasteurized Milk Ordinance—1965 Recommendations of the United States Public Health Service.*



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Diphenylhydantoin: Effects on Calcium Metabolism in the Chick

Abstract. *Rickets, hypocalcemia, decreased duodenal calcium transport, and reduction of calcium binding protein have been produced in chicks treated with diphenylhydantoin. These effects are directly related to diphenylhydantoin dose and inversely related to the intake of vitamin D_3 (cholecalciferol).*

Hypocalcemia, rickets, and osteomalacia develop with variable frequency in patients on long-term anticonvulsant therapy (*1*). Both phenobarbital (PB) and diphenylhydantoin (DPH) have been implicated; several reports suggest that these agents, by altering calciferol metabolism, may produce a state of functional vitamin D deficiency. Our studies in chicks demonstrate that DPH causes a decrease in intestinal calcium absorption, intestinal calcium binding protein (CaBP), serum calcium, and bone ash. Radiographic and histologic evidence of rickets was also seen. These effects were directly related to the DPH dose and inversely related to the amount of cholecalciferol, that is, vitamin D_3 (D_3), given concomitantly.

Cholecalciferol is hydroxylated in the liver to 25-dihydroxycholecalciferol [25-(OH)D_3] and in the kidney to 1,25-$(OH)_2$-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] (*2*). Each step increases polarity and biological activity. The same sequence obtains for ergocalciferol (D_2), which is the form commonly used for dietary supplementation. Hahn *et al.* (*3*) found decreased serum concentrations of 25-(OH)D_3 in patients receiving either PB or DPH. Concentrations of 25-(OH)D_3 correlated directly with serum calcium and with calciferol intake. Hahn *et al.* (*4*) also reported that the half-life of D_3 in the plasma was shortened in subjects receiving PB and described increased conversion of D_3 and 25-(OH)D_3 to more polar metabolites by hepatic microsomes from PB-treated rats.

Tolman *et al.* (*5*) reported accelerated plasma disappearance rates and increased urinary excretion of both D_3 and 25-(OH)D_3 in patients who received either PB or DPH. In contrast, Schaefer *et al.* (*6*) and Silver *et al.* (*7*) found increased serum 25-(OH)D_3 under similar circumstances. Healing of bone lesions has been reported after administration of large doses of D_3 (*1*) or small doses of 25-(OH)D_3 (*8*). These findings have led to the view that anticonvulsants act as calciferol antagonists by inducing liver enzymes that convert D_3 to inactive or rapidly excreted metabolites (or both).

In the rat, both Koch *et al.* (*9*) and Caspary (*10*) found that absorption of calcium was depressed by DPH and PB. Neither found any significant change in intestinal calcium binding activity, and concluded that the synthesis of the CaBP was not affected. Since CaBP has an absolute dependence on vitamin D (or metabolite or vitamin D active steroid) for its synthesis and maintenance (*11*), the negative findings of Koch and Caspary would have important implications with respect to the pathogenesis of osteomalacia and rickets accompanying anticonvulsant therapy, if verified. Also, Clark *et al.* (*12*) saw no radiographic evidence of rickets in DPH-treated rats.

White Leghorn cockerels (1 day old) were offered a calciferol-free but otherwise adequate diet (*13*). After 5 days they were divided into seven groups whose D_3 and DPH intakes are given in Table 1. Crystalline D_3 was dissolved in sesame oil to give 3 or 6 international units (I.U.) per 0.1 ml, which was administered orally. Group 1 received sesame oil alone. The DPH (diphenylhydantoin, Eastman) was added to the diet to give 1 or 2.5 g per kilogram of diet, and DPH intake was calculated from weekly averages of food consumption and body weight (*W*). The body weight was converted to surface area

(M^2) by the formula $(M^2) = 9.2 \times 10^{-4} \times W^{2/3}$. The calculated intakes were higher than those used therapeutically in humans. At 28 days the duodenal uptake of ^{47}Ca was assessed, by in ligated loop procedures (*13*). The duodenal mucosa was then scraped free of the underlying muscle layers and homogenized in a buffered solution (119 m*M* NaCl, 4.74 m*M* KCl, 13.7 m*M* tris-HCl, *p*H 7.4) with the use of a Potter-Elvehjem homogenizer and Teflon pestle. After centrifugation at 38,000*g* for 20 minutes in the cold, the supernatant was treated at 60°C for 10 minutes, cooled, and centrifuged; its calcium binding activity was measured by the Chelex 100 ion exchange binding assay (*14*). The absolute amount of CaBP in the supernatant was also determined by a specific radioimmunoassay (centrifugal diffusion in gel containing antiserum to CaBP), with the use of a highly purified CaBP as the standard (*15*). Protein concentrations were measured by the Lowry method (*16*). Serum calcium was determined by the procedure of Bett and Fraser (*17*). Serums were then pooled, and the DPH content was measured (*18*). The ash content of the dried tibiae that were extracted with ether was also estimated. At 30 days representative chicks were subjected to radiography.

Serum DPH was 2.0 mg per 100 ml on the lower DPH intake and 4.7 mg per 100 ml on the higher. The radiographic appearance and the effects of DPH on the skeleton were visualized (Fig. 1). Direct measurements of the width of the physes (indicated by the arrows, Fig. 1) show a 50 percent increase in the DPH-treated chicks. Histologic examination of sections taken longitudinally through the epiphyseometaphyseal junction and stained with hematoxylin and eosin showed the characteristic changes of rickets. Irregularity and disarray of all cellular components were present in all of the cellular components of the widened cartilaginous physeal plate; the vacuolating and degenerating cells gave no evidence of mineral depostion; excessive amounts of osteoid were present as broad "seams" without calcification; and those bony trabeculae present in both the epiphysis and metaphysis were thin and sparse. The last-mentioned resulted in compromised capacity of the bone to withstand the stress of weight bearing, leading to "flaring" of the juxtaphyseal regions on x-ray. Severe rickets was produced at 30 days by DPH in the chicks on the lower level of vitamin D_3. The greatest effect was observed at the higher DPH intake, being a combination of severe rickets with stunting of overall growth (group 4). In the chicks receiving the higher dose of D_3 radiologic evidence of early rickets was seen in only one animal on the higher intake of DPH (group 7) Radiologic evidence of severe rickets was present in the group 1 chicks at 23 days. The only other group showing abnormality at this time was group 4, which showed evidence of early rickets.

The terminal body weight, serum calcium concentration, and degree of bone mineralization (ratio of ash weight to dry weight) of the various groups are shown in Table 1. The chicks receiving the lower dose of vitamin D_3 (groups 2, 3, and 4) were most severely affected by DPH. Group 4 chicks receiving 2.5 g of DPH per kilogram of diet showed growth rates, serum calcium concentration, and bone ash values (Table 1) indistinguishable from those chicks raised in the absence of D_3 (group 1). Chicks of group 3 yielded values intermediate between those of group 4 (high DPH) and group 2 (controls). A similar situation occurred with respect to calcium absorption and the vitamin D–induced CaBP (Fig. 2).

Table 1. Effect of diphenylhydantoin on body weight, serum calcium concentration, and tibia ash of chicks on two levels of vitamin D_3.

Group*	Treatment		Terminal body weight (mg)	Serum calcium (mg/100 ml)	Tibia ash (%)‡
	D_3† (I.U./day)	DPH (mg/m²)			
1	0	0	193 ± 11	6.4 ± 1.2	26 ± 1
2	3	0	280 ± 14	9.7 ± 0.1	39 ± 2
3	3	760	258 ± 10	7.9 ± 1.1	34 ± 1
4	3	1800	198 ± 9	6.2 ± 0.2	29 ± 2
5	6	0	305 ± 13	10.0 ± 0.2	41 ± 1
6	6	760	293 ± 6	9.7 ± 0.2	42 ± 1
7	6	1800	260 ± 12	8.5 ± 0.3	36 ± 1

chicks per group; values represent means ± standard error of the means. † Vitamin D_3 given ‡ Percentage of fat-free dry weight.

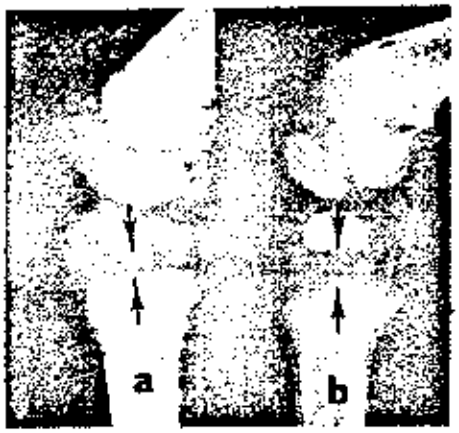

Fig. 1. Tarsometatarsal joints. (a) Group 2, control diet. The chicks were given 3 I.U. of D_3 per day. (b) Group 3, DPH (1 g per kilogram of diet provided 760 mg per square meter per day) was added to the diet, in addition to the 3 I.U. of D_3 given daily.

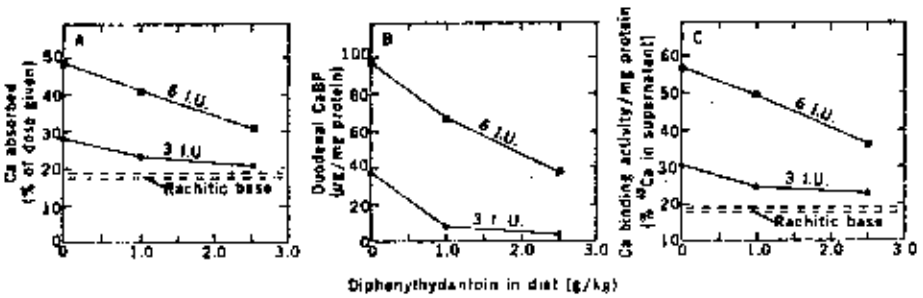

Fig. 2. Effect of diphenylhydantoin and D_3 on calcium absorption, CaBp, and calcium binding activity. Six chicks per group were used; the values represent the means ± standard error of the means. (A) Duodenal absorption of calcium was determined by the in situ ligated loop technique (*13*). (B) Calcium binding protein was determined by the radioimmunoassay (*15*). (C) Solid circles indicate a D_3 dosage of 6 I.U. per day and open circles indicate a D_3 dosage of 3 I.U. per day. Calcium binding activity was determined by the ion exchange resin (Chelex) assay (*14*).

DPH decreased the duodenal absorption of the test dose of ^{47}Ca and duodenal CaBP to near zero (comparable to that in rickets) in a dose-related fashion. A depression in calcium bind- activity, which reflects CaBP, was noted.

Chicks on the higher dosage of D_3 (groups 5, 6, and 7) were affected by DPH but less so than those receiving 3 I.U. of D_3 per day. The ingestion of 2.5 g of DPH per kilogram of diet (group 7) resulted in a significant reduction in growth ($P < .03$), serum calcium ($P < .01$), and tibia ash ($P < .01$) (Table 1). Little or no effect of the lower level of DPH on these parameters was noted. Similarly, group 7 (6 I.U. of D_3; 2.5 g of DPH per kilogram of diet) displayed a reduction in ^{47}Ca absorption (Fig. 2A), vitamin D–induced CaBP (Fig. 2B), and calcium binding activity (Fig. 2C). At the lower intake of DPH (1 g per kilogram of diet), the only significant effect observed was a reduction in CaBP (Fig. 2).

Group 7 chicks maintained calcium absorption and CaBP levels equal to those of group 2, but were hypocalcemic. Bone analysis showed no significant difference between group 2 and group 7 ($P = .25$) (Table 1 and Fig. 2). calcemia without bone involve- has been noted clinically (*1*); no explanation is apparent at the present time.

Our data showing a depression in CaBP by DPH treatment is in disagreement with the reports of Koch *et al.* (*9*) and Caspary (*10*). These investigators assessed CaBP by a nonspecific calcium binding assay, using the ion exchange resin technique (*14*), which measures total calcium binding activity and not that due exclusively to CaBP. In our study, both the nonspecific ion exchange resin procedure and the specific radioimmunoassay indicated a depression in intestinal CaBP (Fig. 2). Since Koch *et al.* (*9*) and Caspary (*10*) used rats, there is a possibility of a species difference in responsiveness to DPH.

It is apparent that, in chicks, DPH administration causes readily demonstrable effects on calcium metabolism which provides support for clinical observations on epileptic patients given nvulsant therapy. The effects dose dependent and related inversely to the amount of calciferol given. The fact that the vitamin D–induced CaBP also changed in proportion to the change in calcium absorption provides further support for the contention that the DPH-dependent defect is related in some way to the abnormal metabolism of or responsiveness to D_3. There is no evidence for a direct effect of DPH on the calcium absorption mechanisms per se, as thought to pertain to cortisone treatment (*19*). Our findings support suggestions that close attention should be paid to calciferol intake in patients requiring seizure therapy. They also indicate that the chick may be a useful model for testing drugs suspected of being calciferol antagonists.

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THE PROVITAMIN D OF CHOLESTEROL

I. THE ANTIRACHITIC EFFICACY OF IRRADIATED CHOLESTEROL

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The discovery in 1927 that ergosterol could be antirachitically activated to a high degree (1) must be regarded as a most noteworthy event in the development of our knowledge concerning the antirachitic vitamin. Since that time a truly enormous amount of work has been carried out on the chemistry and physiological effects of this remarkable substance and its transformation products. The results of these investigations are too well known to require comment here other than that they may well be said to include some of the most notable achievements in the field of vitamin research. In consequence, the antirachitic properties of irradiated ergosterol have been firmly established and it has become an accepted article of commerce with highly prized therapeutic virtues.

As is well known, the way to the above discovery was pointed by the investigations into the nature of the provitamin constituent of cholesterol (1–3), which resulted in the accepted identification of this constituent with ergosterol. Although certain investigators (4–6) obtained results suggesting that the presence of ergosterol did not account for all of the activity of certain samples of irradiated cholesterol, the identity of the main provitamin constituent became definitely accepted. This offered a ready explanation of the known antirachitic effect of ultra-violet light applied to the animal body and of the fact that these same rays induce activation in practically all plant and animal tissues. Consequently, it has become almost universally accepted that ergosterol is the main,

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if indeed not the sole, parent substance of antirachitic activity throughout the plant and animal worlds.

While the antirachitic effect of irradiated ergosterol has been completely established, nevertheless, there has been an increasing number of anomalous results brought to light which have not been satisfactorily explained. It has been definitely shown, for example, that quantitative differences exist between this supplement and others, such as cod liver oil, in their ability to cure and prevent rachitic manifestations, particularly in certain species. Baby chicks are especially sensitive to these different supplements (7–10), receiving protection against leg weakness from much less cod liver oil than irradiated ergosterol on the basis of rat units fed. The difference between these two supplements is so marked as to have caused one group of investigators (9) to conclude that the antirachitic factor was not the same substance in both. The early clinical results on infants receiving irradiated ergosterol (11) brought out a similar quantitative difference although of lesser degree. This relative inefficiency of irradiated ergosterol has been difficult to understand in view of the reported efficacy of direct ultra-violet light applied to the animal body (12–16). More recent clinical experiments (17, 18), dealing with the ability of vitamin D-rich milks to cure or prevent infantile rickets, have yielded results which further complicate the problem. Not only have these milks been reported as being more efficacious than other supplements but also as differing in their efficacy depending on the method by which they are enriched with vitamin D.

In addition to the above, data have appeared within the last few years indicating that ergosterol is poorly, if at all, absorbed from the digestive tract, a situation not easily understood if it is normally a constituent of the animal body. Schönheimer (19) has called attention to the non-absorbability of plant sterols in general and has found that ergosterol behaves in very much the same way. In extensive experiments carried out on mice, rats, rabbits, and dogs fed with ergosterol or ergosteryl acetate (20, 21) he and his associates found no evidence of absorption although, as they have pointed out, the spectrophotometric methods for estimating ergosterol are very sensitive. Only in the laying hen (22) was any indication of absorption obtained as determined by a slight increase in the ergosterol content of the egg yolk after feeding large

amounts of ergosterol. Similar results on this latter species were reported by Menschick and Page (23). These authors have also reported an increase in the ergosterol content of the brain cholesterol of rabbits fed with large amounts of ergosteryl acetate (24). Their results, however, are not very convincing since, as Schönheimer *et al.* (21) have noted, the increase was very small and the values reported all lay within the normal limits of the ergosterol content of cholesterol. Beumer and Hepner (25) were also unable to demonstrate any appreciable absorption of ergosterol by mice after feeding ergosterol, ergosterol plus cholesterol, or ergosterol plus cholic acid.

During the past 3½ years this laboratory has been engaged in studies which have a direct bearing on the problems discussed above. In this paper we wish to present certain data which have been obtained from further investigations into the nature of the constituent of cholesterol which may be antirachitically activated. These data, it is believed, will not only suggest a much more satisfactory explanation of some of the above anomalous findings but will also be of extreme significance for the further development of our knowledge in this field. Our results, in brief, indicate that the provitamin present in cholesterol is not identical with ergosterol, a finding which is in direct conflict with a fundamental concept on which much of the modern theory of vitamin D activity is based.

Methods

The basis of procedure throughout the experiments to be reported was the correlated assays of our various antirachitic preparations on both rats and chicks. The rat assays were carried out according to the procedure of Steenbock *et al.* (26) with use of the well known line test method applied to the split ends of the radius and ulna; and the results were expressed in Steenbock rat units.[1]

Our chick tests were of the prophylactic type carried out in a large heated basement room with small glass windows, those windows near the chicks being shaded with a colored translucent window glass substitute. The birds were kept in a specially constructed all-metal brooder, each cage being equipped with a wire

[1] Steenbock (9) has reported that 1 such unit approximates 2.7 international units.

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mesh bottom and suspended above a large metal pan. In the main, we used white Leghorn chicks either purchased when day-old from reliable poultrymen or hatched in our own laboratory from eggs always obtained from a small flock of hens maintained on a uniform diet. Each group of chicks contained usually fifteen to twenty individuals.

The basal ration which we used most (basal Ration I) had the following composition: ground yellow corn, 40; wheat bran, 20, wheat middlings, 20; alfalfa leaf meal, 5; dried buttermilk, 4; meat scrap, 4; fish meal, 4; ground oyster shell, 2; and salt, 1. This ration was chosen in our early experiments because we wished to determine the effect of the various supplements used under what might be termed practical conditions. Effectively supplemented with vitamin D, it supported excellent growth; unsupplemented it produced good growth during the first 3 or 4 weeks, following which noticeable evidences of leg weakness appeared and growth declined. With this ration, our chick experiments were usually of 8 weeks duration. In many experiments we added the various supplements after the chicks had been on this basal ration for 1 or 2 weeks.

Later, in an attempt to make our experimental conditions more rigorous and so reduce the length of the experiments, we used another ration (basal Ration II). This was the one described by Hart, Kline, and Keenan (27) which had the following composition: ground yellow corn, 59; wheat middlings, 25; crude casein, 12; $CaCO_3$, 1; Ca phosphate, 1; dried yeast, 1; salt, 1. This diet was much more severe than basal Ration I and when unsupplemented with a potent antirachitic preparation soon led to pronounced leg weakness with accompanying poor growth and a very low percentage bone ash. On the basal ration alone or with ineffective supplements many of our chicks did not survive an experimental period of 6 weeks.

In making additions to the basal diets, except where cod liver oil was added, we followed the practise of dissolving or suspending the preparations in corn oil in such concentration that 1 per cent of the oil mixed with 99 per cent of the basal ration introduced the desired amount of supplement. The cod liver oil was added to the basal rations in the amounts indicated (usually 0.25 or 0.5 per cent). The irradiated ergosterol which was used in our experiments

was obtained from large supplies activated and carefully assayed in this laboratory. The cod liver oil used throughout was from one sample of high grade medicinal oil purchased before our experiments were initiated. This oil assayed 40 rat units per gm.

Throughout the experiments the chicks were weighed weekly and observations made as to their physical appearance and behavior. The first appearance of leg weakness, indicated by an unsteady and staggering gait and a tendency to squat, was recorded. At the end of the experiment, the chicks were killed and one bone, the tibia, was dissected out from each. In order to minimize the labor involved in carrying out the ash determinations all the bones from each group were combined into one large sample and an average figure for the group obtained in this way. The bones were crushed, wrapped in large filter papers, extracted with hot alcohol for 36 to 48 hours in a large modified Soxhlet apparatus, dried, and ashed in an electric muffle furnace.

