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NICKEL POISONING

III. Procedures for Detection, Prevention, and Treatment of Nickel Carbonyl Exposure Including a Method for the Determination of Nickel in Biologic Materials

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This is the third paper in this series concerned with the toxicology of nickel carbonyl. The first described our studies in experimental animals;[11] the second dealt with our clinical observations.[17] In addition, more than 8 years of laboratory experience and 2 years of manufacturing plant operation have provided an opportunity for the evaluation of procedures and equipment intended for the prevention, detection, and treatment of nickel carbonyl exposure. These procedures and their usage are described in this paper.

Nickel carbonyl, $Ni(CO)_4$ is readily formed from carbon monoxide and metallic nickel or a nickel compound which can be reduced under the preparative conditions. It is a colorless liquid with a boiling point of 43 C. and a specific gravity of 1.31. The pure vapor decomposes spontaneously above room temperature but is stable at high temperatures if held in a pressure vessel in the presence of excess carbon monoxide. Decomposition is rapid under the influence of oxidizing agents and the dilute vapor in air is 50 per cent decomposed in a few minutes at room temperature. On the other hand, the reaction with water is slow. The liquid frequently ignites spontaneously on contact with air and spontaneous ignition also occurs with certain vapor-air mixtures at or above room temperature.[3]

Operations Involving the Use of Nickel Carbonyl

Hazards from exposure to nickel carbonyl may arise from a variety of operations in refineries, manufacturing plants, and research laboratories. For the isolation of pure nickel from its ores nickel carbonyl has been in use for more than a half century. In this application carbon monoxide is passed through nickel ore at atmospheric pressure. The formed nickel carbonyl vapor is subsequently heated to release metallic nickel and carbon monoxide.

A recent industrial development is the use of nickel carbonyl as an intermediate in organic syntheses.[15, 19] In this application large quantities of liquid nickel carbonyl and its solution may be handled—sometimes at high pressures.

Accidental exposure to nickel carbonyl may occur during industrial operations

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following its inadvertent formation. This is especially likely to take place if carbon monoxide comes in contact with an active form of nickel, such as the nickel catalysts used for hydrogenation.[14] Exposure under these circumstances is likely to be especially hazardous because the presence of nickel carbonyl may not be recognized.

The Acute Effects of Nickel Carbonyl Exposure

Nickel carbonyl is highly toxic by inhalation as judged by both clinical experience and experimental work with animals. Fourteen fatalities in human beings are known to have occurred[11, 17, 18] as well as hundreds of cases of nonfatal poisoning. The LD_{50} values for 30-minute exposure for mice, rats, and cats are 10, 35, and 270 parts per million (ppm) by volume, respectively.

The symptoms which follow exposure to nickel carbonyl are of 2 types, initial and delayed. The initial symptoms are generally mild and disappear quickly when the subject is brought into uncontaminated air. In some cases the patient may feel well for as long as several days after the initial symptoms disappear and before the onset of the more serious delayed reaction. In other cases the 2 stages may merge.[17] In any event the slowness of the development of symptoms is such that the victim will usually be aware of exposure only after the inhalation of a dosage sufficient to cause serious delayed effects.

The Chronic Effects of Nickel Carbonyl Exposure

In addition to the acute effects, nickel carbonyl is also suspected of causing a high incidence of carcinoma of the respiratory passages after long exposure to low concentrations.[5, 10, 12] Thus, Barnett[5] noted that from 1923 to 1948 inclusive, 49 cases of cancer of the nose with 46 fatalities and 82 cases of cancer of the lungs with 72 fatalities occurred among nickel workers in England. The average period of time worked before the development of cancer was 23 years for cancer of the nose and 25 years for cancer of the lung. While nickel carbonyl has not been positively incriminated as the responsible agent, we believe this to be highly probable.

PREVENTION OF EXPOSURE

Allowable Exposure Limits

The selection of rational measures for the prevention of exposure to a toxic agent is dependent upon reliable knowledge of the tolerance of man to acute, subacute, and chronic exposure to the material. In the case of nickel carbonyl, reliable values of the acute toxicity are available for mice, rats, and cats. The usage of these data to predict a tolerance limit for man is complicated by the great differences between the LD_{50} values for the different species. An estimate, based on size,[11] leads to a low estimate for the toxicity of nickel carbonyl for man. We have rejected this value and have selected, for safety guidance, a value approximately the same (30 ppm) as that found for rats (35 ppm for 30 minutes' exposure). Following common practice, it has been assumed that an exposure of one tenth this amount will not produce serious acute symptoms. The

selection of this tolerance limit (3 ppm for 30 minutes) is arbitrary, but is believed to be conservative.

For the usage of this exposure tolerance of 3 ppm for 30 minutes ($ct = 90$, where c is concentration in ppm and t is time in minutes), it is necessary to know the maximum value for t which leads to the same effect as an equivalent dosage given in a shorter time. It is also necessary to know if sensitization, for which allowance must be made, is likely to occur. The maximum value for t which can be regarded as a single acute exposure may be roughly estimated from the observed lapse of time before the appearance of the delayed symptoms. This delay ranges from a minimum of about 10 hours to a maximum of about 8 days and is typically 24 to 48 hours.[17] Thus it is probable that the choice of the 5-day, 40-hour work week as the single dosage interval is conservative. This choice, combined with the previously selected concentration × time product of 90, leads to a maximum average concentration, to avoid *acute* effects, of 0.04 parts per million (0.0003 mg. per liter) during the course of the normal 5-day, 40-hour work week.

Experiments with mice, rats, and cats[11] have shown that previous exposure does not increase sensitivity to nickel carbonyl for these species when multiple exposures are given over a period of several weeks. On the contrary, increased tolerance is observed to develop. It is, therefore, inferred that sensitivity, owing to previous exposure, is unlikely to occur.

It would be desirable to have a reliable estimate of the tolerance of man for exposure to low concentrations of nickel carbonyl over many years. Unfortunately, no information is available on the exposure thought to have produced nose and lung cancer among nickel refinery workers. We have therefore adopted the attitude that any detectable exposure is excessive and must be eliminated. In practice, our experience indicates that exposures too small to produce acute symptoms can be detected by diagnostic methods discussed later in this paper. The precautions taken to avoid chronic effects are thus more rigorous than those for the avoidance of acute symptoms.