Preliminary Experiments

Our interest in this problem dates from 1930 when we set out to study the reported inefficiency (7) of irradiated ergosterol in preventing leg weakness in baby chicks. In our first experiments irradiated ergosterol in solution in corn oil was compared with cod liver oil on the basis of so many rat units per 100 gm. of chick diet. A series of levels of each supplement was fed because we realized that only at the minimum protective dose could exact quantitative comparisons be obtained. Our results confirmed those of others in showing that the irradiated ergosterol was much less effective than cod liver oil.

In later experiments cod liver oil and other less potent fish body oils (menhaden, cod) were reinforced with varying amounts of irradiated ergosterol and a series of levels of each of these preparations was fed and compared with irradiated ergosterol dissolved in corn oil. This was done to find out whether there existed in the fish oils any factor, other than vitamin D, which might be responsible for the greater efficiency of the latter or which might supplement irradiated ergosterol and so increase its effectiveness. In these additions account was taken of the amount of the antirachitic factor from each source and in no case was any evidence found that the fish oils enhanced the effect of the irradiated ergosterol

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beyond the very small amounts of vitamin D which they, themselves, introduced.

In these same experiments groups were included which received the non-saponifiable fraction of cod liver oil dissolved in corn oil. These additions were equally as effective as the cod liver oil.

In one experiment we also injected subcutaneously an oil solution of irradiated ergosterol, believing that poor absorption from, or destruction in, the digestive tract might hold the explanation for the low efficiency of the irradiated ergosterol. Further, we fed a series of levels of the dry irradiated mycelium from *Aspergillus niger* (known to contain ergosterol) to determine if, possibly, in addition to ergosterol, there might be other substances present capable of activation which would increase the antirachitic effect. Our results revealed no advantage from injecting the factor and while our experience with the irradiated mycelium was not sufficiently extensive to say whether or not slight differences existed between it and irradiated ergosterol, it was definite that both possessed a potency of the same order.

In all of these experiments we found that 10 rat units per 100 gm. of diet as cod liver oil or other fish oil yielded good protection against leg weakness while 25 or more times that number of rat units of irradiated ergosterol were required to afford equal protection with the ration which we were using. It seemed to us, therefore, that the vitamin D of fish oils and irradiated ergosterol must be quite different in nature since their effect was so different in this species. Other investigators have published similar findings (8–10).

Experiments with Irradiated Crude Cholesterol

While we were in the midst of the above experiments and reflecting upon this difference between the two supplements, we were struck by the great efficacy of direct ultra-violet light upon the body of chicks in preventing symptoms of vitamin D deficiency as reported in the work of others. Scott, Hart, and Halpin (11) had, for example, reported that as little as 3 minutes per week of direct exposure to a quartz mercury vapor lamp protected to a remarkable degree baby chicks subsisting on a severe rickets-producing diet. Russell and his associates (14) had also shown a marked curative effect of short exposures to the light from a mercury vapor lamp

filtered through an ultra-violet transmitting material and had found (15) that as little as 1 day's exposure to winter sunlight, transmitted through the same material, resulted in a demonstrable protection. Hess and Supplee (16) had also observed that chicks respond to mild intensities of ultra-violet light. It seemed to us that here was a suggestion for a further attack on the problem. Clearly, the irradiation of the precursor in the skin of the chick led to a very efficient vitamin. Therefore, we reasoned, perhaps the photochemical reaction followed a different course in the skin and produced a different substance than was the case when ergosterol was irradiated in the laboratory, or perhaps some other substance, besides ergosterol, was present in the skin and reacted with ultra-violet light to produce a very potent antirachitic factor.

On the basis of this theory it was decided to investigate the potency of an irradiated animal extract which would contain besides cholesterol some of the substances which normally accompany this sterol. Consequently, we purchased commercially[2] a quantity of crude cholesterol which contained, according to the manufacturer, some fat and lecithin. This product was a brownish, semicrystalline preparation with a slight rancid odor and had been obtained by extracting the dried animal tissues (which tissues were not stated) with acetone, concentrating the extract till crystallization began, filtering, and subsequently crystallizing the precipitate once from alcohol.

Successive 10 gm. portions of the crude cholesterol were irradiated by spreading out each in a thin layer 10 inches below a hanging 110 volt Y-type quartz mercury vapor lamp (Cooper Hewitt poultry treater lamp). The total time of exposure was 1 hour, the material being scraped together and redistributed every 15 minutes to present new surfaces and to break up the small lumps. Several portions were irradiated in this way and combined to make a supply for assay on both rats and chicks.

In our first experiment we tested the irradiated crude cholesterol on chicks by adding it at levels of 15, 60, and 240 mg. per 100 gm. of the basal diet. These additions, suspended in corn oil in such proportion that the diet of each group contained 1 per cent of the oil suspension, introduced 20, 80, and 320 rat units per 100 gm. of

[2] The Wilson Laboratories, Chicago.

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diet respectively, since the unit rat dose was found to be approximately 0.75 mg. In this experiment were included also a group receiving 10 rat units of cod liver oil and four groups receiving, respectively, 10, 40, 160, and 640 rat units as irradiated ergosterol, all these amounts being present in 100 gm. of the diet. The results from this experiment, which was of 8 weeks duration, showed that all the levels of irradiated cholesterol yielded excellent protection against leg weakness and the growth and outward appearance was quite comparable to the cod liver oil-fed group. In contrast, the irradiated ergosterol additions afforded very little protection at the lower levels, the three groups receiving 10, 40, and 160 rat units showing distinct leg weakness. The group receiving 640 rat units appeared normal. The figures representing bone ash percentages substantiated these observations although in this experiment considerably more variation in these figures was observed than usual.

In view of these exceedingly interesting results we were naturally stimulated to repeat the experiment and to extend our observations. One of our first thoughts was that the increased efficiency, which we had obtained from irradiated crude cholesterol in the above experiment, might have been due to the presence of the relatively large amount of cholesterol which possibly aided in the absorption of the antirachitic factor. Hence, in planning for a further experiment, it was decided to include certain groups which would receive irradiated ergosterol solutions to which had been added non-irradiated crude cholesterol. A second supply of the irradiated crude cholesterol was prepared in the same way as before.

The plan of this second experiment and the results obtained will be easily understood from a study of Table I. Three groups (Groups 1, 2, and 9) may be considered as controls, since they received supplements which previous experience had taught us would produce definite predictable results. They illustrate respectively, the poor performance on the basal diet alone, the slight improvement from 90 rat units of irradiated ergosterol, and the definite antirachitic protection of 10 rat units of cod liver oil. Against these may be measured the performance of the three groups receiving the various levels of irradiated crude cholesterol and the three groups which received the different levels of a mixture of irradiated ergosterol and unirradiated crude cholesterol.

It will be seen that the antirachitic factor of irradiated crude cholesterol was clearly of the effective type since 20 rat units per 100 gm. of basal ration prevented all symptoms of leg weakness.

TABLE I

Summary of Experiment 7 Showing Difference in Efficacy of Irradiated Crude Cholesterol and of Irradiated Ergosterol Alone and in Presence of Non-Irradiated Crude Cholesterol

Group No.	Supplement to basal Ration I*	Rat units per 100 gm. ration	Average weight at 8 wks.	Bone ash at 8 wks.†	Remarks
			gm.	per cent	
1	None		285	35.2	All symptoms of leg weakness evident; all squat
2	0.25 per cent cod liver oil	10	626	43.7	Chicks healthy; very active
3	10 mg. irradiated crude cholesterol	20	633	44.6	" "
4	30 mg. irradiated crude cholesterol	60	600	43.0	Chicks healthy; condition excellent
5	90 mg. irradiated crude cholesterol	180	614	43.5	" "
6	Irradiated ergosterol + 10 mg. crude cholesterol	10	364	38.5	More than half of chicks squat
7	Irradiated ergosterol + 30 mg. crude cholesterol	30	311	35.4	About ⅔ of group squat; condition poor
8	Irradiated ergosterol + 90 mg. crude cholesterol	90	350	36.7	" "
9	Irradiated ergosterol	90	362	36.9	All but 3 squat; condition poor

* All chicks were maintained on the basal diet for 1 week before additions, as noted, were made.

† Figures for percentage of bone ash were obtained on ten chicks from each group.

It may be interjected here that it was planned to feed this supplement at a level equivalent to 10 rat units but only preliminary rat assays were available when the chicks were started on experiment.

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Later more complete rat assays indicated that 10 mg. introduced approximately 20 rat units. In comparison with Groups 3, 4, and 5 it may be seen that the presence of crude cholesterol did not enhance the antirachitic effectiveness of the irradiated ergosterol fed to Groups 6, 7, and 8.

It will be understood that with these further results at hand a number of questions presented themselves for answer. The most immediate of these was whether the increased efficacy was to be ascribed to the impurities known to be present in the crude cholesterol or whether irradiated pure cholesterol[3] would also possess equally potent antirachitic effects on chicks. Assuming that the latter was the case, was the provitamin of cholesterol actually different from ergosterol or did it, rather, undergo a different photochemical reaction? Was it possible that cholesterol itself, aside from any known provitamin, could be activated by ultra-violet light to yield a substance very active for chicks but not demonstrable by rat tests?

In attempting to find answers to these questions it was necessary to carry out many experiments. Because of the nature of the chick experiments and the time consumed in carrying them out it was, of course, advantageous to investigate simultaneously the various lines of work suggested above; and in most of our experiments groups were included which bore on each phase of the problem. Out of several available, we have chosen for detailed presentation some of our later experiments which were of this nature and which well illustrate the type of data we secured. In some of these experiments certain groups were included which received supplements not germane to our present discussion and these groups have not been included in Tables II to V presented.

While these problems were studied simultaneously, for purposes of clarity and ease of presentation we propose to discuss our findings under the headings which follow.

Irradiated Pure Cholesterol

As a start on the study of these problems we decided to purify a quantity of the crude cholesterol used in the above experiments.

[3] Pure cholesterol is used here and throughout this paper in the ordinary sense, as referring to good color, crystal structure, melting point, etc., and not in the sense of being free from provitamin.

50 gm. were dissolved in 500 cc. of hot ethyl alcohol containing 40 gm. of KOH, and refluxed for 30 minutes. The solution was cooled and the precipitated cholesterol filtered off and washed with alcohol. This procedure was repeated on two further portions yielding a total of 114 gm. from the three saponifications. This amount was then dissolved in 1.5 to 2 liters of alcohol, 10 gm. of carbon[4] were added, and the solution digested for several hours. The warm solution was filtered through a hot water funnel and the filtrate refrigerated. The precipitated cholesterol was filtered off, taken up in ether, and again filtered to remove the last traces of carbon, the ether evaporated off, and the cholesterol finally recrystallized from ethyl alcohol. The air-dried yield of beautifully white cholesterol was 90 gm., m.p. 148–149°.

A quantity of this purified cholesterol was irradiated dry in the same manner as had been done with the crude cholesterol. When later assayed on rachitic rats, this preparation was found to be of an activity such that 0.5 mg. contained 1 rat unit.

Our experience with this irradiated pure cholesterol when fed to chicks was a repetition of that obtained earlier with the irradiated crude cholesterol. In an experiment with white Leghorn chicks, details of which need not be presented here, levels of 10, 20, and 30 mg. (equivalent to 20, 40, and 60 rat units) per 100 gm. of diet were fed and compared with, among other supplements, 10 mg. of the irradiated crude cholesterol. The results showed excellent growth and calcification brought about by all the levels of the irradiated purified cholesterol, indicating that this preparation was equally as efficacious as the irradiated crude cholesterol.

In a later experiment (Experiment 13, Table IV) this same preparation of irradiated pure cholesterol was included at levels equivalent to 10, 20, and 30 rat units and compared with other antirachitic supplements. In this experiment both basal Ration I and Ration II were used and it will be seen in Table IV (Groups 8, 9, 10, and 17) that all these levels served excellently to protect the chicks against symptoms of leg weakness. Even on basal Ration II, 20 rat units of the irradiated pure cholesterol were as effective as the same number of units from cod liver oil and from an active fraction of the irradiated crude cholesterol, all three producing symptom-free birds (Groups 15, 16, and 17).

[4] A commercial product known as darco.

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In addition to the purified cholesterol which we prepared as described above, we have also used commercial samples[4] of pure cholesterol and obtained similar results.

Fractionation Experiments

We have already described an experiment in which unirradiated crude cholesterol was added to solutions of irradiated ergosterol to see whether by so doing the effectiveness of the latter could be enhanced. Another attack on this same problem was made by dissolving a portion of the irradiated crude cholesterol in hot ethyl alcohol, allowing the solution to cool, and filtering off the precipitated cholesterol. We were guided to this procedure by the suggestive results of Rosenheim and Webster (2) and by the well known fact that the irradiation products of ergosterol are much more soluble in the common organic solvents than is the unchanged sterol. It seemed possible, therefore, that by simple crystallization of the irradiated cholesterol from ethyl alcohol the bulk of the cholesterol might be removed from the active fraction which, it was thought, might remain in the mother liquors. This, indeed, proved to be the case and we found that such filtrates possessed all the antirachitic activity while the precipitated cholesterol, if well washed, possessed no such activity. In some preparations we crystallized the activated cholesterol twice from alcohol and combined the filtrates.

Our further procedure was to add corn oil to these mother liquors and then to distil off the alcohol under reduced pressure and so obtain an oil solution of the active fraction. Such a preparation was made from irradiated crude cholesterol and used as additions to Groups 5, 6, and 7 in Experiment 13 (Table IV). It is not to be thought that such preparations were entirely free from unchanged cholesterol since the filtrates contained a small amount in solution but it was relatively easy to remove more than 90 per cent of the cholesterol, showing again that it was not necessary to have the bulk of the cholesterol present to obtain the beneficial effects.

This method of fractionating and removing the bulk of the cholesterol also offered us a means of determining whether the constituent in cholesterol capable of being activated might be composed of more than one substance. As already mentioned, it

[4] C.P. cholesterin, The Wilson Laboratories, Chicago.

was conceivable that, in addition to the known provitamin in cholesterol, which had been identified as ergosterol, there was a second substance which, when irradiated, was very effective for calcification in chicks. Might it not be that cholesterol itself, aside from any previously known contaminating provitamin, could give rise, when irradiated, to a vitamin-like substance needed by chicks? It is to be recalled in this connection that Bills, Honeywell, and MacNair (4) and Koch and his associates (5, 6) had previously entertained the possibility of the presence in cholesterol of more than one substance which could be activated.

To investigate this point we proposed to irradiate repeatedly a sample of cholesterol, and to separate each time, in the above manner, the active fraction from the bulk of the cholesterol and use the recovered cholesterol for the succeeding irradiation. We reasoned that this procedure ought soon to exhaust the so called ergosterol contaminant since it was present in cholesterol in small amount and should be removed by the first irradiations. Our filtrate fractions from the first irradiations, therefore, should contain the antirachitic activity from the "ergosterol" provitamin, while fractions from later irradiations should contain only such substances as were produced by the activation of cholesterol itself. In the event that the latter substances were responsible for the effectiveness of irradiated cholesterol then the later fractions should be as potent as the first when fed to chicks.

For the purposes of this experiment we used a commercial C.P. grade of cholesterol. This was irradiated in the manner we have already described, a total of nine portions, approximating 10 gm. each, being used. The whole of the irradiated cholesterol was then dissolved in about 600 cc. of hot ethyl alcohol and allowed to crystallize in the refrigerator. The precipitated cholesterol was filtered off, washed, and then allowed to air-dry. To the filtrate and washings corn oil was added and the alcohol distilled off under good vacuum and with the aid of a capillary delivering pyrogallol-washed nitrogen. The resulting oil solution was made up to a definite volume on the basis of the amount of cholesterol irradiated.

This procedure was repeated a total of five times, the recovered cholesterol from the previous irradiation being used for the succeeding one. In each case the alcoholic mother liquors were concentrated and made up in oil solution to a volume based on the amount

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of cholesterol irradiated so that we finally obtained five such preparations which we referred to as the filtrate fractions.

As was to be expected, when four of these filtrate fractions were later assayed on rachitic rats, the first showed by far the greatest activity. The first was found to contain 1 rat unit in an amount, equivalent to 0.5 mg. of the cholesterol irradiated while the fifth

TABLE II

Summary of Part of Experiment 10 Showing Difference in Efficacy of First, Second, and Third Filtrate Fractions from Repeated Irradiation of Pure Cholesterol

Group No.	Supplement to basal Ration I*	Rat units per 100 gm. ration	Average weight at 8 wks.	Bone ash at 8 wks.	Remarks
			gm.	*per cent*	
1	First filtrate fraction (= 5 mg.)	10	641	45.0	Chicks healthy
2	Same (= 10 mg.)	20	661	44.6	" "
3	" (= 15 ")	30	719	44.6	" "
4	Second filtrate fraction (= 5 mg.)	<1	582	40.2	Unsteady and staggering
5	Same (= 10 mg.)	<2	544	38.2	"
6	" (= 15 ")	<2	619	43.0	Some unsteady
7	Third filtrate fraction (= 5 mg.)	<1	536	38.1	Marked leg weakness
8	Same (= 10 mg.)	<1	606	40.4	Leg weakness evident
9	" (= 15 ")	<1	588	40.3	6 unsteady

* When this experiment was started our supply of chicks was limited; hence, no group was included which received the basal diet alone. All chicks were maintained on the basal ration 10 days before additions were made.

produced no healing below what corresponded to 100 mg. For the assay of the second and third filtrate fractions only two litters of rats were available for each and the levels fed were too low. It can only be said that the second was inactive at what corresponded to 7.5 mg., while the third was inactive at what corresponded to 15 mg.

With the knowledge that these various filtrate fractions possessed

greatly different potencies for rachitic rats, it remained to test them on growing chicks. In the first experiment in which these preparations were compared, it was possible to include only the first three and each was added at three levels, corresponding to 5, 10, and 15 mg. per 100 gm. of chick diet. The results of this experiment, the details of which are presented in Table II, showed that only the first filtrate fraction was effective at all levels in promoting normal calcification. The three levels of this fraction (introducing 10, 20, and 30 rat units per 100 gm. of diet) produced good sturdy symptom-free birds, while all the levels of the second and third fractions failed to protect completely against leg weakness. The relatively better response of Group 6 may be explained on the basis that possibly the supplement contained sufficient rat units (not more than 2) to bring about an effect with the basal ration used. These results, of course, indicated that the antirachitic activity of irradiated cholesterol for chicks was associated with the same substance as was measured by rachitic rats and that apparently, cholesterol itself, as repeatedly irradiated in the manner described, did not continue to give rise to uniform amounts of antirachitic substances active for chicks.

In a later experiment on chicks (Experiment 12, Table III) this phase of the work was again put to test. Here we compared the first and the fifth filtrate fractions, the first being added at the same three levels as before while the fifth was fed at two levels; these being equivalent to 15 and 50 mg. of the cholesterol irradiated per 100 gm. of the chick diet. In addition, two other groups were included which received the above amounts of the fifth fraction to each of which had been added an oil solution of irradiated ergosterol in an amount sufficient to introduce 30 rat units. This was done to determine whether the fifth fraction contained any substance which might be supplementary to irradiated ergosterol and so enhance its effectiveness.

Our data, as may be seen in Table III, showed no favorable influence from the addition of the fifth filtrate fraction alone (Groups 6 and 7); nor did the same amounts supplement 30 rat units of irradiated ergosterol to an extent sufficient to prevent leg weakness and low bone ash percentages (Groups 8 and 9). These results indicated to us that the repeated irradiation of cholesterol in the manner described did not continue to give rise to appreciable

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amounts of antirachitic substances effective for chicks nor, apparently, any substance which had a supplementing effect on irradiated ergosterol. It seemed apparent, also, that the effective antirachitic activity was associated with the same substance as was

TABLE III

Summary of Experiment 12 Comparing Potency of First and Fifth Filtrate Fractions and of an Irradiated Mixture of Cholesterol and Ergosterol

Group No.	Supplement to basal Ration I*	Rat units per 100 gm. ration	Average weight at 8 wks.	Bone ash at 8 wks.	Remarks
			gm.	per cent	
1	None		351	34.0	Almost all squat
2	0.25 per cent cod liver oil	10	712	44.2	Condition very good
3	First filtrate fraction (= 5 mg.)	10	614	43.3	Good condition
4	Same (= 10 mg.)	20	659	44.4	Very good condition
5	" (= 15 ")	30	626	44.3	" " "
6	Fifth filtrate fraction (= 15 mg.)	<1	418	37.7	More than half squat
7	Same (= 50 mg.)	<1	448	36.6	9 squat
8	Fifth filtrate fraction (= 15 mg.) + irradiated ergosterol	30	479	38.6	9 "
9	Fifth filtrate fraction (= 50 mg.) + irradiated ergosterol	30	479	38.6	6 "
10	Irradiated mixture of cholesterol and ergosterol (95:5)	10	432	36.4	8 "
11	Same	30	422	37.0	8 "
12	"	90	536	36.8	10 "

* All chicks were maintained on the basal ration for 2 weeks before supplements were added.

measured by rachitic rats and was, therefore, the result of the activation of the already recognized provitamin of cholesterol.