Measurement of $Ni(CO)_4$ in Air

Obviously, the simplest method of determining the presence of a noxious material in the air is its detection by odor. However, nickel carbonyl, even in highly hazardous concentrations has only a mild and nonpenetrating odor, often described as "sooty." Thus its presence is likely to be unnoticed by those unfamiliar with it. For example, an accident in which more than 100 persons were exposed to nickel carbonyl occurred without any suspicion that a toxic agent was present until the workmen started becoming acutely ill.[14, 17] Some of these individuals were exposed for as long as 12 hours. In this accident, exposed persons were not familiar with the odor of nickel carbonyl. The ability to detect the presence of nickel carbonyl by odor generally improves substantially with experience. We have found that a few persons, having long experience in working with the material, can usually detect its presence through its odor. However, the odor of nickel carbonyl is readily masked. In addition, individuals vary greatly in their

ability to detect the substance, even with comparable experience; some are fairly precise, others are quite insensitive, while still others report smelling it when it is not present.

We believe that detection by odor has played, and can continue to play, a useful role in laboratory operations where its limitations are understood by the scientifically trained personnel. It has been found to be less useful in plant operations. The working rule in all cases is, "If you can smell it, leave!"

Experience in locations monitored by a continuous detector and experiments intended to determine odor sensitivity[7] have led to the conclusion that under the most favorable circumstances approximately 1 to 3 parts per million of nickel carbonyl can be detected by odor. Our estimate of the minimum concentration detectable by odor is approximately 4 times higher than that given by Armit.[2]

The concentration of nickel carbonyl in air may be determined by a chemical analysis for nickel. A convenient method which has been standardized for use in our laboratories is described in Appendix A. The method is capable of detecting concentrations of a few parts per billion of nickel carbonyl in air provided the sampling period is long (48 hours or more).

An instrumental method[5] in use for the detection and estimation of nickel carbonyl in air is based on the conversion of the vapor to a nickel halide by its reaction with bromine or chlorine vapor. The smoke formed is capable of scattering light. The intensity of the scattered light can be related to the concentration of the nickel carbonyl in the contaminated air. The method has a sensitivity of a fraction of 1 ppm, is almost free of interferences, and is reasonably precise. It has been adapted for the continuous, automatic monitoring of plant areas which may be contaminated. A simple, manual method based on the same principle is also available. It uses visual rather than electronic measurement of the scattered light.

The automatic recording detector plays an important role in our safety programs. In the plant, sampling lines are installed in the ventilating ducts leading from the suspected areas to the exhaust stack. This method of sampling is advantageous in that a representative sample is obtained and the travel time from the monitored area to the detector is short enough (less than 1 minute) to insure against decomposition in transit. The units, as now operated, have a full-scale reading of 9 ppm and an alarm point of 2 ppm. The detectors serve the important purpose of indicating immediately the release of nickel carbonyl in amounts which would be toxic for short exposure. Corrective measures can then be taken without delay. Primary dependence is not placed on these detectors for the prevention of low-level, long-continued exposure. Other means, discussed later, serve this purpose.

Spot detectors[8] may be used to obtain a rapid semiquantitative estimation of nickel carbonyl in the range from about 4 to 70 ppm. These detectors utilize a sample collected in an evacuated bottle containing a halogen. The smoke formed in the presence of nickel carbonyl is compared with a plastic standard of fixed turbidity. Reference to a calibration curve gives the concentration of nickel carbonyl present with an accuracy of about ±25 per cent. This type of detector is useful for sampling areas where the nickel carbonyl concentration is high.

It is also useful for checking the efficiency of decontamination of plant units undergoing repair and may be used for locating leaks in equipment containing nickel carbonyl. Where the accuracy and sensitivity are adequate, this method is superior, because of its speed and convenience, to the chemical method previously described. An experienced operator can carry out approximately 100 air analyses for nickel carbonyl by this method in an 8-hour day. The minimum elapsed time from collection of sample to the end of the determination is about 5 minutes.

Nickel carbonyl in air can also be estimated by virtue of its carbon monoxide content. Carbon monoxide, however, interferes directly with the measurement and this principle for determining nickel carbonyl is therefore not recommended.

ACUTE EXPOSURE

The symptoms immediately following exposure to nickel carbonyl include frontal headache, giddiness, tightness in chest, nausea, weakness, perspiration, cough, vomiting, and shortness of breath. The pulse-respiratory ratio may drop from 4.5 to 3.0 or even lower. Collapse rarely occurs during or immediately following exposure when nickel carbonyl is the sole toxic agent. However, since nickel carbonyl is frequently contaminated with carbon monoxide, early collapse from carbon monoxide poisoning may occur. The immediate treatment for persons exposed to nickel carbonyl is the same as for those exposed to carbon monoxide.[13]

If severe exposure to nickel carbonyl is known to have occurred, we believe that dimercaprol* therapy should be started promptly. If there is some uncertainty regarding the degree of exposure, dimercaprol therapy may be withheld until the severity of exposure can be estimated.

The amount of nickel lost in the urine has proved helpful in judging severity of exposure. The collection of urine is begun immediately after exposure is suspected and is continued for 8 hours. The composite specimen may be analyzed for nickel in accordance with the procedure given in Appendix B. While nickel analyses are in progress, it is important to keep the patient at rest and to treat him symptomatically.

If the concentration of nickel in urine collected during an 8-hour interval immediately after exposure is less than 25 μg. per 100 ml. the exposure may be classified as mild. In this case it would be anticipated that delayed symptoms will either not appear or be relatively mild. These patients are usually able to continue their work. However, should delayed symptoms develop unexpectedly, dimercaprol is given immediately. Clinical experience indicates that some benefit is obtained from dimercaprol therapy even if withheld for as much as 5 to 8 days after exposure.

When the nickel concentration is above 25 μg. but less than 50 μg. per 100 ml. of urine, mild delayed symptoms have been observed.