Experiments with Mixtures of Cholesterol and Ergosterol

As has already been mentioned, one of our early theories, formulated to explain the favorable results with irradiated cholesterol,

was that a small amount of ergosterol in the presence of a large amount of cholesterol might undergo a different photochemical reaction than was the case when it was irradiated by itself. This, we reasoned, might be due to the cholesterol filtering out or, at least, reducing the effect of certain wave-lengths of the ultra-violet on ergosterol, or that the cholesterol interacted in some way with the ergosterol or its transformation products to yield a different antirachitically active substance than when cholesterol was not present. Consequently, the idea of testing irradiated mixtures of cholesterol and ergosterol suggested itself.

Believing, as we did at that time, that the provitamin of cholesterol had been definitely identified as ergosterol, we considered this theory very plausible and, the possible advantages of activating ergosterol in this way being fully appreciated, we chose as our first mixture one containing 95 per cent cholesterol and 5 per cent ergosterol. The cholesterol used was from the supply which we had prepared ourselves by purifying the crude product; the ergosterol used was of high purity (m.p. 163°) prepared in this laboratory from fungus origin. The two sterols were dissolved together in ether, the ether evaporated off, and the dry mixture irradiated for 1 hour in the manner previously described.

In the first testing of this preparation the chicks became available before it had been assayed on rats so that we had no accurate basis for the choice of levels. The levels chosen (2, 6, 10, and 30 mg. per 100 gm. of diet) were later found to be much too high to indicate whether the antirachitic activity was of the irradiated cholesterol type or the irradiated ergosterol type. Actually, it was shown that the lowest level introduced approximately 400 rat units which with our basal ration (basal Ration I) was sufficient to prevent leg weakness even when irradiated ergosterol was used. As a consequence all of the four groups showed good calcification with no symptoms of leg weakness.

Later, after rat assays had been completed, this preparation was tested again on chicks but at levels which introduced 10, 30, and 90 rat units per 100 gm. of diet. As may be seen in Table III (Groups 10, 11, and 12) even the highest level did not serve to prevent leg weakness, the response being the same as when, in similar cases, we fed irradiated ergosterol. These results indicated that the presence of the cholesterol had not apparently influenced the type of activity produced by ultra-violet light acting on the ergosterol.

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Because in the above preparation we had used a great excess of ergosterol as compared with the amount of provitamin which is normally present in cholesterol, we realized that possibly this had prevented the cholesterol from bringing about its full effect. Consequently, in setting out to repeat this experiment, we decided we would mix them more nearly in the proportion in which they presumably occurred in the animal body. Further, since we had in our hands a cholesterol preparation which had been rendered practically provitamin-free by repeated irradiation, we chose this as the sample to be mixed with ergosterol. The cholesterol to which we refer was that which had been recovered from the fifth irradiation in the preparation of the five filtrate fractions already described. In other words, having largely removed the provitamin (presumably ergosterol), we now proposed to restore it to this sample of cholesterol.

To 10 gm. of the provitamin-low cholesterol was added an ether solution carrying 10 mg. of pure ergosterol. More ether was added to bring all the cholesterol into solution, the ether then evaporated away, and the sterol mixture scraped from the beaker, and powdered. The final traces of ether were dissipated by allowing the preparation to stand overnight at room temperature.

This mixture, containing approximately 0.1 per cent ergosterol, was activated under the mercury vapor lamp in the manner already described, the time of irradiation being 1 hour with stirring every 15 minutes. After irradiation the mixture was twice crystallized from 90 cc. of ethyl alcohol in order to remove thoroughly the activated fraction and the combined mother liquors were concentrated under vacuum and made up in corn oil to a definite volume. When this oil solution was carefully assayed on rachitic rats, it was found that what corresponded to 0.75 mg. of the mixed sterols produced healing equivalent to 1 rat unit.

Later, when tested on chicks, it was introduced at levels of 10 and 20 rat units per 100 gm. of diet and was found to be ineffective at both levels in protecting against leg weakness (Groups 11 and 12, Table IV). Although higher levels were not fed it was apparent that it was not nearly so efficient as an equal number of rat units from the activation of the provitamin naturally occurring in cholesterol.

While in neither of the two irradiated mixtures of cholesterol and

TABLE IV

Summary of Part of Experiment 15 Comparing Efficacy of Various Supplements Which Included Irradiated Mixture of Cholesterol and Ergosterol

Group No.	Diet and supplement*	Rat units per 100 gm. ration	Average weight at 8 wks.	Bone ash at 8 wks.	Remarks
			gm.	per cent	
1	Basal Ration I	0	350	36.1	Almost all squat
2	Same + 0.25 per cent cod liver oil	10	642	43.0	Excellent
3	Same + irradiated ergosterol	30	353	36.1	12 squat
4	Same as for Group 3	90	485	39.7	8 "
5	" + filtrate from irradiated crude cholesterol	10	655	45.9	Excellent
6	Same as for Group 5	20	617	43.1	Condition very good
7	" " " " 5	30	755	44.7	Excellent
8	" + irradiated pure cholesterol	10	612	43.7	Condition very good
9	Same as for Group 8	20	565	43.6	" " "
10	" " " " 8	30	614	42.6	Excellent
11	" + filtrate of irradiated cholesterol + 0.1 per cent ergosterol	10	510	40.4	4 squat
12	Same as for Group 11	20	421	37.6	10 "
14	Basal Ration II	0	227	30.7 (D.) (6)† 33.6 (L.) (8)	All squat
15	Same + 0.5 per cent cod liver oil	20	534	40.5	Condition good
16	Same + filtrate from irradiated crude cholesterol	20	618	42.5	" very good
17	Same + irradiated pure cholesterol	20	566	41.6	" good

* All chicks in this experiment were maintained on basal Ration I for 2 weeks, additions then being made as noted. Groups 14 to 17 were changed to basal Ration II at the same time.

† Bones were removed from six chicks which died (D.) during the last week of the experiment and were kept in a separate group. The eight remaining (L.) at the end of the experiment were grouped and the ash was determined separately.

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ergosterol which we have described was evidence found that the antirachitic factor had been rendered more efficient than that from irradiated ergosterol itself, we, nevertheless, decided on a further attempt in this direction. Since Koch, Koch, and Ragins (5) had shown that heating cholesterol under certain conditions produced changes suggesting an increase of provitamin, it was thought that heating mixtures of cholesterol and ergosterol might bring about changes in the ergosterol which would render it more like the natural contaminant in cholesterol. Consequently, we prepared two mixtures of cholesterol and ergosterol in the proportion of 95 per cent of the former and 5 per cent of the latter and heated them in the following manner.

To 4.75 gm. of a commercial C.P. cholesterol in a small Erlenmeyer flask was added 0.25 gm. of pure ergosterol. The two sterols were stirred and mixed and then the flask closed with a rubber stopper carrying inlet and outlet tubes. The flask was then evacuated twice, the vacuum being broken each time with pyrogallol-washed nitrogen. The outlet and inlet tubes were then closed and the flask lowered into an oil bath heated to 190°. Heating at this temperature was continued for 2 hours when the flask was removed and the contents allowed to cool. Later the solidified melt was scraped from the flask and powdered with a spatula. The sterol mixture was then irradiated in the dry condition for 1 hour in the usual manner. After irradiation it was crystallized twice from 50 cc. of ethyl alcohol, the combined mother liquors concentrated, and the vitamin made up in oil solution such that 1 cc. represented 50 mg. of the irradiated mixture.

A second preparation was carried out in exactly the same way except that the heating was continued for 4 hours.

These two preparations were then carefully assayed on rachitic rats and found to contain 1 rat unit in amounts equivalent to 0.011 mg. and 0.02 mg. respectively of the irradiated mixture. Following this, steps were taken to assay these two mixtures on baby chicks.

In Table V we present the detailed data of this experiment; and there our plan and results may be observed. We fed the two irradiated heated mixtures in amounts corresponding to 10, 30, 90, and 270 rat units per 100 gm. of diet and compared these amounts with two levels of cod liver oil (10 and 20 rat units) and three levels of

TABLE V

Summary of Part of Experiment 15 Comparing Efficacy of Irradiated Ergosterol, Two Heated and Irradiated Mixtures of Cholesterol and Ergosterol, and a Fraction of Irradiated Crude Cholesterol

Group No.	Supplement to basal Ration II	Rat units per 100 gm. ration	Average weight at 6 wks.	No. of chicks: At beginning	No. of chicks: At end	Bone ash at 6 wks.*	Remarks
			gm.			per cent	
1	None	0	135	16	4	28.6 (13)	All survivors squat
2	0.25 per cent cod liver oil	10	428	10	15	41.6	Slight unsteadiness in few
3	0.5 per cent cod liver oil	20	410	15	15	41.4	Healthy
4	Irradiated ergosterol	30	164	15	10	30.4 (15)	All except 3 squat
5	Same	90	209	15	13	31.6 (14)	About ⅔ of group squat
6	"	270	396	15	13	37.2 (14)	4 unsteady, 1 squats
7	Filtrate irradiated, heated (2 hrs.) mixture cholesterol + ergosterol	10	140	14	5	28.7 (9)	All survivors squat
8	Same	30	175	15	4	27.1 (13)	" " "
9	"	90	195	15	14	30.5	" but 3 squat
10	"	270	389	15	15	35.6	4 or 5 unsteady
11	Filtrate irradiated, heated (4 hrs.) mixture cholesterol + ergosterol	10	134	14	5	28.9 (12)	All survivors squat
12	Same	30	194	15	10	30.3 (15)	Almost all squat
13	"	90	239	15	14	33.1 (15)	About half "
14	"	270	432	15	15	39.6	Slight unsteadiness in few
18	Filtrate from irradiated crude cholesterol	2	175	15	13	30.9 (15)	All squat
19	Same	5	361	15	15	40.2	Slight unsteadiness in few
20	"	10	391	15	15	41.1	" "

* Since most of the fatalities occurred during the last 10 days of the experiment, bones were removed from those chicks that died in that time and included with the bones from the survivors in determining percentage bone ash. Figures in parentheses indicate number of bones used. Where there are no parentheses surviving chicks contributed all bones.

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irradiated ergosterol (30, 90, and 270 rat units). We also included in this trial three levels (2, 5, and 10 rat units) of the filtrate fraction from irradiated crude cholesterol as a comparison substance and because we had never fully determined the minimum effective level of this preparation (this was the same preparation as fed to Groups 5, 6, 7, Table IV). Basal Ration II was used throughout this experiment which extended over 6 weeks.

The results which we secured can be seen to show unequivocally that ergosterol heated and irradiated in the presence of cholesterol did not produce any more effective vitamin than that from ergosterol irradiated alone. An amount equivalent to 270 rat units of either of the heated and irradiated mixtures or of irradiated ergosterol itself did not serve to protect completely against the development of leg weakness. This quantity of the irradiated mixtures, particularly that heated 4 hours, undoubtedly contained an appreciable amount of activity referrable to the irradiation of the cholesterol itself which may explain the relatively better performance of Group 14. All the lower levels of the supplements containing ergosterol were decidedly ineffective. Contrasted with this performance it can be observed that both cod liver oil and irradiated crude cholesterol were remarkably efficient. It is to be noted that under the severe régime of basal Ration II 10 rat units per 100 gm. of ration of the effective supplements were close to, or below, the minimum required for complete protection. The efficacy of the factor from irradiated crude cholesterol was particularly brought out by this experiment, as small an amount as 5 rat units affording remarkable protection.

On the basis of the above results, together with our earlier data on irradiated mixtures of cholesterol and ergosterol, it will be evident that ergosterol did not behave as did the provitamin naturally present in cholesterol.

DISCUSSION

To summarize briefly the results which have been presented, it may be said that they show convincingly that irradiated cholesterol possesses a substance which is much more effective in the prevention of rachitic manifestations in chicks than is an equivalent number of rat units of irradiated ergosterol. This substance is at least as effective as the vitamin D of cod liver oil. Our

results also show that the increased effectiveness cannot be explained by the presence of other or supplementary factors hitherto unrecognized but that the high efficiency is, apparently, a property of the already well known provitamin. Further, we have found that when ergosterol is mixed with cholesterol and irradiated it does not yield an antirachitic substance of greater potency than when irradiated alone. In view of these findings it seems apparent that *the provitamin of cholesterol cannot be identical with ergosterol.*

The importance of such a conclusion will be at once obvious for it is directly opposed to the widely held belief regarding ergosterol as the main provitamin of the animal body, which has been the basis of all the modern developments in this field. However, since our results have been at hand for some time, we have had an opportunity to reflect upon and weigh them in the light of past work. We feel that, not only may the evidence on which this belief is based be reinterpreted so as to bring it into closer agreement with our conclusion, but also that our results may offer an explanation of many hitherto anomalous observations and to that extent receive confirmation.

A detailed discussion of the evidence which has been adduced in establishing the identity of ergosterol as the provitamin of cholesterol would extend far beyond the limits of this paper and, further, would appear unnecessary since it has been the subject of many reviews. Loofbourow (28), in particular, has critically and extensively considered the data and the methods and reasoning followed. It may be said that these data showed unmistakably a great similarity between the two substances as indicated by their absorption spectra in the ultra-violet region, and by their reaction to treatment with ultra-violet light and chemical agents. This, together with the fact that pure ergosterol showed such remarkably high potency when irradiated, naturally offered excellent presumptive evidence that the two substances were identical. However, in view of our more direct evidence to the contrary, it would seem that this work must now be interpreted on the basis that they are remarkably *similar* but not *identical*.

In connection with the work just discussed it is to be recalled that Bills, Honeywell, and MacNair (4) and Koch and his associates (5, 6), while accepting ergosterol as the main provitamin of cholesterol, offered evidence that it did not account for all the activity

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of their irradiated samples, and to this extent directed attention to a difference between these two sources of vitamin D activity. From the view-point presented in this paper it will be apparent that their results, and others which we shall mention, are more easily understood and take on an added significance.

If the provitamin constituent naturally present in cholesterol is not to be regarded as ergosterol, there is no evidence available to show that the latter substance is normally present to any extent in the animal body. The non-absorbability of ergosterol by animals, which has been observed by Schönheimer and his associates (21) and by Beumer and Hepner (25), may now be understood on this basis. Schönheimer in his excellent review (19) has pointed out that plant sterols in general are poorly, if at all, absorbed from the digestive tract; and the weight of the evidence would indicate that ergosterol is no exception to this rule. The apparent slight absorption of ergosterol by laying hens, as observed by Schönheimer and Dam (22), and by Menschick and Page (23), would indicate, however, that this point requires further investigation. A distinct difference between plant and animal sources of the provitamin is, nevertheless, suggested.

While our results show that irradiated cholesterol is as potent as the vitamin D of cod liver oil in preventing leg weakness in chicks, we have no further evidence that the two factors are the same. It is significant, however, that both are of animal origin and that the active factor of fish oils is always found associated with cholesterol. Steenbock, Kletzien, and Halpin (9) have concluded that the vitamin D of cod liver oil is a different substance from irradiated ergosterol; and it is of interest to note in this connection that Hess, Bills, Weinstock, and Imboden (29) found no evidence that in the cod ergosterol could be activated when it was fed or injected intramuscularly.

The reports of clinical experience with irradiated cholesterol, carried out mainly before ergosterol became available, are not without interest in view of our favorable results with baby chicks. In 1927 Hess (30) reported that both an olive oil suspension of irradiated cholesterol and an irradiated dried brain preparation brought about rapid calcification in rachitic infants. In commenting on his own results and those of others which he reviewed, Hess stated, "It will be seen that a considerable literature has

already developed in relation to this subject, and that opinions are unanimous as to the antirachitic efficacy of irradiated cholesterol." It is not possible, however, to determine the dosage which was fed in terms of present day units and, therefore, no quantitative comparison as to the efficacy of this supplement may be made.

Redman in 1929 (31), in studying the hydrogen ion concentration and the calcium and phosphorus content of the feces of rachitic children receiving various antirachitic supplements, among which were cod liver oil, irradiated ergosterol, and irradiated cholesterol, noted that, "The best clinical results were obtained by the use of irradiated cholesterol. . ." No data, however, were given as to the amount of the various supplements fed; and since only two infants received irradiated cholesterol, it is again impossible to obtain quantitative comparisons. In view of the great similarity in sensitivity to lack of vitamin D exhibited by infants and baby chicks, it is to be expected that irradiated cholesterol will possess a high clinical potency and the above observations are significant. It will be of interest to await further clinical tests of irradiated cholesterol.

The value of irradiated milk, either fluid or dry, in the prophylaxis and cure of infantile rickets has been well recognized in the past and the more recent comparisons (18) of this supplement with others have further emphasized its merit. This high potency may now be understood on the basis that it contains the activated provitamin present in cholesterol. However, it has been found that milk enriched by the feeding of irradiated supplements to cows also has a very high clinical efficacy and this situation is more difficult to understand. While the evidence is neither extensive nor conclusive, it would seem that the milk produced by cows receiving irradiated ergosterol or irradiated yeast contains the same type of vitamin as that fed to the cows and that it has been little, if at all, changed in character by its passage through the cow. Thus, Krauss, Bethke, and Monroe (32) have assayed on both rats and chicks the butter fat from cows fed irradiated ergosterol. While the highest amount of this supplement which they fed to chicks was 12.5 rat units per 100 gm. of ration, this amount did not produce as high a bone ash percentage as did 3.3 rat units from cod liver oil. The nature of the vitamin in butter fat from cows fed irradiated ergosterol has also been investigated in our laboratory

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(unpublished data) and, while the results are not extensive, they show that the factor was not as efficacious for chicks as that from cod liver oil. McDonald and Massengale (33) have found that the laying hen, when fed massive doses of irradiated ergosterol, deposits in the egg yolk an antirachitic factor much less potent for chicks than cod liver oil. In view of the relative inefficiency of irradiated yeast and irradiated ergosterol it remains to be shown what other factors are operating to render milks, activated by feeding these supplements to the cow, so potent clinically. It is not excluded, of course, that the ingestion by infants of any of the vitamin D factors uniformly distributed throughout milk may possess virtues still not understood but which may be explained, for the moment, on the assumption of better absorption, etc. It must also be considered that, while the clinical evidence is ample to show qualitatively the usefulness of these various vitamin D-rich milks, one may question whether the data are sufficiently extensive to justify the quantitative interpretations which have been drawn from them. This criticism is pertinent in view of the difficulty in showing small differences by animal experimentation which is susceptible to much greater control than clinical experiments. This may explain the placing of cod liver oil below the activated milks when arranged in order of their efficacy for infants.

It will be understood that, in establishing a difference between ergosterol and the provitamin of cholesterol, a vast number of problems are immediately suggested for investigation. One of these concerns the origin of the provitamin and its relation to cholesterol and the sterol metabolism of the body. This is a question on which we prefer to postpone discussion but to which we hope soon to return. It will also be obvious that, before satisfactory answers are obtained to many of the questions raised by the above discussion, the cooperation of many laboratories will be necessary. It is for this reason and to direct the efforts of other workers along the lines suggested by our results that this paper has been presented.

SUMMARY

1. Evidence is presented to show that irradiated cholesterol is much more efficacious in preventing rachitic manifestations in chicks than is an equivalent number of units of irradiated ergosterol. The antirachitic factor of irradiated cholesterol is as potent for chicks as the vitamin D of cod liver oil.

2. It is shown that this high potency is a property of the activated provitamin already well recognized and previously identified as ergosterol, and that it is not to be explained on the assumption that other substances are present which may be activated.

3. Pure ergosterol, mixed with cholesterol and irradiated, does not give rise to an antirachitic factor more efficacious than ergosterol irradiated alone.

4. It is, therefore, concluded that the provitamin constituent of cholesterol is a substance different from ergosterol, a conclusion not in agreement with the widely held belief that ergosterol is the main precursor of vitamin D activity in the body.

5. The significance of these findings in interpreting many past results and suggesting further investigations is briefly discussed.

It is a pleasure to acknowledge our gratitude to Dr. A. Zimmerli, formerly of this company, for stimulation and encouragement in the early stages of this work, to Mr. W. H. Allen for valuable suggestions as to the hatching and brooding of baby chicks, and to Dr. E. H. Killheffer, president of this company, for permission to publish these results.