*Dimercaprol is available commercially as a 10 per cent solution in a mixture of peanut oil and benzyl benzoate. The dose for a person weighing 70 Kg. is 1.75 ml. of the 10 per cent solution, given intramuscularly. Dimercaprol may be obtained from Hynson, Wescott and Dunning, Inc., Baltimore, Maryland.

If the concentration of nickel in the urine exceeds 50 μg. per 100 ml. the delayed symptoms are likely to be severe and it is our present feeling that dimercaprol therapy should be started immediately as follows: Six doses of 2.5 mg. per Kg. of body weight respectively, given intramuscularly at 4-hour intervals. This schedule of dosage is repeated if the concentration of nickel in urine exceeds 25 μg. per 100 ml. for the 8-hour period beginning 24 hours after administering the last dose of the first series.

Single doses of dimercaprol larger than 2.5 mg. per Kg. of body weight should be avoided. Eagle and Magnuson[9] have found that only 1 per cent of patients will suffer a reaction from dimercaprol at this dosage. If the dosage is increased to 4 mg. per Kg. of body weight, it is estimated that one patient in 7 will have a reaction. If the dosage is increased to 5 mg. per Kg., toxic symptoms will develop in more than 50 per cent of subjects. These effects include sweating, burning sensation of the skin, lacrimation, salivation, tachycardia, dyspnoea, and a sense of constriction over the precordium. Symptoms of dimercaprol intoxication are likely to appear within a few minutes after injection. They reach their height within 10 to 30 minutes and then subside. Ephedrine may be used as an antidote for treating the symptoms resulting from administration of dimercaprol. It should be noted that dimercaprol is a powerful reducing agent and will produce an increased amount of reducing substances in the blood for several days after its administration. This has led on occasion to the erroneous diagnosis of diabetes mellitus.

In cases of very severe exposure, labored breathing may be observed within a few hours after exposure. When this occurs, pressure oxygen therapy may be required and the administration of dimercaprol may be started.

Recovery from acute exposure to nickel carbonyl is frequently protracted. Many patients complain of fatigue and have high pulse rates 2 months after exposure. Cyanosis and tenderness over the hepatic and splenic areas are often observed during convalescence.[17] These observations were made on patients who received dimercaprol starting the fifth day after exposure. No data are available for evaluating the effect of prompt dimercaprol therapy on this slow convalescence. The high pulse rates, cyanosis, and persistent fatigue are consistent with the assumption that loss of aerating tissue persists after the acute delayed symptoms have subsided.

Barnes and Denz[4] studied the changes occurring in the tissues of rats and rabbits following the inhalation of nickel carbonyl in lethal or near-lethal quantities. They observed that the normal thin alveolar wall in the lungs of rats was replaced by a thick, fibroblastic infiltration. This infiltration reached a maximum about 3 months after exposure and then slowly resolved. Barnes and Denz found essentially normal lung tissue in rats examined a year or more after exposure.

CHRONIC EXPOSURE

The prevention of chronic effects of exposure among nickel carbonyl workers is dependent upon operating procedures which will reduce exposure to a practical minimum. A method of detecting exposure to nickel carbonyl, in the absence

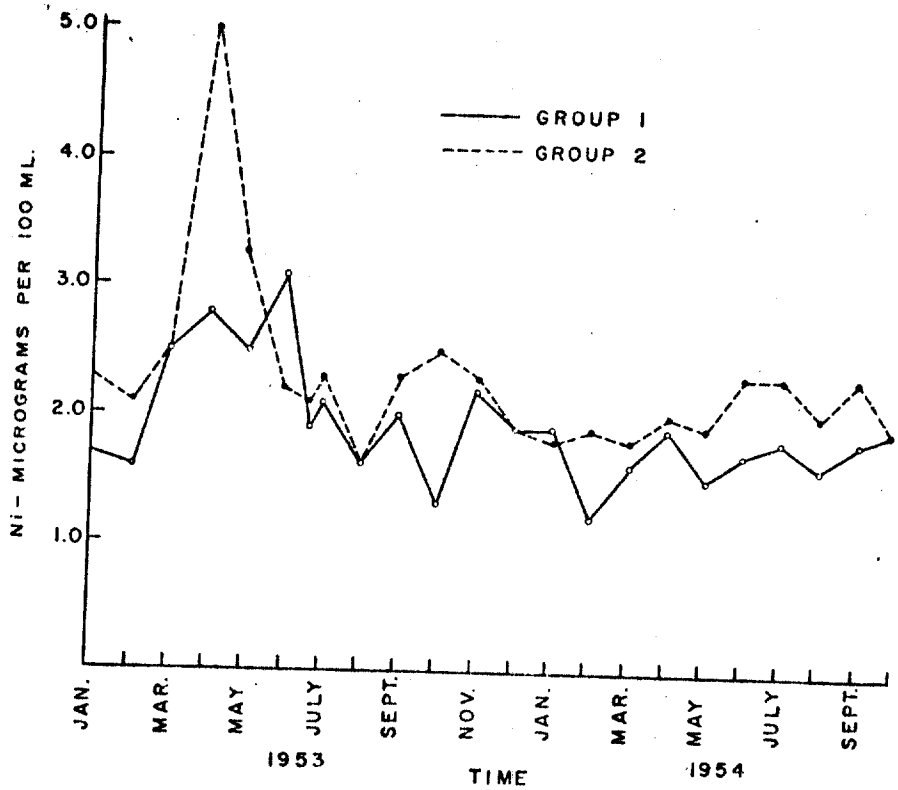

FIG. 1. Nickel in urine in nickel carbonyl workers

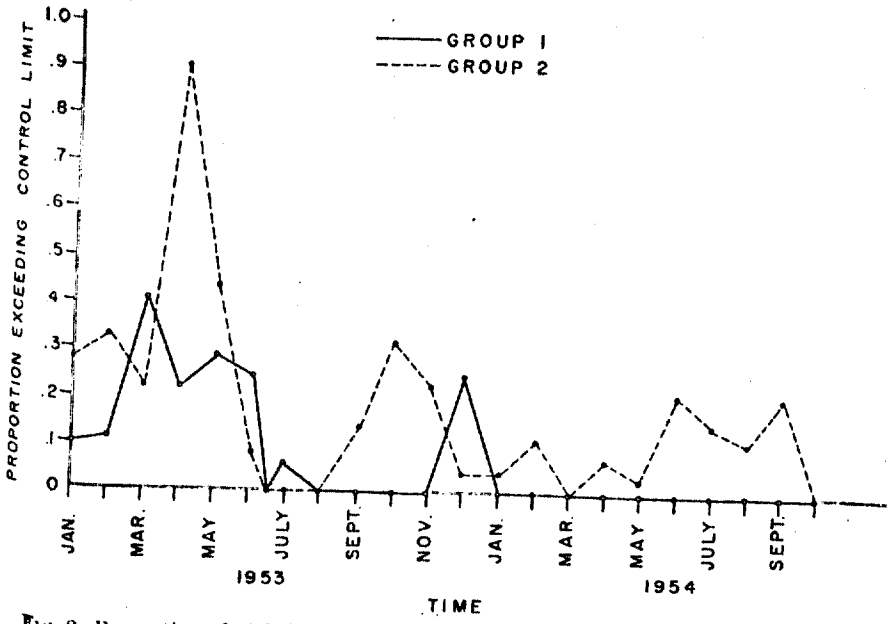

FIG. 2. Proportion of nickel carbonyl workers in which concentration of nickel in urine exceeded 3.0 μg. per 100 ml.

of acute symptoms, is needed. Such a method has been developed based upon the determination of the concentration of nickel in urine.

The analytical method used is essentially that of Alexander and co-workers.[1] Modifications have been devised for the measurement of nickel in blood and urine. Details of the procedure are given in Appendix B.

Analytical data for a group of 69 normal persons have yielded a normal value of 1.1 μg. per 100 ml. (S.D. ±0.9) for the concentration of nickel in urine. These data have led to the conclusion that only one specimen in 50 selected from a normal population will be found to exceed a value for nickel in urine of 3.0 μg. per 100 ml. This value has therefore been selected as the upper limit of normal.

Experience is available for a number of cases in which circumstances indicated that exposure to nickel carbonyl had occurred, but in which no acute symptoms developed. In these cases the concentration of nickel in a specimen of urine collected during an 8-hour period, starting shortly after exposure, was always found to be above 3.0 μg. per 100 ml. Several values fell in the range between 10 and 20 μg. per 100 ml. We therefore conclude that the presence of excess nickel in urine is a more sensitive criterion for exposure than is the appearance of acute symptoms.

Data are available for the concentrations of nickel in urine collected from 2 groups of individuals subject to nickel carbonyl exposure. Collections were obtained from workers at monthly or semimonthly intervals. Group 1 consisted of individuals for whom exposure was possible but rather unlikely. Group 2 consisted of individuals whose work required their presence in areas where exposure was always possible. The number of determinations per month for each group ranged from 6 to 30. The data are summarized in Figures 1 and 2. Figure 1 shows the average value for the concentration of nickel in urine for each group over the period from January 1953 through October 1954. Figure 2 shows the proportion of all measurements in which the value exceeds the control limit of 3.0 μg. per 100 ml. Determinations in which the circumstances suggested exposure have been eliminated.

It is clear from Figure 1 that the average concentration of nickel in urine for Group 2 persistently exceeds that for Group 1. Figure 2 shows the persistence with which the proportion of values exceeding the control limit is greater for Group 2 than for Group 1. A feature of the curves is the improvement which occurred in June 1953. Prior to that time work was permitted in potentially contaminated areas without the use of air masks, provided the detectors did not indicate the presence of nickel carbonyl and there was no evidence of leakage from the equipment. After this date the wearing of air masks was required at all times when workers were in suspected areas. The decrease in exposure, as indicated by nickel analyses, indicates the value of this precaution. The data for the period after the adoption of the improved safety practices suggest the possibility of low-level exposure for Group 2. However, it should be mentioned that these workers were in areas where solutions of nickel chloride were handled in large quantities. Absorption of nickel through the skin or gastrointestinal tract may be responsible for the observed difference between the 2 groups.

APPENDIX A, DETERMINATION OF NICKEL CARBONYL IN AIR

The chemical method of determining nickel carbonyl in air has a wide range of application because of the ease with which the size of the sample can be varied in accordance with the concentration of nickel carbonyl present. The method was developed for use at concentrations below those to which the automatic detector (mentioned in the text) will respond. It may also be used at higher concentrations.[6]

The sampling apparatus consists of an air pump which is used to collect the sample, a rotometer, and an absorber. A needle valve on the inlet of the pump may be adjusted to control the flow at 100 ml. per minute as measured on the rotometer. The sampling time will vary according to the concentration expected.

Procedure I, for samples from 0.5 to 10.0 mg. of $Ni(CO)_4$. Charge the absorber with 75 ml. of an alcoholic iodine solution (5 Gm. of iodine per liter of absolute ethanol). Connect the absorber, rotometer, and pump and adjust the needle valve so that air is pumped through the absorber at the rate of 100 ml. per minute. If the air to be sampled contains from 0.5 to 10 mg. of nickel carbonyl per liter, sample the air for 10 minutes (1-liter sample). Transfer the contents of the absorber to a 500-ml. volumetric flask. Use a 5-ml. portion of ethyl alcohol, followed by a 100-ml. portion of distilled water for rinsing the absorber. Collect all washings in the volumetric flask. Add 1 ml. of glacial acetic acid and mix. Simultaneously, prepare a blank containing 75 ml. of the alcoholic iodine solution, 5 ml. of ethyl alcohol, 100 ml. of distilled water, and 1 ml. of glacial acetic acid in a 500-ml. volumetric flask.

Treat the blank and sample as follows: Add 0.1 N sodium thiosulfate carefully while swirling the contents of the flask until the iodine color approaches a pale yellow. Then add the thiosulfate carefully drop by drop to disappearance of the yellow color. Add 10 ml. of concentrated ammonium hydroxide and mix well. Add 10 ml. of freshly prepared saturated bromine water. Add 2 ml. of a 2 per cent solution of dimethylglyoxime in 95 per cent ethanol. Dilute to the 500-ml. mark with distilled water. Make readings in a photoelectric colorimeter at 440 mμ after adjusting the instrument to zero by means of the blank. The concentrations are determined by reference to a previously constructed calibration chart.