Addendum—Since the above manuscript was written we have seen the publications by Romeson (34) in which he puts forward the view, on the basis of studies on the physical and chemical properties of cholesterol, that it is capable of existing in a keto and an enol form and that the keto form may be regarded as vitamin D. He believes that the keto form is formed by the action of ultra-violet light from the enol form. He further concludes that the provitamin of cholesterol is not identical with ergosterol. While it would appear that this author's data are hardly sufficient to establish his views, it is of interest to note his conclusion with regard to the identity of the provitamin of cholesterol in view of our more definite results.

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ESTIMATION OF VITAMIN D IN BLOOD SERUM

II. LEVEL OF VITAMIN D IN HUMAN BLOOD SERUMS

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In a previous communication a method for the estimation of vitamin D in blood serum was described.[1] It was shown that the level of vitamin D in the serum of animals can be raised by a single administration of a large dose of viosterol and that the surplus of vitamin D disappears from the serum within a few weeks. In addition, the results of a number of estimations in serums of children and adults were reported.

In the present study we wish to supplement the data concerning the level of vitamin D in human serums. It seems desirable to know not only the average value of the vitamin D in human serums but the variations which may be expected. Since those variations, which may be due to physiologic fluctuations or to the limitations of the method, are rather large, a knowledge of the fluctuations observed in the blood of normal human beings is necessary for the evaluation of results with abnormal serums. It will be shown in subsequent communications that the variations found under various abnormal conditions are far beyond the limits of normal variations.

The present report is based on 608 line tests obtained by assaying 155 samples of human blood serum over a period of several years.

One hundred and eleven samples of blood were obtained from white and 44 from Negro children and adults. Eighty-eight samples were from children under 14 years of age, 4 from adolescents between 14 and 18 years and 63 from adults between 24 and 76 years. All the adults tested were white. Serums of infants were not included in this study.

From the Children's Hospital Research Foundation and the Department of Pediatrics, University of Cincinnati.

1. Warkany, J.: Estimation of Vitamin D in Blood Serum, Am. J. Dis. Child. **52**:831 (Oct.) 1936.

In this first communication the headings of the last two columns of tables 1 to 3 and 6 to 12 should read "Vitamin D Content of Rabbit's Serum per 100 Cc." instead of "Vitamin D Content of Rat's Serum per 100 Cc." The inscription below the abscissa of chart 2 should read "1.0 cc. of viosterol (1,000,000 U. S. P. units) instead of 0.1 cc. viosterol (100,000 U. S. P. units)." On page 833, line 13 should read "Wheat gluten, 20 per cent," instead of "Wheat gluten, 30 per cent."

There were excluded from this analysis the serums of persons who were seriously ill or who had received treatment by artificial ultraviolet irradiation or with vitamin D, as cod liver oil or in any other form, within the three months preceding the test.

METHODS

Biologic methods must be used for the estimation of vitamin D in body fluids and tissues. Several years ago, when this study was begun, it was thought that the biologic estimation would soon be replaced by a simple and accurate physical or chemical determination. It is apparent, however, that for some time to come this hope will not be realized, since a variety of compounds, differing in physical and chemical properties, have proved effective against rickets.[2] It appears improbable, therefore, that any specific physical or chemical test will replace the highly sensitive biologic test in the near future.

Obviously, the methods generally used for assaying vitamin D in substances such as cod liver oil and milk had to be modified to a certain extent when applied to human blood serum, which is not available in sufficient amounts to allow a satisfactory assay of every sample by such methods. A single value by itself may be unsatisfactory, but a considerable number of assays will furnish satisfactory average figures.

Blood taken from a vein in the arm was allowed to stand for one-half hour to one hour at room temperature, then centrifuged and the serum pipetted off. Amounts[3] of 1.5, 2, 2.5, 3, 3.5, 4 (and sometimes 5) cc. respectively were each mixed with about 40 Gm. of the Steenbock and Black diet[4] no. 2965. After being air-dried, these mixtures were finely pulverized in a porcelain mortar and put into glass bottles.

The rats were fed the rachitogenic diet for twenty-one days. They were then fed the same diet plus serum, added as described, for eight days. For rats that consumed more than 5 Gm. of their diet daily, these mixtures were supplemented with varying amounts of rachitogenic diet. Thus it was possible to distribute the intake of serum over the whole eight day period. After ten days the line tests[5] were performed in the usual manner and rated as follows: 0, no deposit of silver between the zone of cartilage and the intermediate zone; +, a small

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3. The total phosphorus content of human blood serum amounts to approximately 12 mg. per hundred cubic centimeters. By feeding 5 cc. of serum, the maximum amount administered in our assays, an additional amount of approximately 0.6 mg. of phosphorus was added to a rat's diet over the eight day period. This quantity does not disturb the calcium: phosphorus ratio of the diet and has not been considered a factor in the assays.

4. Steenbock, H., and Black, A.: Fat-Soluble Vitamins: Induction of Growth-Promoting and Calcifying Properties in Fats and Their Unsaponifiable Constituents by Exposure to Light, J. Biol. Chem. **64**:263 (June) 1925.

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deposit of silver or an incomplete line between the two zones; ++, a complete and thin line; +++, a heavy line, and ++++, calcification extensive enough to reach the osseous part of the shaft.

When these experiments were begun, the Steenbock unit of vitamin D was still widely used. It was defined as the total amount of vitamin D which would produce a narrow line of calcium deposit in the rachitic metaphyses of the long bones of standard rachitic rats in a period of ten days. Accordingly, we attempted to ascertain the minimum amount of serum required to produce ++ healing in rachitic rats. At the present time, the Steenbock unit has been replaced by the international, or U. S. P., unit. At first, 1 Steenbock unit was considered equivalent to 2.7 international, or U. S. P. XI, units.[6] However, Russell and Taylor[7] considered 1 Steenbock unit equivalent to 3.2 U. S. P. units for their animal colony. Haman and Steenbock,[8] in a study on energy equivalents of vitamin D units, stated that a comparison of the energy equivalents of the Steenbock unit and the international unit "as obtained in independently executed series of assays, and direct comparison of the biological potency of the International Standard Preparation with a preparation produced by a measured amount of monochromatic ultraviolet revealed that one Steenbock unit of Vitamin-D is equivalent to 3.33 International units." Since it is possible that different equivalents can be found for the Steenbock unit under varying experimental conditions, it was thought necessary to ascertain the equivalent under the conditions of our laboratory. In the course of one year various amounts of reference oil (1 Gm. containing 95 U. S. P. units of vitamin D) were fed by pipet to 105 rats. These amounts corresponded to 20.7, 24.2, 27.7, 31.2, 34.7 and 38.2 mg. of oil for the eight day period. After ten days line tests were made. For each amount of reference oil a certain percentage of line tests resulted in ++ healing (or more). This percentage increased with the amount of oil administered, and it was possible to construct a curve which indicated the percentage of ++ line tests obtained from each amount of reference oil. It was found that 35.1 mg. of reference oil (an amount which corresponded to 3.33 U. S. P. units of vitamin D) produced ++ healing in 66 per cent of the line tests. In this study a factor of 3.3 was used, therefore, to convert Steenbock units into U. S. P. units.

6. Steenbock, H.; Kletzien, S. W. F., and Halpin, J. G.: (a) Reaction of Chicken to Irradiated Ergosterol and Irradiated Yeast as Contrasted with Natural Vitamin D of Fish Liver Oils, J. Biol. Chem. **97**:249 (July) 1932; (b) Recent Developments in Pharmacopeial Vitamin Standardization, J. A. M. A. **102**:1877 (June 2) 1934.

7. Russell, W. C., and Taylor, M. W.: Relation Between Vitamin A and D Intake by the Hen and the Output in Eggs, J. Nutrition **10**:613 (Dec.) 1935.

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As a rule, increased healing in a series of rats could be expected when the amount of serum fed was increased from 1.5 cc. to 4 cc., the increment being 0.5 cc. The minimum amount which would produce ++ healing was considered the curative dose, i.e., 1 Steenbock unit.

Figure 1 illustrates the results of such an assay. Two cubic centimeters of serum did not produce any calcification between cartilage and the intermediate zone, but 2.5 cc. produced beginning healing rated as

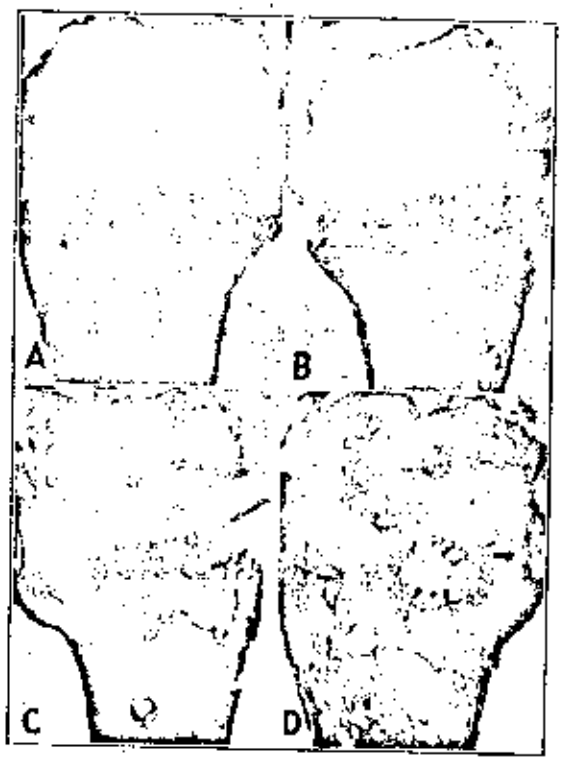

Fig. 1.—Line tests obtained with the administration of increasing amounts of human blood serum. *A* shows negative healing with 2 cc. of serum; *B* shows + healing with 2.5 cc.; *C* shows ++ healing with 3 cc., and *D* shows +++ healing with 4 cc.

+ healing, 3 cc. a complete thin line rated as ++ healing and 4 cc. a thicker complete line rated as +++ healing. In this case one could say that 3 cc. contained 1 Steenbock unit. The level of vitamin D in this serum could be estimated as 33.3 Steenbock, or 110 U. S. P. units per hundred cubic centimeters. Such a test can be considered "complete." Although not too much confidence should be placed in a single estimation, a large number of such complete tests permits the establishment of a reliable average figure.

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Results similar to those illustrated in figure 1 could not always be expected, since in the course of some assays difficulties arose which prevented their completion. Some of these difficulties may be cited:

1. Establishment of a complete assay series was impossible because of lack of serum.

2. Sometimes 1 or more rats of a series lost more than 2 Gm. of weight during the test period. Line tests from such animals were discarded, and the valid tests of the series were not sufficient for a complete assay.

3. Sometimes all the rats of a series showed ++ healing (or more), indicating a high level of vitamin D in the serum. If a reserve of serum was not at hand, it could not be ascertained whether the smallest amount of serum fed was actually the smallest dose capable of producing ++ healing.

4. Conversely, none of the rats of some series manifested ++ healing. In this case also the minimum curative dose could not be established unless a reserve of serum was at hand.

5. Occasionally, it happened that in a series a smaller amount of serum produced a higher degree of healing than a larger one, thus disrupting the continuity of the assay.

Under such conditions the assays could not be considered complete, and it was not possible to express the vitamin D content of the assayed serum in units per hundred cubic centimeters. Yet it was felt that the valid tests of these "incomplete" assays should be taken into consideration for the final calculation of the average content of vitamin D of human blood serum. Otherwise, there is the possibility that an exclusion of incomplete tests might lead to a false interpretation of the results.

One example may illustrate this point. If, for instance, a sample was not completed because doses of 1.5 to 4 cc. of serum did not result in ++ healing (and tests with 5 and 6 cc. could not be made because of lack of additional serum), one was probably dealing with a serum low in vitamin D content. If a large number of such serums were among the "incomplete" assays, a large number of serums low in vitamin D would be excluded from the calculation of the average value.

In order to avoid such a possibility, all of the valid line tests obtained from children and adults were considered as derived from one "pooled" sample of blood serum, and the average dose required to produce at least ++ healing in two thirds of the test animals was ascertained. It will be shown later that a fair agreement exists between the results obtained from the complete assays and those derived from all valid line tests.

RESULTS

From a total of 155 blood samples, 89 "complete" assays were obtained. The values found in these assays are presented in table 1.

The values ascertained in the complete tests ranged between 66 and 165 U. S. P. units per hundred cubic centimeters of serum. The majority of samples contained 110 or 132 U. S. P. units per hundred cubic centimeters. The average level of vitamin D in the serum of all the persons tested was 116.4 U. S. P. units per hundred cubic centimeters.

No striking differences were noted when the results obtained in different subgroups were separated. The only noticeable difference was that between the values for white and those for Negro children. The average value of 122.6 units found for white children was about 15 per cent higher than the average value of 106.5 units calculated for Negro children. This difference is of interest and is in accord with the familiar assertion that in dark-skinned races rickets develops more easily than in those with lighter skins. Since, however, the average for Negro persons was based on a rather small number of tests, a generalization based on the foregoing results does not yet seem justified.

TABLE 1.—*Frequency Distribution of Persons According to Levels of Vitamin D in Blood Serum*

U. S. P. Units per 100 Cc. of Blood Serum	Total No. Tested	White Adults	White Children	Negro Children
165	3	2	5	1
132	27	10	11	6
110	31	11	10	7
94	6	2	4	2
83	13	1	3	9
66	1	1	0	0
Total	89	30	34	25
Average vitamin D level, units per 100 cc.	116.4	117.0	122.0	106.5

There was no seasonal variation found by separating the assays of serums taken from May to October, the months in which the effect of sunshine should become manifest, and those taken from November to April.

As previously pointed out, it was considered advisable to check the average value calculated from the complete assays by another method which takes into consideration all the valid line tests obtained by complete and incomplete assays.

Six hundred and eight valid line tests were available for this purpose. Forty-four of these were obtained from rats fed 1.5 cc. of serum; 134 were procured with 2 cc., 149 with 2.5 cc., 160 with 3 cc., 62 with 3.5

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cc. and 59 with 4 cc. of serum. In each group a certain percentage of line tests showed ++ healing or more. This percentage increased with the amount of serum administered, as is shown by table 2.

TABLE 2.—*Results of Line Tests Obtained After Feeding Varying Amounts of Normal Human Serums*

Cc. of Serum Fed	Total Number of Line Tests	Line Tests Resulting in ++ Healing (Or More)	
		Number	Per Cent
1.5	44	6	13.6
2.0	116	30	26.0
2.5	149	64	43.0
3.0	109	107	68.9
3.5	62	50	80.6
4.0	59	49	83.1

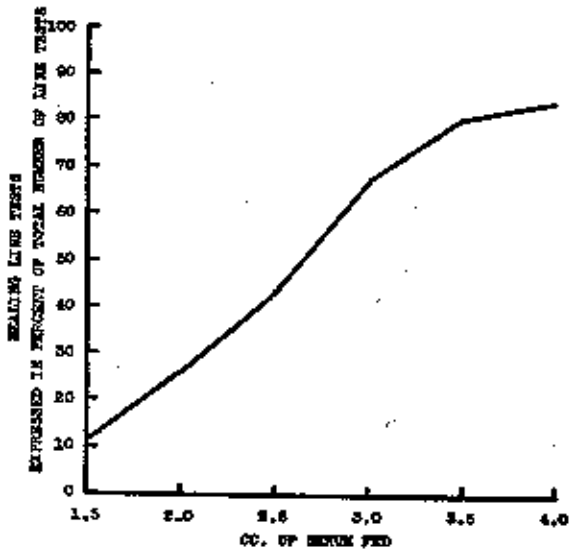

Fig. 2.—Curve showing the relation between the amount of serum administered and the percentage of healing line tests found in all valid line tests.

The curve in figure 2 shows graphically how increasing amounts of serum cause an increasing percentage of healing line tests.

If the curative dose of serum is considered to be that amount which is capable of producing ++ healing in two thirds of the line tests,[9] this amount can be found on the abscissa of the graph in figure 2, at the

9. The curve resembles that obtained with increasing amounts of reference oil described previously. Since 35.1 mg. of the reference oil as well as 3 cc. of serum caused ++ healing in two thirds of the experimental animals as shown by the line test, it can be assumed that these two amounts contain the same quantity of vitamin D. Since 35.1 mg. of the reference oil contains 3.33 U. S. P. units of vitamin D, 3 cc. of serum likewise contains 3.33 U. S. P. units and 100 cc. of serum contains 110 U. S. P. units.

point where the curve reaches the ordinate of 66.6. This value is almost exactly 3 cc. and corresponds to a vitamin D level of 33.3 Steenbock units or 110 U. S. P. units per hundred cubic centimeters of serum. This result agrees satisfactorily with the average obtained from the complete line tests.

COMMENT

From 89 "complete" bioassays of human blood serum an average vitamin D content of 116 U. S. P. units per hundred cubic centimeters was calculated. When the results of all the valid line tests obtained in this study were taken into consideration, an average vitamin D level of 110 U. S. P. units per hundred cubic centimeters was calculated. It is of interest to compare this value, which is based on 608 line tests, with the vitamin D content of other physiologic materials or foodstuffs.

The substance most frequently tested for its vitamin D content is cod liver oil. Although the oil from livers of individual cod fish may show enormous variations,[10] the average vitamin D content of medicinal cod liver oil can be rated as 10,000 or 15,000 U. S. P. units per 100 Gm.[11] It can be stated, therefore, that human blood serum has approximately one one hundredth the potency of medicinal cod liver oil.

The yolk of hen's eggs has been reported to contain in February and June, respectively, 140 U. S. P. units and 390 U. S. P. units per 100 Gm.[12] Thus the average vitamin D content of egg yolk would be twice that of human blood serum. Butter, which has a vitamin D content of 120 U. S. P. units per 100 Gm.,[11b] has an antirachitic potency equal to that of human blood serum.

Mammalian liver is reported to contain from 10 to 50 U. S. P. units of vitamin D per 100 Gm.,[13] a rather small amount compared to that of blood serum. (The vitamin D content of the serums of a number of mammals tested did not differ greatly from that of man).[14]

The level of vitamin D in cow's milk varies from 0.5 to 4 U. S. P. units per hundred cubic centimeters according to the season.[15] Human

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milk apparently contains no appreciable amount of vitamin D.[16] Muscle, kidneys and other glandular organs, with the exception of the liver, likewise contain no vitamin D.[17]

It seems, therefore, that blood serum has the highest vitamin D content of all the mammalian materials so far tested.

SUMMARY

An average vitamin D content of 110 U. S. P. units per hundred cubic centimeters of human blood serum was calculated from the results of 608 line tests. In assays of individual blood samples based on a small number of line tests, values between 66 and 165 U. S. P. units should be considered normal. No considerable difference was found in the vitamin D content of serums of adults and children. Marked seasonal variations could not be demonstrated. The values found for white children were about 15 per cent above those obtained for Negro children.

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Science, 183: 1092-94, 1974

Calcium Absorption and Calcium-Binding Protein Synthesis: Solanum malacoxylon Reverses Strontium Inhibition

Abstract. *The ingestion of diets containing high concentrations of stable strontium inhibits calcium absorption and intestinal calcium-binding protein synthesis and, as shown by others, does so by inhibiting the conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol, the active form of vitamin D. The addition of the South American plant* Solanum malacoxylon *to strontium-containing diets counteracts the inhibitory action of dietary strontium, thereby indicating that the plant contains a factor which can mimic the action of 1,25-dihydroxycholecalciferol and representing the first such factor identified in a botanical source.*

A disease of cattle in the Argentine known as "Enteque seco" is characterized by extensive soft tissue calcification and is due to the ingestion of the plant *Solanum malacoxylon* by the grazing animal (*1*). Controlled studies with different species, including cattle (*2*), rabbits (*3*), sheep (*4*), and guinea pigs (*4*), verified the etiology of the disease and showed that the plant or an extract of the plant caused an increased absorption of calcium and phosphorus. Analogy between the active principle in *S. malacoxylon* and vitamin D has been made, and it was shown that the unknown factor seems to act faster and fade faster than a massive dose of vitamin D (*3*) and appeared to cause an increase in calcium-binding activity in rabbit ileum (*5*). The similarity between the activity of vitamin D and *S. malacoxylon* was confirmed by the report by O'Donnell and Smith (*6*). However, from the reported symptoms and signs of Enteque seco, it was more likely that the active principle in *S. malacoxylon* might have biological properties similar to the active form of vitamin D, 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$]. 1,25-$(OH)_2D_3$ is formed in the kidney by the 1-hydroxylation of the precursor metabolite, 25-hydroxycholecalciferol (25-OHD_3), the latter resulting from the 25-hydroxylation of vitamin D_3 (cholecalciferol) in the liver (*7*).

To test the hypothesis that the *S. malacoxylon* factor had activity similar to that of 1,25-$(OH)_2D_3$, use was made of the established observation that a high stable strontium intake by chicks blocks the conversion of 25-OHD_3 to 1,25-$(OH)_2D_3$ by the kidney enzymes (*8*). The lack of 1,25-$(OH)_2D_3$ production in the presence of strontium results in a depressed absorption of calcium and the inhibition of synthesis of the intestinal vitamin D–dependent calcium-binding protein (*9*). The inhibitory action of strontium, as would be predicted, can be overcome by the administration of 1,25-$(OH)_2D_3$ per se but not by 25-OHD_3 or cholecalciferol (*8*), and we now report here that the inhibitory effect of dietary strontium can also be reversed by *S. malacoxylon*—evidence that the *S. malacoxylon* factor does, indeed, have activity similar to that of the active metabolite of vitamin D_3, 1,25-$(OH)_2D_3$.

Day-old White Leghorn cockerels were fed a commercial chick diet (Agway) for 23 days; at this time (designated day 0), they were placed on either a control diet containing 1.3 percent Ca, 0.65 percent P, and 1200 international units (I.U.) of vitamin D_3 per kilogram or a diet containing 2.5 percent Sr, 0.2 percent Ca, 0.65 percent P, and 1200 I.U. of vitamin D_3 per kilogram. On day 4, some of the chicks on the high strontium diet were continued on this diet, and the others were fed the same high strontium diet without vitamin D_3 but supplemented with either 0.2 or 0.5 percent of *S. malacoxylon* powder. The basal diet is the rachitogenic diet (*9*). On days 0, 4, and 7, six chicks from the appropriate groups were anesthetized with ether, and the degree of absorption of ^{47}Ca from a ligated duodenal segment in situ was determined (*10*). The 1-ml test dose contained NaCl, 150 m*M*; $CaCl_2$, 25 m*M*; and ^{47}Ca, 0.05 μc/ml, *pH* 7.2. After an absorption period of 15 minutes, the chicks were bled by cardiac puncture and killed with an overdose of sodium pentabarbitol. The duodenum was excised and the radioactivity therein was counted immediately in a well-type scintillation detector against reference ^{47}Ca. The ^{47}Sc contribution was excluded by the use of a single channel analyzer. The segment, after counting, was thoroughly rinsed and slit open lengthwise; the mucosa was removed from the underlying muscle coats (*11*). The duodenal mucosa was further processed and the supernatant

from the mucosal homogenate was assayed for the vitamin D–dependent calcium-binding protein (CaBP) by radioimmunoassay (*12*). Protein was measured by the method of Lowry *et al.* (*13*). Absorption data are expressed as the percentage of the administered dose of ^{47}Ca, and CaBP as micrograms per milligram of total protein in the supernatant of the mucosal homogenate.

Calcium and strontium in the plasma were determined by atomic absorption spectrometry, and plasma phosphate was determined by a modification of the procedure of Fiske and Subbarow (*14*), with the use of an autoanalyzer (Technicon). The *S. malacoxylon* plant was made available by Dr. H. R. Camberos, Ministry of Agriculture, Buenos Aires, Argentina, and Dr. G. K. Davis, University of Florida.

The experimental data on the response of strontium-fed chicks to *S. malacoxylon* powder are presented in Fig. 1. At days 4 and 7, the strontium diet significantly ($P < .001$) inhibited ^{47}Ca absorption (Fig. 1A) and reduced duodenal CaBP levels to nearly zero (Fig. 1B). Those chicks placed on the diets free of vitamin D_3 but containing *S. malacoxylon* recovered absorption and CaBP capacities (Fig. 1, A and B) in a dose-related fashion, and the correlation coefficient between ^{47}Ca absorption and CaBP was .90. This reversal of strontium inhibition by the plant was replicated in two other studies.

Clearly, the South American plant does have vitamin D–like activity as demonstrated by the induction of the vitamin D–dependent protein in our study and in vitamin D–deficient, rachitic chicks (*15*). The fact that *S. malacoxylon* can overcome the inhibitory effect of dietary strontium indicates that the factor in the plant mimics the action of $1,25\text{-}(OH)_2D_3$ since, as previously noted, convincing evidence is available showing that, under similar conditions, the conversion of $25\text{-}OHD_3$ to $1,25\text{-}(OH)_2D_3$ is blocked (*8*). This effect cannot be due to the calciferol-like activity of the plant alone, which, by bioassay, would contribute about 600 I.U. of vitamin D_3 equivalents per kilogram of diet. The high strontium control diet already contained 1200 I.U. of vitamin D_3 per kilogram, and certainly the inhibitory effect of strontium was evident in the presence of this and greater quantities (*9*) of vitamin D_3 (Fig. 1).

It is conceivable that the *S. malacoxylon* factor is a potent stimulator of the kidney 1-hydroxylase system and allows the $25\text{-}OHD_3$ hydroxylation to proceed even in the presence of strontium. This possibility seems unlikely as indicated from the following experiment. *Solanum malacoxylon* powder was extensively extracted with a mixture of methanol and chloroform to remove bona fide vitamin D. An aqueous extract of the residue was prepared and found effective in stimulating calcium absorption and inducing CaBP synthesis in the vitamin D–deficient chick. Since the only source of vitamin D–like potency was the extract, this indicated that the factor was not acting by stimulating 1-hydroxylation of $25\text{-}OHD_3$ that preexisted in the animal. This same aqueous extract also reversed the inhibitory effect of high levels of dietary strontium. However, an unequivocal approach to the above proposition will be the determination of the effectiveness of *S. malacoxylon* in rachitic nephrectomized animals or in an isolated intestinal organ culture system (*16*), or both.

It is also unlikely that *S. malacoxylon* operates directly on the intestinal calcium transport system, similar to the antibiotic filipin (*17*), since the direct incorporation of *S. malacoxylon* aqueous extract into the ^{47}Ca-labeled dosing solution was ineffective in stimulating ^{47}Ca absorption, and such a factor would not likely cause the synthesis of the vitamin D–induced calcium-binding protein.

The effect of the various treatments on plasma Ca, Sr, and inorganic phosphate concentrations, and on body weights, are shown in Table 1.

The present results bear on the fundamental nature of the soft-tissue calcification disease Enteque seco (*18*). In the normal animal, the calcium absorption mechanism is reasonably well controlled, with the efficiency of absorption changing in response to such variables as dietary calcium concentration, age, pregnancy, and growth (*19*).

Table 1. Plasma calcium, phosphate, and strontium concentrations, together with body weights of chicks on the experimental diets. The strontium diet alone decreased growth ($P < .001$) and plasma Ca ($P < .001$). The values given are for experimental day 7 only and represent the mean ± S.E.M. of six chicks per group. In any given column, the different superscript letters indicate that the values differ at $P < .05$, the comparisons being made between groups. SM, *Solanum malacoxylon* powder.

Group	Diet	Body weight (g)	Plasma Ca (mg/100 ml)	Plasma P_i (mg/100 ml)	Plasma Sr (mg/100 ml)
1	Control	319 ± 5[a]	11.5 ± 0.04[a]	5.2 ± 0.3[a]	
2	High Sr	285 ± 3[b]	9.7 ± 0.1[b]	4.5 ± 0.1[b,c]	6.0 ± 0.3[a]
3	High Sr + 0.2% SM	260 ± 19[b]	10.0 ± 0.2[b,c]	4.7 ± 0.2[b]	6.0 ± 0.5[a]
4	High Sr + 0.5% SM	255 ± 21[b]	10.4 ± 0.3[c]	4.2 ± 0.1[c]	7.7 ± 0.3[b]

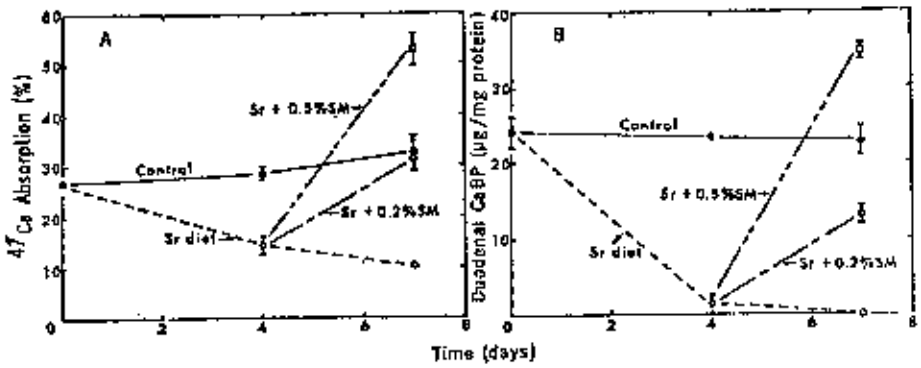

Fig. 1. Reversal of the inhibitory effect of dietary strontium on calcium absorption and net synthesis of the vitamin D–dependent calcium-binding protein (CaBP) by *S. malacoxylon* (*SM*). Chicks at 3½ weeks of age were fed a normal diet for 7 days or a diet containing strontium (2.5 percent) for 4 days. On day 4, some of the strontium-fed chicks were continued on that diet or given the same diet without vitamin D_3, but with the addition of *S. malacoxylon* powder (either 0.2 or 0.5 percent) for 3 days more. The degree of duodenal absorption of a test dose of ^{47}Ca is shown in (A) and net synthesis of CaBP in (B). Each point represents the mean of six chicks. The standard error of means rest within the confines of the symbol or are as designated by the range lines.

The modifiable reaction appears to be the degree of conversion of 25-OHD_3 to 1,25-$(OH)_2D_3$ by kidney enzymes (*20*); the more 1,25-$(OH)_2D_3$ produced, the greater the efficiency of calcium absorption. Since the *S. malacoxylon* factor effectively bypasses (or conceivably stimulates) the controlling point, that is, the kidney hydroxylase step, calcium absorption (and probably other aspects of calcium metabolism) occurs at a high rate and essentially out of control, thus accounting for the pathological symptoms of the disease. This is supported by the finding that *S. malacoxylon* elicits no gross toxicity in chicks on a low calcium diet but is deleterious when a normal calcium diet is fed (*15*), suggesting no direct action of the plant factor alone on the soft tissues.

The *S. malacoxylon* factor is water soluble and readily extracted from the plant by aqueous or other highly polar solvents (*21*) and this has been confirmed at our laboratory. This important property tends to eliminate the factor as being vitamin D or even 1,25-$(OH)_2D_3$ per se. It could, however, be a derivative of the metabolite containing a highly polar moiety, thereby accounting for its solubility properties. Required is the isolation and characterization of the factor in order to understand the relation of the factor to the calciferol series, if any, and to aid in elucidating its mode of action. Its therapeutic potential in certain human and animal disease states remains to be determined.

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Toward better therapy for resistant rickets

THE OBSERVATION of Stickler and associates, recorded elsewhere in this issue, that administration of vitamin D in high dosage to a patient with resistant rickets was associated with the continuing presence or worsening of the rachitic lesion has not been paralleled in the experience of others. The authors observed that one of a pair of identical twins given 800,000 I.U. of vitamin D weekly developed pseudofractures and had histologic evidence of rickets in a bone biopsy while the other twin, receiving no vitamin D, had no evidence of these changes. As the authors state, such a paradoxical effect does nothing to increase one's esteem for vitamin D as the sole agent for the treatment of this disease. Obviously the usual effect of vitamin D is to increase bone density and prevent further deformity, but all who manage patients with this disease are aware of the hazards of its use and the limitations of its value in treatment.

Fortunately, 2 recent developments may go far to ease the way of the rickets therapist and to improve the outlook for these patients. About 15 years ago Fraser and associates[1-3] demonstrated that constant infusion of phosphate would heal either resistant or deprivational rickets without recourse to therapy with vitamin D. This observation was of great interest but had little practical value until it was shown[4,5] that administration of a phosphate supplement orally would allow healing of rickets when vitamin D was given concurrently in doses in the range of 25,000 to 75,000 I.U. daily, a range which rarely results in intoxication. In 2 of the studies,[4,5] the effect of the oral phosphate has been dramatic in healing long-standing, severe rickets and osteomalacia. Continuing the regimen of phosphate and vitamin D after the rickets have healed has been successful in preventing recurrences.[5] The result is much smoother management of the patient; management with vitamin D alone frequently required such large doses that intoxication occurred with healing of the rickets and, as the dose was reduced, the rickets was reactivated with the risk of producing further deformity. These 2 extremes can now be avoided. Long-term use of the combined phosphate vitamin D regimen in our clinic has resulted in virtual disappearance of rachitic deformity in one patient who had very severe rickets as a small child. In addition, recent observations of our patients treated over long periods of time (as yet unpublished[6]) indicate that in some, acceleration of growth may be produced by combined therapy, providing a dose of vitamin D is given which is somewhat greater than that needed to heal or maintain the healing of the rachitic lesions. The main disadvantage of the phosphate therapy is the frequency with which doses of the medication must be given. The optimal regimen is probably a dose 6 times daily but one often has to settle for 4 or 5 doses daily. No toxic side effects have been noted except for mild-to-moderate diarrhea appearing in the first few days after the therapy is started and disappearing despite continuing the regimen.

See related article, p. 68.

The second development which may revolutionize therapy is a much more recent one and not as yet fully evaluated. It has been found by Blount and associates[7] that the active metabolite of vitamin D is 25-hydroxycholecalciferol (25-HCC). In some if not all patients with vitamin D–resistant rickets, this metabolite is formed only in

limited quantities or not at all when vitamin D_3 is given.[8] Whereas it has been reported[9] that administration of this metabolite over periods of one month has no effect on calcium or phosphate balance in patients with D resistance, others[7, 11] have reported that it does influence calcium metabolism and is effective in producing healing of the lesions. These reports[7, 10] indicate that its duration of action is short as compared to that of vitamin D_3. Discontinuing administration of the agent causes rapid reversion to a negative calcium balance and thus a therapeutic regimen employing the agent should be subject to rapid, fine adjustments as compared to the slow response to changes in vitamin D dosage. Toxicity should therefore be avoidable or quickly reversible. Whether use of this agent will promote growth in these children has not been determined.

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Evaluation of Vitamin D_3 Supplements by Biological Assay[1] Using the Turkey

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ABSTRACT Twenty-six vitamin D_3 supplements were assayed biologically using the young turkey. Four of the samples showed low relative biopotencies, i.e., 38.9, 40.1, 41.9, and 55.8%, using bone ash as the response criterion. Nineteen of the samples were satisfactory (above 80%). The respective average chemical assay values for the four low samples were 223, 91, 85, and 114% of stated potencies. The results of chemical assay did not reflect the biopotencies accurately, suggesting that the USP chemical assay measures biologically inactive as well as active compounds. In an additional study, 22 vitamin D_3 supplements were assayed biologically using toe ash as the response criterion. Nine of the samples had significantly lower biopotency than the standard; of these, three were under 60%. Several biological response criteria were compared in a study of assay precision and correlation. Growth; femur bone ash; bone length, breaking strength, and rigidity; plasma alkaline phosphatase; and plasma inorganic phosphorus correlated well with vitamin D status. Bone ash gave the greatest assay precision. *J. Nutr. 103:* 1187–1194, 1973.

INDEXING KEY WORDS vitamin D_3 · turkeys

Vitamin D_3 is required by turkeys for proper bone mineralization; its deficiency results in rickets. Highly potent vitamin D_3 concentrates are prepared commercially by irradiation of animal sterol (7-dehydrocholesterol). The potencies of supplements are usually established by chemical assay and the extent to which biological assay correlation is required has been uncertain.

Bone ash and growth have been principal criteria for vitamin D_3 bioassay of turkeys (1–3) and chicks (4, 5). Toe ash also gives a good response to vitamin D_3 activity (6–8). A relationship between plasma alkaline phosphatase activity and vitamin D has been reported (9). The assay of vitamin D_3 by a method based on activity of this enzyme has been attempted (10, 11). Plasma calcium and inorganic phosphorus were observed to be dependent on dietary vitamin D_3 (12, 13).

Occasionally rickets appears in turkey flocks. If it is not a result of low dietary calcium or phosphorus, it is usually assumed (albeit unfairly) that vitamin D is deficient. Vitamin D is rarely assayed in feeds since the biological assay is expensive and time consuming. Further, it is probably meaningless to determine the vitamin in the feed currently supplied, as the feed responsible was fed 1 to 3 weeks earlier during the depletion period.

This study was conducted with young turkeys to evaluate vitamin D_3 supplements in current use. It was deemed that insight into the reliability of vitamin D supplements could be gained by testing a random assortment of supplements in current use. The results would provide a basis for judging whether unexplained cases of rickets may be caused by vitamin D deficiency, or whether some other causative agent must be sought. Several biological response criteria were measured in the assay: bone and

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toe ash; growth; bone length, width, breaking strength, and rigidity; and plasma alkaline phosphatase, calcium and inorganic phosphorus.

MATERIALS AND METHODS

This study consisted of four experiments. Day-old, Broad White, male turkeys (Nicholas strain [4]) were caged by group in electrically heated battery brooders with raised wire-mesh floors. The windowless room was held at a temperature of 23 to 24°. Feed and water were supplied ad libitum. The diet (table 1) was a corn-soybean meal type. No added vitamin D was fed during a depletion period which followed receipt of the poults from the hatchery. At the times indicated below, the diet was supplemented with various levels of vitamin D_3 in assaying vitamin D_3 supplements from different sources. All vitamin D supplements were stored at −20°.

Experiments 1 and 2. Blood samples were taken from the heart with a heparinized syringe and plasma was separated by centrifugation. An automated continuous flow system (AutoAnalyzer [5]) was used to analyze the plasma constituents. Alkaline phosphatase activity was determined by a modified method of Marsh et al. (14).[6] Calcium and inorganic phosphorus were analyzed simultaneously [7] using the principles of methods by Kessler and Wolfman (15) and Fiske and Subbarow (16), respectively.

Femora were dissected from the separated right legs and cleared of adhering tissues. Measurements of bone length were made using a vernier caliper. Femur length was considered as the distance from the upper part of the great trochanter to the lower curvature of the condyles. Femur width was calculated as the average of the antero-posterior and medial-lateral width measured on the shaft at the equidistant point from the upper part of the great trochanter and from the lower curvature of the condyles.

Bone breaking strength and rigidity were determined with the aid of a modified shear press.[8] The force applied to the femur to break the bone was calibrated in kilograms. Rigidity, a measure of bone compressibility, was expressed as the ratio of the breaking strength (force) of the femur to time (distance on the recording chart) from the point of the pressure application to that of cracking.

The determination of bone ash was similar to the AOAC chick assay method (5). The split bones were dried in a vacuum oven at approximately 65° for 24 hours, extracted with 95% ethanol–benzene mixture (2:1, v/v) for 24 hours in a Soxhlet apparatus, ground, and ashed in a muffle furnace at 550° for 24 hours. The ash was expressed as a percentage of the dried, fat-free bone.

The vitamin D_3 supplement samples were packed in sealed polyester envelopes and sent to three industrial laboratories for chemical analysis of vitamin D_3 potencies. The analyses were performed essentially according to the U. S. Pharmacopoeia (1970) method (17).

TABLE 1

Composition of basal diet

Ingredients	%
Corn, ground yellow	36.5
Soybean meal, dehulled	45.0
Fish solubles (100% equivalent-dried on soybean meal)	3.0
Alfalfa meal, dehydrated 17% protein	3.0
Dried whole whey	3.0
Corn oil (refined)	3.0
DL-Methionine	0.1
Dicalcium phosphate	2.5
Calcium carbonate	1.0
Salt, iodized	0.5
Trace mineral mixture[1]	1.0
Vitamin mixture[2]	1.0
Vitamin D_3 premixture[3]	1.0
Calculated analysis	
Protein, crude %	27.89
Metabolizable energy, kcal/kg	2520
Calcium, %	1.21
Phosphorus, total, %	0.89
Phosphorus, inorganic, %	0.61

[1] Trace mineral mixture: (% of diet) $MnSO_4 \cdot H_2O$, 0.0254; ZnO, 0.0144, with ground corn carrier. [2] Vitamin mixture supplied the following per kg diet: vitamin A, 10,000 IU; vitamin E acetate, 22 IU; menadione sodium bisulfite complex (63%, USP), 4.41 mg; niacin, 40 mg; riboflavin, 6.63 mg; Ca D-pantothenate, 6.62 mg; choline chloride, 360 mg; folic acid, 3 mg; vitamin B_{12}, 22 μg; and biotin, 100 μg; ground corn carrier. [3] Vitamin D_3 from the supplements under assay well-premixed in corn carrier.