Procedure II, for samples from 0 to 0.5 mg. of $Ni(CO)_4$. When low concentrations of nickel carbonyl are present, a sample may be obtained over a period of hours or days in the same manner described under *Procedure I*. Approximately 0.00001 mg. of $Ni(CO)_4$ per liter of air (2 parts per billion by volume) can be detected by sampling the air for 50 hours. This value has been derived from the known sensitivity of the method. Values approaching this limit have been actually observed.

After obtaining the sample, rinse the contents of the scrubber into an evaporating dish using ethyl alcohol and evaporate to dryness. Dissolve the black residue of nickel iodide in 10 ml. of distilled water and transfer to a 25-ml. volumetric flask. Rinse the dish twice with 5 ml. of water, adding the rinse water to the volumetric flask. A blank is prepared in like manner. To the blank and sample add the following 3 reagents, mixing between additions: (1) 10 drops of saturated bromine water, freshly prepared; (2) 1 drop of concentrated ammonium hydroxide; and (3) 5 drops of 2 per cent dimethylglyoxime. Bring the total volume to 25 ml. with distilled water. Determine the optical density in the same manner as described under *Procedure I*.

APPENDIX B, DETERMINATION OF NICKEL IN BIOLOGIC MATERIALS

This method for the estimation of nickel in biologic materials employs two reactions of nickel. After the sample is wet-ashed, nickel is separated from interfering elements by chloroform extraction of nickel dimethylglyoxime. This compound is extracted from chloroform with dilute hydrochloric acid. The nickel is converted to the diethyldithiocarbamate complex, and extracted with isoamyl alcohol. The nickel is determined in this solu-

tion by spectrophotometric measurement of the concentration of the yellow-green complex at 385 mμ.

In measurements of nickel in urine and blood for diagnostic purposes, it is our practice to run recovery tests with each group of unknowns. This is accomplished by adding 10 μg. of nickel to one or more specimens from each set. If a recovery of less than 8 or more than 12 μg. is obtained, the unknowns are re-analyzed. Any value for an unknown which exceeds the statistical control limit is rechecked.

This method has been successfully applied to the analysis of urine and blood samples, and to several organic samples of unknown composition. The sample size should be limited to an amount giving not more than 50 μg. of nickel.

Sampling and preservation of samples. Urine collections are deposited directly into scrupulously clean, 1-pint, wide-mouthed, screw-capped bottles. Toluol is added as preservative and the specimen is placed in a refrigerator.

Blood samples are discharged into test tubes containing 0.04 Gm. of potassium oxalate for each 20 ml. of blood. The tubes should be shaken vigorously and stored under refrigeration. Every precaution should be taken to avoid possible contamination from needles, sterilizers, and other equipment.*

Reagents

1. Dimethylglyoxime solution. Dissolve 0.25 Gm. C.P. dimethylglyoxime in 50 ml. of 95 per cent ethanol and dilute to 250 ml. with distilled water.

2. Sodium diethyldithiocarbamate solution. Dissolve 1 Gm. of C.P. sodium diethyldithiocarbamate in 100 ml. of distilled water. Filter and dilute to 500 ml.

3. Reagent A. Dissolve 200 Gm. of diammonium citrate in 600 ml. of water; adjust to pH 9.0 to 9.5 with ammonium hydroxide, and transfer to a 1-liter separatory funnel. Add 10 ml. of dimethylglyoxime solution and make 3 extractions with 30-ml. portions of chloroform. Filter the solution and dilute to 1 liter.

4. Reagent B. Dissolve 200 Gm. of diammonium citrate in 600 ml. of water, adjust to pH 9.0 to 9.5 with ammonium hydroxide, and transfer to a 1-liter separatory funnel. Add 10 ml. of sodium diethyldithiocarbamate solution and extract with 20-ml. portions of carbon tetrachloride until the carbon tetrachloride layer is colorless. Vigorous agitation is required. Add 5 ml. more of the reagent and again extract with carbon tetrachloride. If the solvent layer is colorless the extraction is complete; if it remains yellow, add more sodium diethyldithiocarbamate solution and continue the extraction. When the extraction is complete, filter the solution and dilute to 1 liter.

5. Hydrochloric acid, approximately 0.5 N. Dilute 40 ml. of hydrochloric acid (sp. gr. 1.18) to 1 liter with distilled water.

6. Nickel standard. Dissolve 0.5 Gm. of pure nickel in 20 ml. of nitric acid (1:1) and dilute to 1 liter. Prepare a working standard solution by diluting 10 ml. of this solution to 1 liter with 0.5 N hydrochloric acid. One ml. of the working standard solution contains 5 μg. of nickel.

7. Ammonium hydroxide, 1:50. Dilute 20 ml. of concentrated ammonium hydroxide to 1 liter with distilled water.

8. Ammonium hydroxide, 1:1. Dilute 500 ml. of concentrated ammonium hydroxide to 1 liter with distilled water.

9. Mixed acid solution. Mix 1 part of sulfuric acid (sp. gr. 1.84) with 1 part of perchloric acid (70 to 72 per cent) and add 3 parts of nitric acid (sp. gr. 1.42).

Preparation of samples. Transfer 100 ml. of urine or 10 ml. of whole blood to a 250-ml. erlenmeyer flask. Add to the sample in the flask 25 ml. of concentrated nitric acid, cover with a watch glass, and evaporate on a hot plate. Urine samples may be taken to complete dryness, but blood samples are removed when charring begins.

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* Nickel-free needles may be obtained from Becton, Dickinson and Co., Rutherford, New Jersey.

Cool the samples and add 25 ml. of the mixed acid solution. After the initial reaction subsides, digest on the hot plate until fumes of sulfur trioxide evolve. If the mixture darkens or chars at this point, add concentrated nitric acid dropwise until the sulfuric acid is refluxing freely with no further charring or darkening. Increase the heating until the volume is reduced to 2 to 3 ml. Cool the flasks and dilute with a little water. Bring the mixture to boiling, and transfer to a 100-ml. beaker and rinse flasks with a minimal volume of distilled water.