[4] Peterson Turkey Hatchery, Cannon Falls, Minn.
[5] Technicon Instruments Corp., Tarrytown, N. Y.
[6] Technicon AutoAnalyzer methodology: N-method file N-26.
[7] Ibid.; N-method file N-28.
[8] Allo-Kramer shear press, Model No. SP 12.

In experiment 1, 26 vitamin D_3 supplement samples were obtained in May, 1970, from feed manufacturers and colleges. Levels of 100, 300, and 900 ICU of vitamin D_3 (according to manufacturers' stated potencies) per kilogram feed were fed to poults 8 to 24 days of age. Each treatment (level) consisted of 10 birds in a single group. Blood samples and bones for the assay were obtained from five of the 10 birds per group at 24 days of age.

In experiment 2, eight vitamin D_3 supplement samples, selected from those tested in experiment 1, were assayed. Poults were fed 300 or 900 ICU of vitamin D_3 per kilogram feed from 8 to 21 days of age. Each treatment (level) consisted of two replicate groups of eight birds each. Five of the eight birds per group were used for blood and bone analysis.

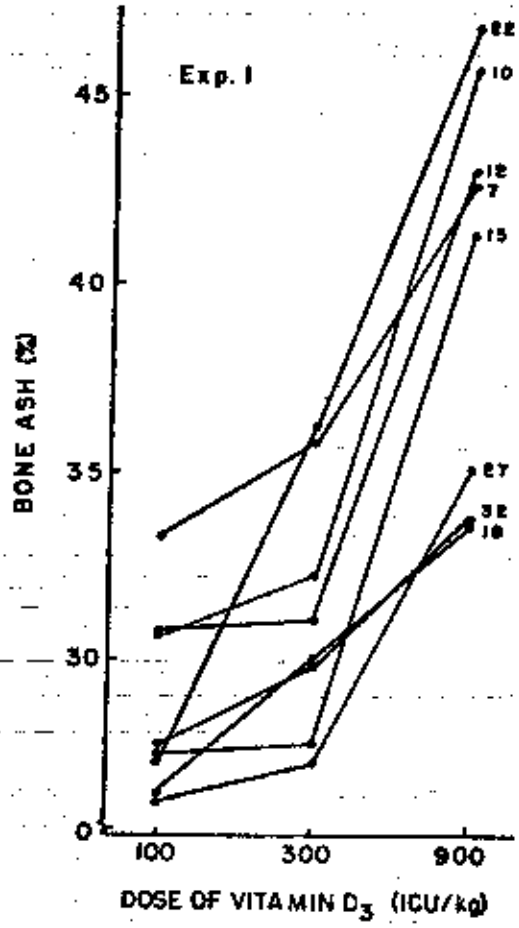

Fig. 1 Bone ash response of turkeys fed graded levels of selected vitamin D_3 supplements (identified by number) in experiment 1. Each point represents average of five turkeys.

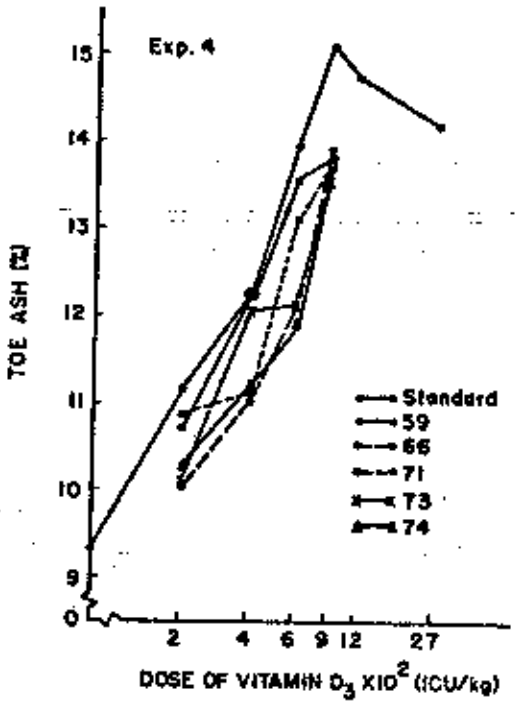

Fig. 2 Toe ash response of turkeys fed graded levels of ANRC standard and samples in experiment 4. Each point represents average of two replicates; each replicate value is a pool of toes of six turkeys.

Experiments 3 and 4. Poults were fed only the basal diet with no vitamin D_3 supplementation until 6 days of age in experiment 3 and 7 days of age in experiment 4. The center toe of the right foot was removed at the fourth joint from the claw with a scalpel. All the toes within each replicate of each treatment were pooled, dried, and ashed as described above. The results are expressed as the percentage ash in the dry unextracted toe. The ANRC Vitamin D_3 Standard[9] (2,200 ICU/g) was used for reference.

In experiment 3, 22 vitamin D_3 supplement samples from the field, including the USP Vitamin D Reference Standard Oil (400 μg/g; for chick assay),[10] were col-

[9] Animal Nutrition Research Council Vitamin D Reference Standard form Hoffmann-LaRoche Inc., Nutley, N. J. (courtesy of Dr. W. L. Marusich).

[10] From the United States Pharmacopoeia Convention, Inc., Bethesda, Md.

lected in May, 1971, and assayed biologically. All poults were fed 600 ICU of vitamin D_3 (sample no. 59) on days 7 and 8 following the 6-day depletion period. The samples under test were fed on days 9 to 25 of the experiment. Each treatment (level) consisted of three replicates of five birds each.

In experiment 4, five vitamin D_3 supplements selected from those tested in experiment 3 were assayed. The poults were fed 200, 400, 600 or 900 ICU vitamin D_3 from each sample per kilogram feed from 8 to 24 days of age. The turkeys receiving the standard source were fed 200, 400, 600, 900, 1,200, or 2,700 ICU vitamin D_3 per kilogram feed. Each treatment (level) of the sample had two replicates of six birds each.

RESULTS AND DISCUSSION

A. *Evaluation of vitamin D_3 supplements*

The percentage of ash versus log dose of vitamin D_3 of the samples assayed in experiments 1 and 4 are shown in figures 1 and 2, respectively. Many samples did not give maximum responses at the level of 900 units of vitamin D_3 per kilogram of diet, which is the requirement as estimated by the NRC (1971) for turkey poults (18).

Sample no. 32 was obtained for use as a standard in experiment 1. It, however, resulted in low biological responses and was obviously unsatisfactory. In making a substitute standard, it was considered that adopting an average value of several samples would be preferred to using a value from a single sample. The average results of samples no. 7, 10, and 22 were selected for the "substitute standard" as they showed chemical assay potency of about 96% of the stated and expected biological responses. The validity of this assumption was supported by the results of experiment 3 (table 3), in which sample no. 63 (same as sample 22 in experiments 1 and 2) resulted in approximately the same response in toe ash as the ANRC Standard.

TABLE 2

Estimated relative biopotencies of vitamin D_3 supplements using femur ash as response criterion (experiments 1 and 2)

Sample no.	Experiment 1		Experiment 2	
	Potency	Confidence[1] limits	Potency	Confidence[1] limits
	%			
7	93.3	131.6– 66.1	88.2	105.3– 73.9
8	92.0	114.5– 73.9	—	—
9	85.3	100.0– 72.2	—	—
10	90.6	118.3– 69.4	89.5	101.4– 79.8
11	85.4	104.2– 70.0	—	—
12	73.2	96.2– 55.8	69.6	84.5– 57.3
13	96.8	120.5– 77.8	—	—
14	81.1	94.4– 69.6	—	—
15	55.8	66.0– 47.2	47.7	56.4– 40.3
16	114.9	125.3–105.3	—	—
17	83.3	99.9– 69.4	—	—
18	41.9	50.9– 34.5	31.7	37.2– 26.9
19	102.9	127.7– 82.9	—	—
20	93.5	125.6– 69.6	—	—
21	66.5	72.9– 60.6	—	—
22	118.3	131.6–106.4	126.8	149.3–107.8
23	94.6	119.7– 74.8	—	—
24	103.4	135.6– 78.8	—	—
25	133.9	147.7–121.3	—	—
26	97.6	117.2– 81.2	—	—
27	38.9	48.1– 31.5	58.9	67.4– 51.3
28	94.5	111.2– 80.4	—	—
29	119.4	121.7–102.9	—	—
30	78.8	99.0– 62.7	—	—
31	93.5	106.5– 82.1	—	—
32	40.1	46.0– 34.9	38.4	46.7– 31.7

[1] At the 95% confidence limit.

TABLE 3

Effect of different vitamin D_3 supplements on body weight and toe ash of turkeys (experiment 3)

Sample no.	Level of vitamin D_3	Weight at 25 days of age[1]	Toe ash[2]
	ICU/kg	*g*	*%*
ANRC Reference Standard	0	370	9.94
	600	592	14.79
	2700	604	15.26
51	600	582	14.54
52	600	606	15.15
53	600	562	14.36
54	600	571	13.72**
55	600	573	14.15
57	600	604	15.18
58	600	582	15.07
59	600	516**	13.68**
60	600	555	13.81**
63	600	545	14.89
64	600	562	13.89**
65	600	559	14.90
66	600	400**	11.97**
69	600	600	14.94
70	600	547	14.35
71	600	526**	13.68**
72	600	545	13.93**
73	600	489**	12.95**
74	600	526**	13.64**
75	600	563	15.70
76	600	552	15.12
77	600	566	15.30

[1] $s_{\bar{x}} = 12.7$ [2] $s_{\bar{x}} = 0.254$. ** Significantly lower potency compared to the standard at $P < 0.01$.

The relative biopotencies of the samples and their confidence limits were calculated using published equations (19–21). The calculated relative biopotencies of the vitaman D_3 supplements and their confidence limits are presented in tables 2 and 4. Table 3 shows the screening data of the second series of samples. The results showed that some vitamin D_3 supplements were inadequate in respect to their claimed potencies.

Samples numbered 15, 18, 27, and 32 out of 26 supplements in experiments 1 and 2 showed low relative biopotencies using femur ash as the response criterion; samples 71, 73, and 74 out of 22 supplements tested in experiments 3 and 4 also showed low relative biopotencies, based on toe ash. They had relative biopotencies below 60%.

The results of chemical assay of the samples used in experiment 1 are presented in table 5 and the average values are plotted against respective biopotencies in figure 3. Chemical analyses did not reflect accurately the respective biopotencies. The poor agreement suggests that biologically inactive components of the samples respond in the chemical assay. Freed (22) stated that the chemical method of analysis for vitamin D adopted in the sixteenth revision of the Pharmacopoeia of the United States (23) had been found satisfactory for the assay of many pharmaceutical preparations. However, it was also mentioned that disagreement with biological values may occur in the analysis of samples containing biologically inactive irradiation product(s) or vitamin D breakdown products which may result from storage.

The chemical assay data indicated considerable difference among the laboratories. Low potency samples tended to yield higher assay potencies in laboratories 2 and 3. Different modifications in the chemical analysis doubtless contributed to the variation.

The usual USP chemical determination of vitamin D_3 is apparently not capable of predicting the biological response with complete accuracy. It was recently sug-

TABLE 4

Estimated relative biopotencies of vitamin D_3 supplements using toe ash as response criterion (experiment 4)

	Sample no.					
	Standard	59	66	71	73	74
Potency (%)	100.0	78.5	67.2	58.2	58.9	56.1
Confidence[1] limits (%)	108.9–91.8	87.9–70.3	75.0–61.6	64.4–52.5	68.5–50.4	61.5–50.9
M[2]	0.000	−0.105	−0.173	−0.235	−0.230	−0.251
$s_{\bar{x}}$ M[3]	0.0171	0.0226	0.0223	0.0210	0.0316	0.0191
Slope[4]	5.94	5.30	5.34	5.72	5.12	5.38

[1] Confidence limits at the 95% level. [2] Estimated relative biopotencies in logarithms. [3] Standard errors of potencies in logarithms. [4] Combined slope of log dose–response curve.

TABLE 5

Potencies of vitamin D_3 supplements by chemical assay (experiments 1 and 2)

Sample no.	Stated[1] ICU/g	Lab 1	Lab 2	Lab 3	Average
			% of stated		
7	200,000	104.0	110.5	106.4	106.9
8	8,818	88.0	144.0	110.6	114.2
9	200,000	89.0	112.7	97.6	99.1
10	200,000	93.0	105.0	90.5	98.2
11	200,000	93.5	119.3	—	106.4
12	200,000	79.0	95.5	—	87.2
13	200,000	75.0	118.0	74.3	89.1
14	24,800	81.1	114.1	102.8	99.3
15	1,500	74.4	146.6	120.4	113.8
16	200,000	90.0	124.0	122.7	112.2
17	200,000	73.5	101.5	102.1	92.4
18	440,000	78.7	90.4	86.1	85.1
19	1,653	88.9	139.1	—	114.0
20	200,000	85.0	115.0	103.9	101.3
21	200,000	67.0	106.0	—	86.5
22	34,000	53.6	77.0	123.6	84.7
23	325,000	82.8	91.7	—	87.2
24	325,000	84.9	82.5	—	83.7
25	4,409	126.0	356.1	155.2	218.6
26	441	—	—	—	—
27	2,646	67.3	370.4	229.4	223.4
28	1,470	95.2	938.7	224.8	419.6
29	2,204	103.4	276.7	101.4	160.5
30	1,102	—	—	—	—
31	551	—	—	—	—
32	220,000	83.2	90.9	100.0	91.4

[1] Stated potencies of samples.

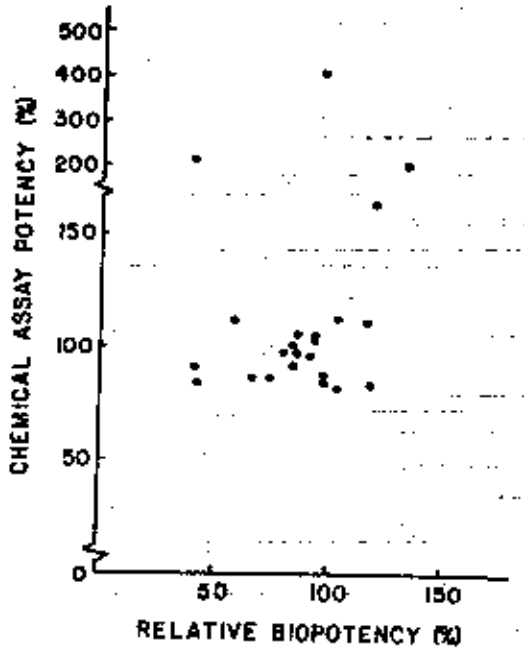

Fig. 3 Comparison of chemical and biological potency responses of the vitamin D_3 supplements employed in experiments 1 and 2.

gested (24) that subjecting vitamin D to derivatization and then gas–liquid chromatography may improve the accuracy of the determination. An approach (25) holding promise to improve the correlation between biological and chemical responses involves the removal of tachysterol by maleic anhydride and the prevention of certain other interferences during the chemical analysis.

From the results of this study it was not possible to discern whether the reduction in vitamin D_3 biological activity was due to unsatisfactory activation of the vitamin D_3 during irradiation or to losses following production. The ANRC Vitamin D_3 Standard in beadlet form was reported[11] to have excellent stability and retained 93% of its potency after storage for 12 months at room temperature (23°). This information was, however, derived from results obtained using the USP chemical method.

The results of this study suggest that the vitamin D_3 allowances (requirement × safety factor) in turkey rations should be 3 to 4 times the requirement rather than the lower margin of safety usually accepted earlier. A dietary level of this magnitude is not believed to represent an overdosage. A vitamin D_3 level of 27,000 ICU/kg (30 times the requirement) did not show any deleterious effect in turkeys.[12]

B. Comparison of assay response criteria

Several biological criteria other than growth or bone ash were measured in response to vitamin D_3.[13] Low vitamin D resulted in decreased plasma calcium and phosphorus; decreased femur ash, length, breaking strength, and rigidity; and increased plasma alkaline phosphatase. The simple correlation coefficients among the various measurements are presented in table 6.

The precision (λ) of measurement as expressed by the ratio of standard devia-

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[13] Data may be found in thesis by Yang, H. S. 1971 Evaluation of vitamin D_3 supplements by biological assay with young turkeys. M.S. Thesis, University of Minnesota.

TABLE 6

Simple correlation coefficients among various measurements in response to dietary vitamin D adequacy in turkeys (Experiment 1)

Measurements	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
X_1	1.00										
X_2	0.47	1.00									
X_3	0.82	0.77	1.00								
X_4	0.87	0.67	0.95	1.00							
X_5	0.82	0.68	0.89	0.90	1.00						
X_6	0.42	0.46	0.52	0.53	0.47	1.00					
X_7	0.88	0.49	0.80	0.85	0.79	0.53	1.00				
X_8	0.87	0.46	0.77	0.82	0.76	0.43	0.93	1.00			
X_9	−0.75	−0.46	−0.70	−0.73	−0.67	−0.42	−0.74	−0.74	1.00		
X_{10}	0.48	0.22	0.35	0.39	0.35	0.19	0.41	0.39	−0.38	1.00	
X_{11}	0.68	0.41	0.66	0.70	0.62	0.34	0.65	0.64	−0.60	0.28	1.00

X_1: Femur ash
X_2: Body weight at 14 days of age
X_3: Body weight at 21 days of age
X_4: Body weight at 24 days of age
X_5: Femur length
X_6: Femur width
X_7: Femur breaking strength
X_8: Femur rigidity
X_9: Plasma alkaline phosphatase activity
X_{10}: Plasma calcium
X_{11}: Plasma phosphorus

TABLE 7

Precision of vitamin D_2 biological assay according to various measurements as expressed by λ (standard deviation in terms of log dose) (Experiments 1 and 2)

	sp (S)[1]		Slope (B)[2]		λ (S/B)		
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Average
Femur ash	3.94	4.22	21.4	18.9	0.18	0.22	0.20
Growth 1[3]	62.58	57.03	203.8	84.6	0.30	0.68	0.49
Growth 2[4]	77.05	69.44	332.3	194.3	0.23	0.35	0.29
Femur length	0.294	0.323	0.83	0.34	0.35	0.95	0.65
Femur width	0.057	0.053	0.09	0.04	0.64	1.32	0.98
Femur breaking strength	8.12	8.84	52.5	25.1	0.15	0.35	0.25
Femur rigidity	2.15	3.04	16.4	11.1	0.13	0.32	0.22
Plasma alk. phosphatase	62.35	62.16	−254.0	−146.7	0.24	0.42	0.33
Plasma calcium	1.57	1.66	1.27	0.89	1.24	1.87	1.55
Plasma phosphorus	1.31	1.33	3.75	3.25	0.35	0.41	0.38

[1] Standard deviation about dosage-response line. [2] Slope of dosage–response line of the standard. [3] Body weights at 21 days of age. [4] Body weights at 24 days of age.

tion to slope (dosage expressed logarithmically) was estimated (19, 21) and is presented in table 7. A smaller value of λ indicates greater inherent precision of an assay method. Femur ash showed consistently the most precision. Toe ash was not included among the measurements in this experiment.

Some response criteria were highly correlated to and showed precision comparable with bone ash. They were growth, bone length and breaking strength, activity of plasma alkaline phosphatase, and plasma inorganic phosphorus. Growth proved to be a more precise criterion when the experimental period was longer.

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VASCULAR ENZYME ACTIVITY CHANGES ACCOMPANYING THE INDUCTION OF EXPERIMENTAL ATHEROSCLEROSIS

PART I. RATS FED HARTROFT'S DIET

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INTRODUCTION

It is well-established that in comparison with many other animal species the rat is very resistant to experimental atherosclerosis induced by simple cholesterol feeding.

Proceeding on the basis of previous work[1–5], THOMAS AND HARTROFT[6] in 1959 described a diet containing cholesterol, cholate, thiouracil and a large excess (40 %) of butter. Rats fed this diet for a period of about 14 months develop arterial lesions which seem to correspond quite closely to the early and moderately advanced lesions found in man[7]. It is also well known that in many rats fed this diet, thromboses and myocardial infarctions may appear considerably earlier than the development of distinct atherosclerotic lesions. Blood coagulation studies in these animals reveal certain abnormalities[8].

GRESHAM AND HOWARD[9] presented evidence that the typical arterial lesion in rats fed such a diet (containing butter) was characterised by an accumulation of lipids in the macrophages of the intima, without any vascular connective tissue reaction. Since the induction of experimental arterial lesions is usually accompanied by more or less typical changes in the activity of certain vascular enzymes[10], it seemed to us important to investigate the above experimental model, in order to identify enzymic changes occurring independently of connective tissue reactions. On the other hand, the study of aortae of calciferol-intoxicated rats provides an opportunity to investigate those enzyme activity changes that accompany connective tissue reactions[11].