Isolation of nickel. To the prepared sample or aliquot add 10 ml. of Reagent A and dilute with distilled water to a volume of approximately 50 ml. Using a pH meter, adjust the pH of the solution to 8.5 with ammonium hydroxide. Transfer the solution to a 125-ml. separatory funnel and when cool, add 5 ml. of dimethylglyoxime solution. Shake for about 30 seconds and add 10 ml. of chloroform. After a few minutes standing, stopper securely and shake vigorously for 1 minute. After separation, draw off all but a few drops of the solvent layer into another 125-ml. separatory funnel containing 25 ml. of dilute ammonium hydroxide (1:50). Repeat the extraction with an additional 5 ml. of chloroform. If the chloroform layer from this extraction is colorless, draw off the solvent layer as completely as possible into the second funnel. If the solvent layer is still colored, continue the extractions as before with additional 5-ml. portions of chloroform, drawing off completely only the final colorless layer. Shake the combined solvent extracts and dilute ammonium hydroxide vigorously for 1 minute, allow the phases to separate cleanly, and draw off the aqueous layer through a pipet attached to a vacuum line. Wash down the sides of the funnel with distilled water, and again remove the aqueous layer as before.

Add to the solution 25 ml. of 0.5 N hydrochloric acid. Shake vigorously for 1 minute and allow the phases to separate. Draw off and discard the solvent layer. Add a few ml. of carbon tetrachloride, and shake briefly to extract any dissolved chloroform. Draw off the solvent as completely as possible, being careful to dislodge and remove the drops that float on the surface.

Color development. To the acid solution add 5 ml. of Reagent B and a small piece of red litmus paper. Add dilute ammonium hydroxide (1:1) until the solution is alkaline to litmus, followed by 10 ml. of isoamyl alcohol and 5 ml. of diethyldithiocarbamate solution. Stopper the funnel and shake vigorously for at least 2 minutes. After standing for 15 minutes, transfer the solvent layer to a 1-cm. cuvet and determine the optical density at a wavelength of 385 mμ. A reagent blank is prepared in the same manner. If the solutions become turbid due to the separation of dissolved water, they may be placed momentarily in hot water.

Calculations. The optical density readings of the samples are referred to the calibration curve to determine the concentration. If the optical density indicates more than 50 μg. of nickel, the analysis should be repeated taking a smaller aliquot.

Preparation of calibration curve. A calibration curve is prepared from appropriate dilutions of the working nickel standard with 0.5 N hydrochloric acid.

COMMENTS

Ashing. Dry-ashing procedures were found to give erratic results. This was attributed to sporadic contamination from the nichrome wire in the electric furnace. Metallic devices, such as forceps and tongs, should be used with extreme care, especially during the ashing step, to avoid contaminating the sample with nickel.

Extraction. It was not found necessary to oxidize the nickel with bromine prior to the dimethylglyoxime extraction, as reported by some investigators.[16]

The efficiency of extraction is almost independent of temperature through the range from 20 to 45 C., although slightly better results were obtained at the lower temperature. A temperature under 30 C. is recommended.

The recovery of nickel was found to be a function of pH. Recovery was low at pH 7.5 and 10.0; optimum results were obtained at pH 8.5.

The reaction between nickel and dimethylglyoxime was shown to be complete after only a few minutes' contact. However, dimethylglyoxime may be extracted by the chloroform and good mixing is essential before the extraction is carried out.

Color development and measurement. No effect of pH on color development was found through the range from 7.5 to 9.5.

The use of 5 instead of 10 ml. of isoamyl alcohol has been shown to give equally good precision and greater sensitivity.

The color developed was found to be stable at room temperature for at least 2 hours.

Chemicals in the quantities herewith listed were found to cause no interference with recovery of nickel: Na_2SO_4, 1.5 to 10 Gm.; Na_2SO_4, 6 Gm. + $NaNO_3$, 1 Gm.; $(NH_4)_2SO_4$, 7 Gm.; $(NH_4)_2SO_4$, 6 Gm. + $NaNO_3$, 1 Gm.; toluol, 1 Gm.; urotropin, 0.5 Gm.; dimercaprol (BAL), 0.5 Gm.; mercury, 0.1 Gm.; cobalt, 5 μg.; manganese, 5 μg.

SUMMARY

Procedures for the detection, prevention, and treatment of exposure to nickel carbonyl are described. Our studies led to the selection of a concentration of 0.04 parts per million by volume of nickel carbonyl vapor in air as the maximum allowable concentration for the avoidance of acute effects in man. For the avoidance of chronic effects, a lower value is favored.

Methods for the measurement of nickel carbonyl in air are described.

Methods are given for the estimation of nickel in biologic fluids. The use of these methods to detect and estimate the severity of exposure is illustrated. It is shown that abnormally high amounts of nickel appear in urine following exposures that are too small to cause acute effects.

The administration of dimercaprol is recommended for the treatment of acute poisoning from nickel carbonyl and a schedule of dosage is proposed. Criteria for the use of dimercaprol are discussed.

The minimum level of nickel carbonyl detectable by odor has been found to be 1 to 3 parts per million under the most favorable circumstances.

SUMMARIO IN INTERLINGUA

Es describite procedimentos pro le detection, prevention, e tractamento de exposition a carbonylo de nickel. Nostre studios duceva nos a stipular que un concentration de 0,04 per million (in volumines) de vapor de carbonylo de nickel in aere es le maximo permissibile pro evitar effectos acute in homines. Pro evitar effectos chronic, plus basse valores es a recommendar.

Es describite methodos pro mesurar carbonylo de nickel in aere.

Es presentate methodos pro estimar le contento de nickel in fluidos biologic. Nos illustra le empleo de ille methodos in deteger e estimar le severitate de exposition. Es demonstrate que anormalmente alte nivellos de nickel appare in le urina post expositiones non sufficientemente forte pro causar effectos acute

Le administration de dimercaprol es recommendate pro le tractamento de invenenamento acute per carbonylo de nickel. Un programma de dosages es proponite. Nos discute criterios pro le uso de dimercaprol.