MATERIAL AND METHODS

Albino rats from the Konárovice farm, 3 months of age, weighing 190–200 g, were used. Control rats were fed an ordinary laboratory diet. The experimental rats were fed a diet in which the basal dry component included saccharose 47 %, casein

47 %, salt mixture 5 %, and vitamin mixture 1 %. Of this basal dry component, 50 % was mixed with cellulose powder 2 %, cholesterol 5 %, sodium cholate 1 %, methylthiouracil 1.2 %, and butter 40 %. The vitamin mixture contained, for every 1000 g of the dry basal diet, 2500 mg of cod liver oil, 100 mg of thiamine hydrochloride, 40 mg of sodium riboflavin-3-phosphate, 400 mg of nicotinamide, 30 mg of pyridoxine, 50 mg of calcium pantothenate, 250 mg of tocopherol, 5000 mg of choline hydrochloride, 1500 mg of β-aminobenzoic acid, and 500 mg of inositol. The diet of the control rats consisted of wheat pollard 70 %, dry skimmed milk 10 %, casein 17 %, alfalfa powder 2 %, and calcium carbonate 1 %. For every 1000 g of this basal diet, 80 mg of margarine, 7500 mg of cod liver oil, and 250 mg of salt mixture were added.

The durations of the feeding experiments were one, two and eight weeks, respectively. Some of the rats in the eight-week experiment were given, in connection with another kind of experiment, 3 U vasopressin and 30 NIH units thrombin per 200 g weight intravenously 60 min before killing. As the findings concerning enzyme activities in these animals did not differ from the findings in the other rats from the same group, these animals were included in some of the results (Figs. 5 and 6).

Both experimental and control rats were killed by decapitation. Immediately after killing, the aortae were removed, rinsed several times in ice cold saline to remove adherent blood, and carefully freed of perivascular tissue and of virtually the entire adventitia. Aortic samples of about 50 mg weight were sectioned on a freezing microtome in 20–30 μ thicknesses, and afterwards a 1 % homogenate in saline (adjusted to pH 7.0) was prepared by means of a Potter–Elvehjem glass homogeniser.

The homogenate was extracted with ice cold saline for 15–16 hours and then centrifuged at 3000 r.p.m. (8200 g) for 5 min. The supernatant was used for the determinations of enzyme activities. This procedure proved to be satisfactory for comparative purposes, and in particular made possible the simultaneous determination of a large number of enzyme activities. This is an important advantage in studies of this kind, where only a very small amount of tissue is available. It must be also stressed that all enzyme activities were calculated on the basis of the protein content of the extract, determined by the method of LOWRY *et al.*[12]. All reagents used were prepared with glass-redistilled water.

For the determinations of enzyme activities, the following methods were used. Phosphomonoesterase I and II activities were measured using slight modifications of the methods described by KAPLAN AND NARAHARA[13,14]. Determination of 5′-nucleotidase activity was performed by a slightly modified procedure of AHMED AND REIS[15], and aortic adenylpyrophosphatase activity, by a modification of the method of BANGA AND NOWOTNY[16]. The group-specific carboxylic esterase activity was assessed by the method of SELIGMAN *et al.*[17], using β-naphthol acetate as the substrate. Details of the above methods (including the modifications) can be found in our previous communications[10].

For determination of the activity of NAD-linked dehydrogenases, spectrophotometric methods ("optical methods") based on the estimation of changes in the concentration of reduced NAD (read at 340 or 366 mμ, according to the principle

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of WARBURG AND CHRISTIAN[18]) were used. Using such a technique, lactate dehydrogenase (LHD) was determined essentially by the method of KUROWITZ AND OTT[19], as modified by BERGMEYER *et al.*[20], but using the aortic extracts (0.1–0.2 ml) instead of serum as described in the above methods. Optical densities were read at minute intervals for 3–5 min, and the results were calculated in units according to WRÓBLEWSKY AND LADUE[21] and expressed as units per 100 γ of protein in the extract. The same principle was applied in assays of malate dehydrogenase activity (MDH), and the method of OCHOA[22] in the modification described by BERGMEYER AND BERNT[23] was used. In both "optical tests" for dehydrogenases, reagents and enzymatic preparations (made by the Boehringer firm) were utilised. The activity of glutamate oxalacetate transaminase (GOT) was determined spectrophotometrically, using the method described by BERGMEYER AND BERNT[24]. Results, as in all the above determinations, were calculated on the basis of the protein content of the extract.

All the methods were used in a range where a good linearity between the amount of extract and the optical density readings could be obtained, and all methods were carefully checked for reproducibility.

RESULTS

Findings in the aortae of 21 rats killed one, two or eight weeks respectively after the start of the feeding experiment, together with findings in the aortae of 22 control rats, are summarised in Figs. 1–5.

As seen from Figs. 1 and 2, the aortae of these animals revealed a decrease in

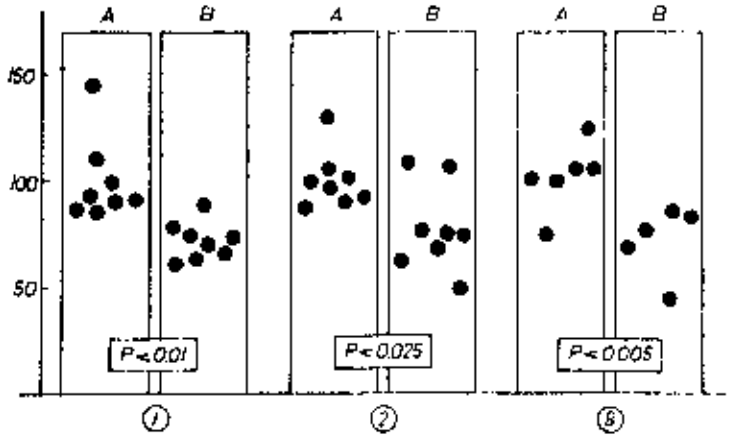

Fig. 1. The activity of aortic malate dehydrogenase in rats on a cholesterol–butter–cholate–thiouracil diet (HARTROFT's diet). A: control rats, B: rats fed diet for a period of one, two or eight weeks. Average activity in aortae of control rats, 214.65 WRÓBLEWSKI units per 100 μg protein of aortic extract. Results expressed as percentage differences.

the activity of malate dehydrogenase and glutamate oxalacetate transaminase as early as one week after the start of feeding with HARTROFT's diet. After one mon-

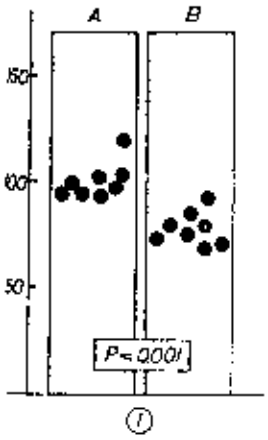

Fig. 2. The activity of aortic glutamate oxalacetate transaminase in rats fed HARTROFT's diet for a period of one week. Average activity in aortae of control rats: 62,26 WRÓBLEWSKI units per 100 μg of protein of aortic extract. Results expressed as percentage differences.

week there also appeared a decrease in the activities of phosphomonoesterase I (Fig. 3) and phosphomonoesterase II (Fig. 4), and an increase in the activity of

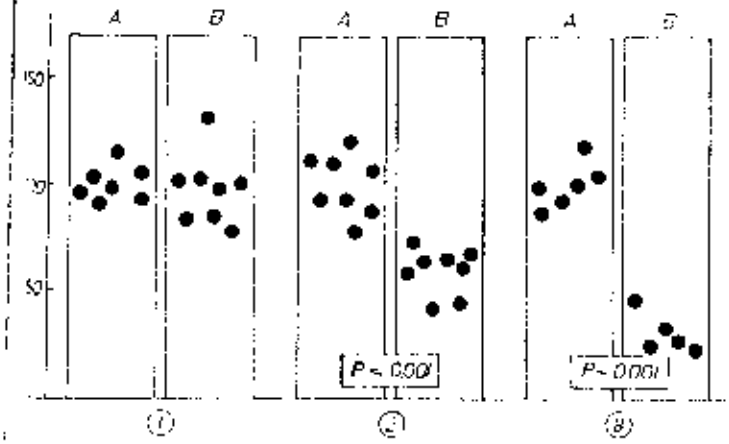

Fig. 3. The activity of aortic phosphomonoesterase I in the same rats as in Fig. 1. Average activity in aortae of control rats: 9.95 units (μmoles of phenol liberated/h/1 mg protein of aortic extract). Results expressed as percentage differences.

aortic adenylpyrophosphatase (Fig. 5). It is important to note that in these early stages of the experiment the aortae of these animals did not reveal any accumulation of lipids, or any other microscopic changes attributable to atherosclerotic or thrombotic types of lesion. At later stages, *i.e.* in the 8 week experimental series, a decrease in the activity of 5′-nucleotidase could be observed as well (Fig. 6). The activity of group-specific carboxylic esterase did not change in any of the experimental series.

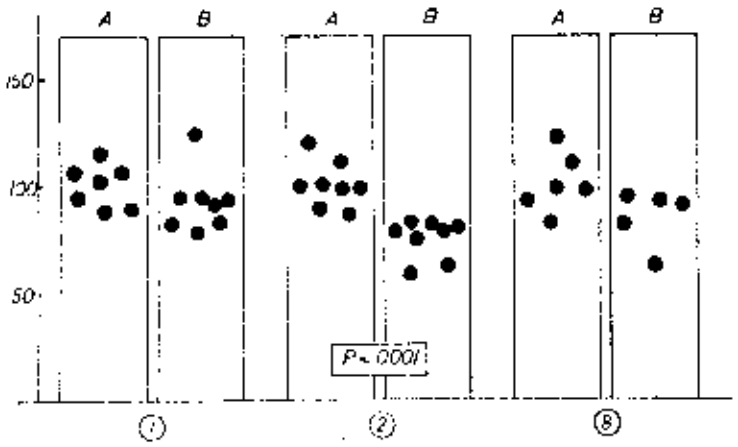

Fig. 4. The activity of phosphomonoesterase II in the same rats as in Fig. 1. Average activity in aortae of control rats: 1.08 units (μmoles of phenol liberated/h/1 mg protein of aortic extract). Results expressed as percentage differences.

DISCUSSION

An outstanding feature of these experiments is the very early decrease in the activity of aortic malate dehydrogenase. It must be pointed out that in other experiments, concerned with early stages of experimental atherosclerosis in rabbits[10,23], we also observed a decrease in the activities of some of the enzymes of the citric acid cycle. Human vessels consistently show lower activities of malate and succinate

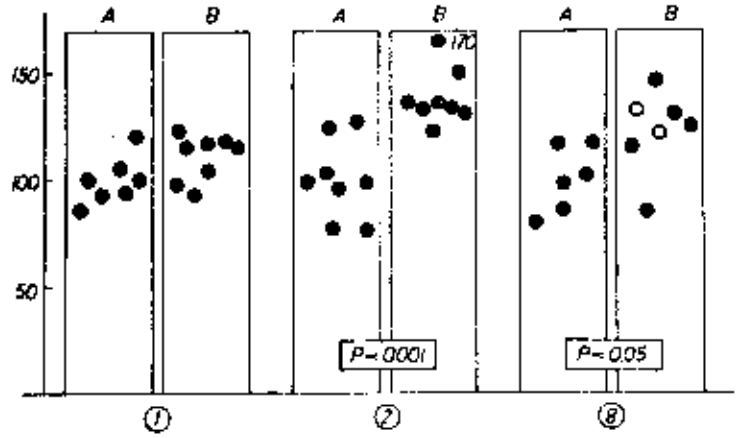

Fig. 5. The activity of aortic adenylpyrophosphatase in the same rats as in Fig. 1. Average activity in aortae of control rats: 7.90 units (μg of phosphate liberated/30 min/100 μg of protein of aortic extract). Results expressed as percentage differences. ○: enzyme activities in rats given 30 NIH units thrombin/200 g body weight 60 min before killing.

dehydrogenase in macroscopically intact aortic segments in which the degree of atherosclerosis is usually high, *e.g.* in the abdominal aorta, as compared to the as-

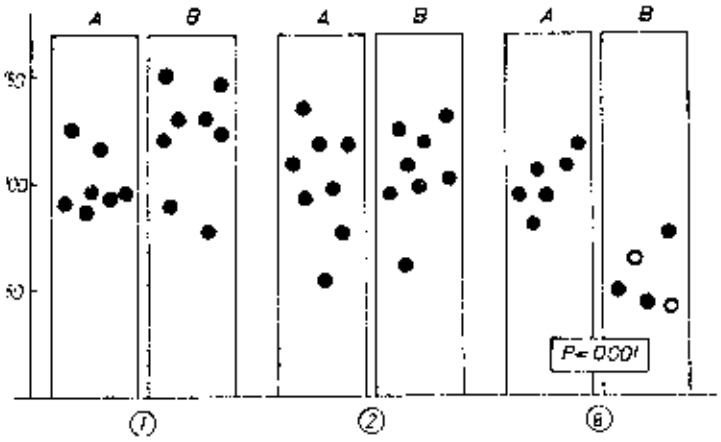

Fig. 6. The activity of aortic 5'-nucleotidase in the same rats as in Fig. 1. Average activity in aortae of control rats, 1.17 units (μg of phosphate liberated per 30 minutes per 100 μg of protein in aortic extract). Results expressed as percentage differences. Enzyme activities in rats given 30 NIH units thrombin/200 g body weight 60 min before killing.

ending aorta of the same individual[26,27]. The activities of these enzymes are also lower in the human aorta than in the pulmonary artery, in which the extent and severity of atherosclerosis is usually very small[26,27]. According to the findings of KIRK[28], all enzyme activities of the citric acid cycle are higher in the latter vessel.

It is therefore reasonable to assume that not only full-blown atherosclerotic lesions (spontaneous and experimental), but also "pre-atherosclerotic" or potentially atherosclerotic situations are characterised by a decrease in the activities of some (or all?) of the enzymes of the citric acid cycle. Perhaps this decrease is a prerequisite for the accumulation of lipids in such vessels.

Another feature of this experimental series was the decrease in the activities of the phosphomonoesterases, including 5' nucleotidase. This is quite in contrast to findings observed in connection with experimental or spontaneous atherosclerosis, where the vessels display an early increase in the activities of these enzymes. Such enzyme activities, especially the increased activities of phosphomonoesterase I and 5'-nucleotidase, probably reflect corresponding changes in connective tissue metabolism, although we have no definite knowledge of the exact physiological significance of these enzymes[19]. It is therefore reasonable to assume that in the aortae of rats fed HARTROFT's diet there is no increase in phosphomonoesterase activity because — as stated in the introduction — there is no connective tissue reaction in these vessels. This, of course, does not answer the question why we observed a decreased instead of an (expected) unchanged activity of these enzymes in these experiments.

A third feature of these experiments was the distinct increase in the activity of aortic adenylpyrophosphatase. Increased adenylpyrophosphatase activity should lead to an increased formation, and possibly accumulation, of ADP in the vessel wall, and there seems to be a close correlation between ADP and the phenomenon of blood platelet aggregation[29]. One is therefore tempted to speculate whether this

feature may not be an additional factor contributing to the increased incidence of arterial thrombosis in these rats*.

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SUMMARY

Male albino rats were fed a diet containing 5 % cholesterol, 1 % sodium cholate, 40 % butter and 1.2 % methylthiouracil. From previous work of other authors, it is known that feeding such a diet (HARTROFT's diet) results in early arterial changes characterised by an accumulation of lipids in the endothelial macrophages, without any vascular connective tissue reaction. In later stages of such experiments, multiple thrombosis and myocardial infarctions can be observed.

The aortae of these rats revealed a very early decrease in the activities of malate dehydrogenase and glutamate oxaloacetate transaminase, followed by decreases in the activities of phosphomonoesterase I, phosphomonoesterase II and 5'-nucleotidase, and by an increase in the activity of aortic adenylpyrophosphatase.

The possible relationship of these enzymic changes to the accumulation of lipids, connective tissue reactions, and thrombogenesis, is discussed.

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* Further results of work dealing with the question of ATP-ase activity in the aortae of such rats were presented at the *Symposium of Thrombosis*, held at at Cambridge, June 10, 1965.

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1α-HYDROXYVITAMIN D_3: BIOLOGICAL ACTIVITY AND METABOLISM TO 1α,25-DIHYDROXYVITAMIN D_3. Joseph F. Zerwekh, Douglas J. Cork, Peter F. Brumbaugh* and Mark R. Haussler (SPON: B.L. Reid) Dept. of Biochem., Arizona Medical Center, Tucson, Arizona 85724.

1α-Hydroxyvitamin D_3 (1α-OH-D_3) is a synthetic sterol with biologic characteristics similar to those of 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3), the hormonal form of the vitamin. 1α-OH-D_3 (courtesy of Dr. M. Fechet) was equal in potency to 1α,25-$(OH)_2$-D_3 and functioned with an identical time course in stimulating calcium absorption in rachitic chicks. In chronic administration studies, the synthetic sterol was slightly more antirachitic than 1α,25-$(OH)_2$-D_3 with both hydroxylated derivatives being 3-5 times more active than vitamin D_3.

However, *in vitro* experiments employing the intestinal receptor for 1α,25-$(OH)_2$-D_3 indicate that 1α-OH-D_3 binds much less effectively than the natural hormone. Since the equipotency of 1α-OH-D_3 and 1α,25-$(OH)_2$-D_3 was not reflected at the molecular level, we examined the possibility that 1α-OH-D_3 is converted to 1α,25-$(OH)_2$-D_3 *in vivo*. Rachitic chicks were given 1α-OH-D_3 and 3 hr later their intestinal chromatin was extracted. The suspected 1α,25-$(OH)_2$-D_3 fraction was isolated by Celite chromatography and a saturating amount (26 pmoles) of 1α,25-$(OH)_2$-D_3 was detected in the chromatin by competitive protein binding assay. Since 1α,25-$(OH)_2$-D_3 was present in the receptor prior to the initiation of increased calcium absorption by 1α-OH-D_3, we conclude that 1α-OH-D_3 is rapidly metabolized to 1α,25-$(OH)_2$-D_3 and probably functions by conversion to the hormone. (Supported by USPHS Grant AM15781.)

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EFFECTS OF LARGE DAILY DOSES OF VITAMIN D ON TEETH AND JAWS OF RATS AND ON HUMANS[1]

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The purpose of this study was to determine the effect of massive daily doses of vitamin D[2] on dentin apposition, bone apposition, tooth eruption, calcification rhythm, skeletal calcification, pulp stone formation, estrus cycle, and toxicity in rats, and pulp stone formation in humans. [Experiments in the treatment of rheumatoid arthritis with massive daily doses of vitamin D have been conducted by a number of investigators (1).]

Schour and Ham (2) reported the effect on the calcification of the incisor dentin of the rat from the administration of a single large dose of vitamin D. Schour and Hoffman (3), in a study of dentin calcification rhythm, reported that severe experimental interferences such as hypophysectomy or parathyroidectomy did not alter the calcification rhythm. Weinmann (4) studied calcification and pulp stone formation by histological section. The methods employed in the present study differed from those used in the aforementioned. To the best of our knowledge, the literature does not report a comparable experiment.

ANIMAL EXPERIMENTS

Thirty-two female, white rats of the Sherman strain, aged 57 to 59 days at the beginning of the experiment, were observed. Twelve served as an untreated group, while 20 were treated experimentally. There were 4 sets of litter-mates, 4 animals to each set, distributed between the untreated and treated groups in equal numbers. The study was set up so as to provide 2 sets of controls; namely, the untreated group of animals, and a control period in each animal. A control period of 2 weeks was followed by an experimental period of 2 weeks. In this way, a safe margin was allowed for the time required for the incisor of the rat to renew itself. Only 4 animals were placed in a cage, thus giving each a better opportunity to obtain ample food. The animals were earmarked and weighed, and each cage was provided with a regular stock diet.[3]

[1] Presented at a meeting of the Jarvie Society, New York, May 1st, 1942.

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[2] Ertron, a vitamin D preparation of exceptionally high potency, prepared by the Whittier process (activation of heat-vaporized ergosterol by electrical energy). The materials were furnished and the investigation was partially supported by a grant from the Nutrition Research Laboratories, Chicago, Ill.

[3] Alfalfa leaf meal, 2%; whole milk powder, 10%; ground whole wheat, 15%; pearled barley, 15%; shelled oats, 15%; yellow corn, 15%; soya bean meal, 15%; meat scraps, 10%; sodium chloride, 2%; calcium carbonate, 0.5%.

The rats were weighed at the beginning of the experiment, at the end of the control period (first 2 weeks), and again at the end of the experimental period (second 2 weeks).