Il esseva constatate que le concentration minimal de carbonylo de nickel detegibile per le odor es 1 a 3 per million sub le conditiones le plus favorabile.

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C.R. Acad. Sci. 186:181-183, 1928

Experimental Pathology—Effect of Nickel and Cobalt Salts on the Hypoglycemic Power of Insulin in Diabetics

By: Marcel Labbé H. Roubeau and F. Nepveux
Presented by: Gabriel Bertrand

In our preceding article, we have shown that the addition to insulin of a trace of nickel or cobalt salt intensifies and prolongs the hypoglycemic effect of insulin injected into the rabbit. Our results confirm those of Gabriel Bertrand and Macheboeuf.

We have wondered if the same kinds of facts could be observed in diabetics treated by insulin along with the salts of nickel or cobalt.

In our first series of experiments, we studied the effects of an isolated injection of nickel or cobalt chloride in isotonic solution on glycemia in diabetics. Our study carried out on diabetics with malnutrition and acidosis, based on series of measurements of glycemia in the hours following the injection showed that the level of blood sugar is not modified by nickel or cobalt salts.

The same injections given to normal subjects also caused no modification in their glycemia.

In a second series of experiments we have studied whether if in diabetics as in animals, the injection of metallic solutions influences the hypoglycemic power of insulin.

We were concerned with eight diabetics having malnutrition and acidosis and one diabetic without malnutrition. The doses of insulin administered were one-half of a clinical unit per kilogram of weight. We varied the doses of injected nickel and cobalt chloride: per kilogram bodyweight, the first series of patients received 0.1 mg of nickel chloride. The second series 1 mg of nickel chloride. The third series 1 mg of cobalt chloride.

We gave to our patients who couldn't maintain an absolute fast: at 7:30 a first meal composed of 150 g of milk and 120 g of brewed coffee. At 10 o'clock

(or 2 1/2 hours latter) we took the first blood sample, then they received the hyoglycemic injection (insulin + salt of Ni or Co) immediately followed by a uniform meal for all of 500 g of milk. Blood was then sampled each hour and the amount of sugar measured by Bang's micromethod. Samples were taken up to 16 hours (4 o'clock); then, we followed the evolution of the glycemia for 6 hours.

For each of the patients, it was possible to make two series of observations: the first with insulin, alone; the second with the same quantity of insulin added to the metallic solution. We then saw that for each patient the two glycemia curves with and without the nickel or cobalt salt were essentially superimposable (table). We could not verify the strengthening of the effect of insulin either in the intensity of the reaction or in its duration.

Conclusion

1. The injection of cobalt and nickel salts without insulin in healthy and diabetic humans was without hypoglycemic effects, a result conforming to those obtained by Gabriel Bertrand and Macboeuf with animals[1].

2. The changes brought on the effect of insulin in diabetic humans by the addition of nickel and cobalt salts does not agree with those observed in healthy rabbits and dogs. This divergence perhaps comes from the differences which exist between the metabolism of glucides in healthy animals and diabetic humans.

Comparative Table of Glycemia in Diabetics under the influence: 1-of insulin; 2-of insulin to which nickel or cobalt chloride was added.

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[1] Gabriel Bertrand and Macheboeuf, Compte Rendus, 182, 1926, p. 1305 and 1504; 183, 192 p. 5 and 25.

TABLE 1

5 Temps.	1 M. Max... : Diabète avec dénutrition.		2 M. Da... : Diabète avec dénutrition.		3 M. Mou... : Diabète avec dénutrition.		4 Mme Ari... : Diabète avec dénutrition.	
	20 unités d'insuline.	+0.006 Ni.	30 unités d'insuline.	+0,006 Ni.	30 unités d'insuline.	+0,006 Ni.	30 unités d'insuline.	+0,05 Ni.
h m								
10.00.....	3,23	3,20	1,98	2,27	2,80	2,99	3,00	3,28
10.15.....	6 Injection hypoglycémiante + repas type : 500cm³ de lait							
11.30.....	4,03	2,93	2,03	2,39	3,14	3,20	2,88	3,19
12.30.....	3,11	2,90	1,98	1,80	2,33	3,05	2,39	2,69
14.00.....	2,79	2,82	1,23	1,32	1,73	2,17	1,55	2,00
15.00.....	2,80	2,34	1,18	0,85	1,50	1,87	1,50	1,95
16.00.....	2,65	2,50	0,95	1,30	1,37	1,73	1,39	1,52
17.00.....								1,55

1-Mr. May. . .: diabetic with malnutrition. 20 units of insulin + 0.006 Ni

2-Mr. Da. . .: diabetic with malnutrition. 30 units of insulin + 0.006 Ni

3-Mr. Mou. . .: diabetic with malnutrition. 30 units of insulin + 0.006 Ni

4-Mrs. Ari. . .: diabetic with nalnutrition. 30 units of insulin + 0.05 Ni

5-Time

5 Temps.	7 Mlle Le... : Diabète avec dénutrition.		8 Mme Le F... : Diabète *sans* dénutrition.		9 M. Sq... : Diabète avec dénutrition.		10 M. Dem... : Diabète avec dénutrition.		11 M. Mé... : Diabète avec dénutrition.	
	20 unités d'insuline.	+0,04 Ni.	20 unités d'insuline.	+0,04 Ni.	30 unités d'insuline.	+0,01 Co.	20 unités d'insuline.	+0,06 Co.	20 unités d'insuline.	+0,05 Co.
h m										
10.00.....	2,21	2,75	1,47	1,57	2,51	2,52	2,08	1,85	2,54	1,90
10.15.....	6 Injection hypoglycémiante + repas type : 500cm³ de lait									
11.30.....	2,27	2,25	1,08	1,28	2,34	2,17	2,08	2,09	2,72	1,85
12.30.....	1,66	1,62	0,81	1,14	2,06	1,69	1,57	1,88	2,03	1,83
14.00.....	1,21	1,30	0,86	1,17	1,23	1,24	1,05	1,27	1,29	0,99
15.00.....	1,00	1,22	0,92	1,16	0,88	0,89	1,01	1,02	1,13	0,93
16.00.....	1,13	1,21	1,00	1,03	0,84	0,88	1,14	0,90	1,01	0,98
17.00.....		1,28								

6-Hypoglycemic injection + meal: 500 cm^2 milk

7-Miss Le. . .: diabetic with malnutrition. 20 units of insulin + 0.04 Ni

8-Mrs. Le F. . .: diabetic without nalnutrition 20 units of insulin + 0.04 Ni

9-Mr. Sq. . .: diabetic with malnutrition. 30 units of insulin + 0.04 Co

10-Mr. Dem. . .: diabetic with malnutrition. 20 units of insulin + 0.06 Co.