Because of the favorable reports of Reed and others (1) on the comparative safety of the Whittier-process type of vitamin D and because it was being tried on patients in the Arthritis Clinic at the Medical Center, Columbia University, this product (Ertron) was chosen as the experimental agent. Each rat of the twenty received daily 10,000 U.S.P. units incorporated in the diet, the mixture fed being weighed and consumption time recorded. There was some loss owing to unavoidable wastage so that the estimated amount ingested daily was about 7,000 U.S.P. units.

The weights of the rats (Table I) vary from 100 to 170 grams, the average starting weight being 128 grams (130 for untreated, 126 for treated animals). The daily dose per kilogram of body weight was between 60,000 to 100,000 U.S.P. units. For the rat of average weight (128 grams) the daily dose per kilogram of body weight was about 78,000 U.S.P. units. No specific attempt was made to determine the minimal toxic dose. It is significant, however, that none of the animals showed grossly observable signs of toxicity or deleterious effects while receiving the vitamin D preparation.

Dentin Apposition Rate

The vital staining method (5) was used for measuring the amount of dentin apposition. Alizarin Red S was injected intraperitoneally, 100 mgm. for each kilogram of body weight, at the beginning of the control period. This was repeated at the beginning of the experimental period, and again at the end of the experimental period.

Ground histologic sections were made of each mandible to study the appositional growth of dentin by means of the alizarin lines. These sections were made by cutting 5 cross-sections from one-half of the mandible and 1 longitudinal-section from the other half in such a way that the incisor tooth and jaw bone were visible in each section. From each slide, sections were selected where 3 adjacent alizarin lines were present in the dentin, indicating the dentin apposition during the control and experimental periods. The distance between these lines was measured along the dentinal tubules, measuring between the pulpal sides of the alizarin lines with an eye-piece screw micrometer.

Results. There was an increase in the dentin apposition rate in both groups of rats during the experimental period. In the untreated animals the increase was slight but significant (0.94 micra per day), whereas the treated animals showed an accelerated dentin apposition rate (2.33 micra per day) (Tables II and VI).

There was no observable change in quality of dentin as observed microscopically with methods of preparation described above (*figs. 1, 2, 3, 4*).

Bone Apposition

The vital staining method, as described under *Dentin Apposition Rate*, was employed. Bone measurements were made perpendicular to the surface in sec-

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TABLE I

Weight in grams

RAT	CAGE	(CONTROL PERIOD) (EXPERIMENTAL) Start	Mid	End
Untreated animals				
Plain	2	110	150	145
NL	2	135	160	155
NR	2	130	150	170
*SR	2	140	165	175
Plain	4	135	155	165
NL	4	140	150	170
NR	4	140	140	160
SR	4	125	155	170
Plain	8	130	150	175
NL	8	130	160	175
NR	8	125	150	185
SR	8	125	150	175
Totals		1565	1835	2020
Mean		130	153	168
Mean gain		22	15	
σ_m		3	3	
Critical ratio		8.0	4.7	
Treated animals				
Plain	1	130	150	155
NL	1	130	150	160
NR	1	140	155	165
SR	1	130	155	130
Plain	3	140	150	170
NL	3	140	160	165
NR	3	145	150	150
SR	3	145	165	170
Plain	5	105	130	140
NL	5	100	120	135
NR	5	150	165	165
SR	5	120	135	135
Plain	6	110	130	135
NL	6	115	135	150
NR	6	140	155	170
SR	6	100	125	135
Plain	7	100	150	165
NL	7	110	140	155
NR	7	110	125	135
SR	7	170	195	200
Totals		2530	2940	3085
Mean		126	147	154

TABLE I—*Continued*

RAT	CAGE	(CONTROL PERIOD) (EXPERIMENTAL) Start	Mid	End
Treated animals—*Continued*				
Mean gain		20	7	
σ_m		2	2	
Critical ratio		4.4	3.4	
*Treated-untreated		−2	−8	
σ_m		3	4	
Critical ratio		0.6 (not significant)	2.1 (not significant)	

* Note: While the *rate* of weight increase declines during experimental period as compared with control period, and the rate of decline is a little bit greater in treated animals, when the differences are compared between the treated and untreated animals, the result is insignificant.

tions where 3 adjacent alizarin lines were present, indicating the bone formation during the control and experimental periods.

Results. A marked *increase* in bone formation was observed in the treated animals during the experimental period as compared to the amount of apposition seen during the control period when no vitamin D was administered. No such increase occurred in the untreated rats. The bone formation in the 12 untreated animals showed a slight but significant *decrease* in amount during the experimental period as compared to that found during the control fortnight. Since bone is not formed at an equal rate in all areas, increases were determined in terms of percentages rather than in terms of absolute figures so as to compensate for these appositional differences. Statistical examination at this phase of the study revealed the percentage increase to be significant—34.1% (Tables III and VI).

There was no observable change in the quality of bone as observed microscopically (*figs. 5* and *6*).

Calcification Rhythm

To observe the calcification rhythm in the dentin, the perpendicular distance between successive contour lines of Owen was measured. Measurements were made of the dentin formed during the control period as well as that formed during the experimental period, determined by the presence of the alizarin lines.

Results. Calcification rhythm was found to vary both in the untreated and the treated animals from 10.0 to 19.8 micra, with the median at about 15 micra. The differences in calcification rate during the control and experimental periods in both groups of animals were computed (Table IV). While variations in measurements were noted in both groups, the measurements for each animal were comparatively constant. These results indicate that the experimental procedure had no significant influence on calcification rhythm (Table VI).

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TABLE II

Dentin apposition rate

RAT	CAGE	CONTROL PERIOD	EXPERIMENTAL PERIOD	DIFFERENCE
Untreated animals				
		micra/day	*micra/day*	*micra/day*
Plain	2	14.2	16.7	2.5
NL	2	15.5	16.1	0.6
NR	2	16.4	16.4	0.0
SR	2	16.4	16.6	0.2
Plain	4	14.9	15.8	0.9
NL	4	15.2	16.3	1.1
NR	4	15.8	17.5	1.7
SR	4	12.5	14.6	2.1
Plain	8	15.8	15.8	0.0
NL	8	16.4	16.6	0.2
NR	8	14.2	15.7	1.5
SR	8	14.4	14.9	0.5
Totals		181.7	193.0	11.3
Mean		15.1	16.1	0.94
σ				0.81
σ_m				0.23
Treated animals				
Plain	1	14.5	16.5	2.0
NL	1	15.2	16.1	0.9
NR	1	14.1	17.2	3.1
SR	1	14.5	15.8	1.3
Plain	3	14.7	17.1	2.4
NL	3	14.1	14.5	0.4
NR	3	13.4	16.2	2.8
SR	3	12.7	16.4	3.7
Plain	5	14.5	17.7	3.2
NL	5	14.5	16.0	1.5
NR	5	13.6	16.8	3.2
SR	5	13.8	14.9	1.1
Plain	6	13.8	18.2	4.4
NL	6	11.5	17.5	3.0
NR	6	—		—
SR	6	15.8	18.0	2.2
Plain	7	14.4	16.0	1.6
NL	7	12.6	15.7	3.1
NR	7	14.4	16.1	1.7
SR	7	14.5	17.1	2.6
Totals		269.0	313.8	44.2
Mean		14.2	16.5	2.33
σ				1.01
σ_m				0.23

Rate of Eruption

In order to measure tooth eruption, 1 hole was drilled each week with an 800 L tapering fissure bur in the distal surface of each upper incisor tooth at the cemento-enamel junction even with the gingival margin. For this procedure, the animals were anesthetized. The eruption distance for the week was estab-

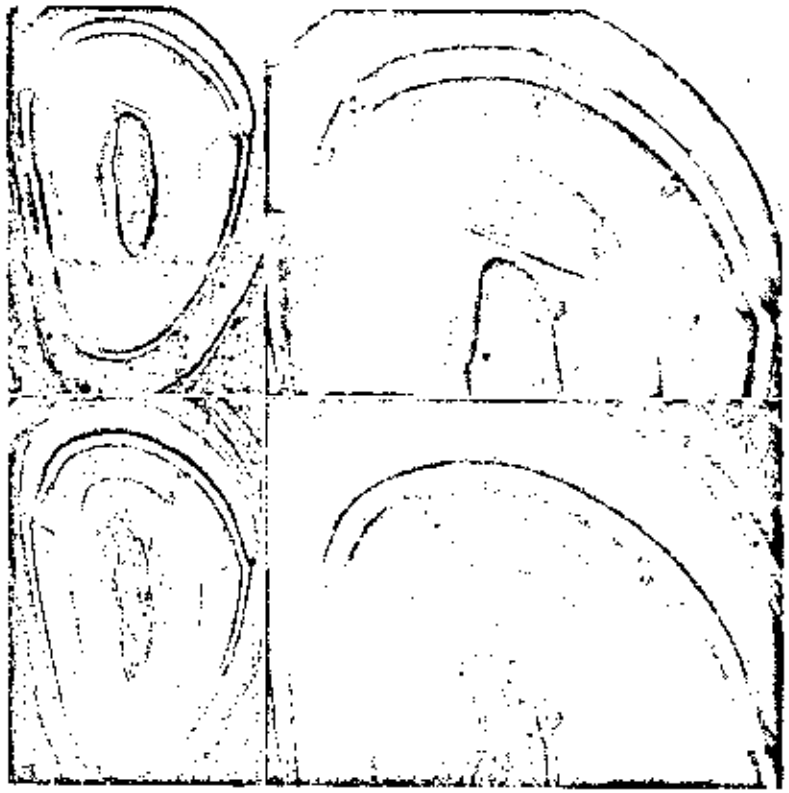

FIG. 1. Rat NL, Cage 2. Untreated animal. Shows 3 black rings in cross-section of incisor. These are "alizarin" red lines seen in original section. There was gain of 0.6 micra per day in dentin apposition rate in experimental period (lines 2 to 3) over control period (lines 1 to 2). × 56 (orig. mag.)

FIG. 2. High power of *fig. 1*. × 130 (orig. mag.)

FIG. 3. Rat NL, Cage 7. Treated animal. Alizarin rings show gain of 3.1 micra per day in dentin apposition rate. × 56 (orig. mag.)

FIG. 4. High power of *fig. 2*. × 130 (orig. mag.) Dentin structure appears normal.

lished by marking off with a divider the distance between the hole in the tooth and the free gingival margin, and measuring this distance by means of a micrometer. The process was repeated each week.

Results. Considerable variation (210 to 285 micra daily) was found in eruption rates of the incisor teeth in all the animals. In the treated series, the rates

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TABLE III

Bone apposition rate

RAT	CAGE	CONTROL PERIOD	EXPERIMENTAL PERIOD	DIFFERENCE	DIFFERENCE
Untreated animals					
		micra/day	*micra/day*	*micra/day*	%
Plain	2	6.06	5.82	−0.24	−4.0
NL	2	5.25	5.21	−0.01	−0.8
NR	2	2.03	2.46	−0.47	−16.0
SR	2	6.97	7.07	0.10	1.4
Plain	4	2.00	1.75	−0.25	−12.5
NL	4	6.00	6.14	0.14	2.3
NR	4	5.10	4.67	−0.43	−8.4
SR	4	3.25	2.82	−0.43	−13.2
Plain	8	4.78	4.92	0.14	2.9
NL	8	—	—	—	—
NR	8	7.36	6.35	−1.01	−13.7
SR	8	4.42	3.94	−0.48	−10.9
Totals		54.12	51.15	−2.97	−72.9
Mean		4.92	4.65	−0.27	−06.6
σ					0.33
σ_m					0.10
Treated animals					
Plain	1	5.50	8.75	3.25	59.1
NL	1	5.35	6.93	1.58	29.5
NR	1	6.14	7.97	1.83	29.8
SR	1	3.57	5.15	1.58	44.3
Plain	3	3.29	4.23	0.94	28.6
NL	3	—	—	—	—
NR	3	4.21	6.11	1.90	45.1
SR	3	4.79	7.32	2.53	52.8
Plain	5	3.00	3.57	0.57	19.0
NL	5	2.57	3.93	1.36	52.9
NR	5	3.14	4.86	1.72	54.8
SR	5	4.32	5.50	1.18	27.3
Plain	6	3.48	5.04	1.56	44.8
NL	6	6.93	10.40	3.47	50.1
NR	6	—	—	—	—
SR	6	4.75	5.79	1.04	21.9
Plain	7	3.54	3.29	−0.25	−7.1
NL	7	5.28	6.21	0.93	17.6
NR	7	4.68	6.22	1.54	32.9
SR	7	4.00	4.57	0.57	14.2
Totals		78.51	105.84	27.30	617.6
Mean		4.36	5.88	1.52	34.3
σ					—
σ_m					0.88
					0.21

of eruption in the upper incisors ranged from 214 to 280 micra daily, with the median about 250 micra. Statistical analysis showed no significant effect on eruption rate as a result of vitamin D administration (Tables V and VI).

Skeletal Calcification

Skeletal calcification was studied by the radiographic method. The animals were placed in a prone position, 2 untreated or 2 treated rats on each film. The whole animal was radiographed at a target distance of 5 feet. Radiographs of the mandibles and entire bodies were examined for increased density by comparing the treated with the untreated animals. No differences were observed between treated and untreated animals.

FIG. 5. Rat NL, Cage 2. Untreated animal. Alizarin lines in bone. Lines 1 to 2 constitute control period; Lines 3 to 4 experimental period. There was loss of .08% in daily rate of bone apposition. × 116 (orig. mag.)

FIG. 6. Rat NL, Cage 7. Treated animal. There was gain of 17.6% in daily rate of bone apposition in experimental period over control period. × 116 (orig. mag.)

Pulp Stones

The mandibles were removed, split in half, debrided of all soft tissue, and radiographed individually, at a target distance of 3 feet, for the detection of pulp stones. Molars and incisors were also examined radiographically. No pulp stones were observed.

Estrus Cycle

Daily vaginal smears were taken on all animals (6, 7, 8). The estrus cycle was found to be unaltered during the experimental period when compared with the control period.

Toxicity

The absence of toxicity in the treated group was judged by such general criteria as: constant weight increase, continued good appetite, silkiness of coat, and activity as contrasted with an earlier test group which received ten times the amount of Ertron and showed a definite toxicity as characterized by weight-loss, lethargy, anorexia, roughness of coat, and increased irritability. None of the

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TABLE IV

Calcification rhythm

RAT	CAGE	CONTROL PERIOD	EXPERIMENTAL PERIOD	INCREASE
Untreated animals				
		micra/day	*micra/day*	*micra/day*
Plain	2	12.6	15.0	2.4
NL	2	15.7	16.9	1.2
NR	2	13.9	15.0	1.1
SR	2	12.2	14.7	2.5
Plain	4	12.7	16.4	3.7
NL	4	15.5	17.8	2.3
NR	4	15.5	18.0	2.5
SR	4	15.4	15.5	0.1
Plain	8	14.3	14.1	−0.2
NL	8	15.6	19.8	4.2
NR	8	14.9	17.1	2.2
SR	8	13.9	13.5	−0.4
Totals		172.2	193.8	21.6
Mean		14.4	16.2	1.80
σ				1.41
σ_m				.41
Treated animals				
Plain	1	14.3	15.3	1.0
NL	1	11.8	16.8	5.0
NR	1	11.3	16.9	5.6
SR	1	11.5	10.0	−1.5
Plain	3	13.5	17.8	4.3
NL	3	13.7	14.1	0.4
NR	3	12.6	17.5	4.9
SR	3	12.5	15.0	2.5
Plain	5	17.9	17.5	−0.4
NL	5	13.5	14.6	1.1
NR	5	13.7	15.0	1.3
SR	5	—	—	—
Plain	6	17.1	15.4	−1.7
NL	6	15.9	14.3	−1.6
NR	6	14.3	14.9	0.6
SR	6	13.9	12.5	−1.4
Plain	7	16.9	15.4	−1.5
NL	7	14.1	15.5	1.4
NR	7	15.5	15.9	0.4
SR	7	12.8	15.3	2.5
Totals		266.8	289.7	22.9
Mean		14.0	15.2	1.21
σ				2.32
σ_m				.53

TABLE V

Eruption rate

RAT	CAGE	CONTROL PERIOD	EXPERIMENTAL PERIOD	DIFFERENCE
Untreated animals				
		micra/day	*micra/day*	*micra/day*
Plain	2	251	220	−31
NL	2	251	253	2
NR	2	251	236	−15
SR	2	258	241	−17
Plain	4	272	220	−52
NL	4	245	236	−9
NR	4	251	244	−7
SR	4	251	214	−37
Plain	8	285	272	−13
NL	8	248	251	3
NR	8	230	244	14
SR	8	244	244	0
Totals		3037	2875	−162
Mean		253	239	−14
σ				18
σ_m				5.2
Treated animals				
Plain	1	265	261	−4
NL	1	251	245	−6
NR	1	265	261	−4
SR	1	251	280	29
Plain	3	251	249	−2
NL	3	258	238	−20
NR	3	258	216	−42
SR	3	258	238	−20
Plain	5	251	242	−9
NL	5	223	220	−3
NR	5	272	216	−56
SR	5	251	186	−65
Plain	6	258	208	−50
NL	6	244	238	−6
NR	6	251	248	−3
SR	6	244	220	−24
Plain	7	251	214	−37
NL	7	251	236	−15
NR	7	224	253	29
SR	7	258	253	−5
Totals		5035	4722	−313
Mean		252	236	−16
σ				24
σ_m				5.4

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enumerated toxic symptoms were exhibited. The rats appeared entirely healthy and continued to increase in weight (Table I).

TABLE VI

Significance tests for differences in mean increase between treated and untreated animals

	DIFFERENCE IN MEAN INCREASES	S.E. OF DIFFERENCE IN MEAN INCREASE	CRITICAL RATIO
Dentin apposition	1.39	0.33	4.2*
Bone apposition	1.79	0.23	7.8**
Calcification rhythm	0.59	0.67	0.88†
Eruption	−2.2	7.5	0.29††

* The difference between the untreated and treated animals in average increase in dentin apposition is statistically significant. The probability that a difference as great as the one observed or greater would arise solely through chance or errors of random sampling, is less than one in a thousand.

** The difference between the untreated and treated animals in average increase in bone apposition is statistically significant. The probability that a difference as great as the one observed or greater would arise solely through chance or errors of random sampling is less than one in a million.

† The difference between the untreated and treated animals in average increase in calcification rhythm is not statistically significant. A difference as large as the one observed or larger would arise about 40% of the time solely through chance or errors of random sampling.

†† The difference between the untreated and treated animals in average increase in eruption rate is not statistically significant. A difference as large as the one observed and larger would arise about 80% of the time solely through chance or errors of random sampling.

TABLE VII

Vitamin D dosage of rheumatoid arthritic patients*

PATIENT	AGE	SEX	TOTAL UNIT DOSAGE	TOTAL DOSAGE PERIOD (DAYS BETWEEN RADIOGRAPHS)
J. W.	33	Female	41,700,000	153
H. S.	47	Female	68,250,000	231
C. P.	71	Female	20,150,000	103
V. B.	55	Female	34,550,000	121
E. W.	29	Female	62,250,000	218
I. R.	61	Female	48,950,000	169
L. T.	48	Male	63,750,000	219
I. P.	48	Female	67,250,000	230

Note: In 3 cases the first set of radiographs was taken a few days after instituting therapy.

* Ertron.

CLINICAL STUDY

Dental radiographs of 8 patients suffering from rheumatoid arthritis were taken before and after daily therapy of approximately 300,000 units of vitamin D (Ertron) was instituted. The pictures were compared for a possible difference in pulp stone formation. No appreciable change was seen (Table VII).

SUMMARY

1. Dentin apposition appeared to be significantly greater in the rats receiving vitamin D (Ertron) than in the untreated group.

2. No difference was observed in the calcification rhythm of the dentin of the lower incisors of the animals fed vitamin D as compared with the untreated group.

3. A significant increase in bone formation occurred during the period when the specific vitamin D preparation was fed as compared to the amount of apposition during the control period. No such increase occurred in the untreated animals.

4. The rate of tooth eruption was not altered significantly in the animals receiving vitamin D as compared with the untreated animals.

5. The skeletal calcification, as observed by comparing the radiographs, was not significantly different in the treated and untreated groups.

6. No pulp stones were seen (in radiographs) in any of the animals, even after the massive vitamin D administration. No appreciable change in pulp stone formation was detected (in radiographs) in 8 humans receiving approximately 300,000 units of vitamin D (Ertron) daily.

7. The daily administration of massive doses of Ertron did not significantly alter the estrus cycle of the rats during the experimental period.

8. Daily massive doses of vitamin D (Ertron) were administered without signs of toxicity.

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