11-Mr. Me. . .: diabetic with malnutrition. 20 units of insulin + 0.05 Co.

399

C.R. Acad. Sci. 186: 181-183 (1928)

PATHOLOGIE EXPÉRIMENTALE. — *Action des sels de nickel et de cobalt sur le pouvoir hypoglycémiant de l'insuline chez le diabétique.* Note de MM. **Marcel Labbé**, **H. Roubeau** et **F. Nepveux**, présentée par M. Gabriel Bertrand.

Dans une Note précédente, nous avons montré que l'adjonction à l'insuline d'une trace d'un sel de nickel ou de cobalt intensifiait et prolongeait l'action hypoglycémiante de l'insuline injectée au lapin. Nos résultats confirment ceux de MM. Gabriel Bertrand et Machebœuf.

Nous nous sommes demandé si des faits du même ordre pouvaient être observés chez les diabétiques traités par l'insuline associée aux sels de nickel ou de cobalt.

Dans une première série d'expériences, nous avons recherché les effets de l'injection isolée de chlorure de nickel ou de cobalt en solution isotonique sur la glycémie des diabétiques. Notre étude, portant sur des diabétiques avec dénutrition et acidose, basée sur les dosages en série de la glycémie dans les heures qui suivent l'injection, montre que le taux du sucre sanguin n'est pas modifié par les sels de cobalt et de nickel.

Les mêmes injections faites à des sujets normaux n'apportent également aucune modification à leur glycémie.

Dans une seconde série d'expériences nous avons recherché si, chez le diabétique comme chez l'animal, l'injection de ces solutions métalliques influençait le pouvoir hypoglycémiant de l'insuline.

Nous nous sommes adressés à huit diabétiques avec dénutrition et acidose et à un diabétique sans dénutrition. Les doses d'insuline administrées ont été d'une demi-unité clinique par kilogramme de poids. Nous avons fait varier les doses de chlorure de nickel et de cobalt injectées : par kilogramme corporel, *la première série de malades reçut* $0^{mg},1$ *de chlorure de nickel. La deuxième série* 1^{mg} *de chlorure de nickel. La troisième série* 1^{mg} *de chlorure de cobalt.*

Nous donnions à nos malades que nous ne pouvions maintenir au jeûne absolu : à 7^h30^m, un premier déjeuner composé de 150^g de lait et de 120^g d'infusion de café. Nous faisions à 10^h (soit 2 heures 30 après) la première prise de sang, puis ils recevaient l'injection hypoglycémiante (insuline + sel de Ni ou Co) immédiatement suivie d'un repas uniforme pour tous de 500^g de lait. Le sang était ensuite prélevé d'heure en heure et le sucre dosé par

la microméthode de Bang. Les prises étaient faites jusqu'à 16h; nous avions ainsi pu suivre pendant 6 heures l'évolution de la glycémie.

Pour chacun des malades, il fut possible de faire deux séries d'observations : la première avec l'insuline seule; la seconde avec la même quantité d'insuline additionnée de la solution métallique. Nous avons vu ainsi que pour chaque malade les deux courbes de glycémie avec et sans sel de nickel ou de cobalt étaient sensiblement superposables (Tableau). Nous n'avons pas constaté de renforcement de l'action de l'insuline soit dans l'intensité de la réaction, soit dans sa durée.

Conclusion. — 1° L'injection des sels de cobalt et de nickel sans insuline a été, chez l'homme sain ou diabétique, sans effet hypoglycémiant, résultat conforme à ceux qu'avaient obtenus Gabriel Bertrand et Macheboeuf sur l'animal (1).

2° Les modifications apportées à l'action de l'insuline sur l'homme diabétique par l'addition de sels de nickel et de cobalt, ne concordent pas avec celles observées chez le lapin et le chien sains. Ces divergences tiennent peut-être aux dissemblances qui existent entre le métabolisme des glucides chez l'animal sain et chez l'homme diabétique.

TABLEAU COMPARATIF DES GLYCÉMIES DE DIABÉTIQUES

sous l'influence : 1° de l'insuline; 2° de l'insuline additionnée de chlorure de nickel ou de cobalt.

	1 M. Maz... : Diabète avec dénutrition.		2 M. Da... : Diabète avec dénutrition.		3 M. Mou... : Diabète avec dénutrition.		4 Mme Ari... : Diabète avec dénutrition.	
5 Temps.	20 unités d'insuline.	+0.006 Ni.	30 unités d'insuline.	+0.006 Ni.	30 unités d'insuline.	+0.006 Ni.	30 unités d'insuline.	+0.006 Ni.
h m								
10.00.....	3,23	3,20	1,98	2,27	2,80	2,99	3,00	3,28
10.15.....	6 Injection hypoglycémiante + repas type : 500cm3 de lait							
11.30.....	4,03	2,93	2,03	2,39	3,14	3,20	2,88	3,19
12.30.....	3,11	2,90	1,98	1,80	2,33	3,05	2,39	2,[illegible]
14.00.....	2,79	2,82	1,23	1,32	1,73	2,17	1,55	1,[illegible]
15.00.....	2,80	2,24	1,18	0,85	1,40	1,87	1,50	1,[illegible]
16.00.....	2,65	2,50	0,95	1,30	1,27	1,73	1,[illegible]	1,[illegible]
17.00.....								1,[illegible]

(1) GABRIEL BERTRAND et MACHEBŒUF, *Comptes rendus*, 182, 1926, p. 1305 et 1364; 183, 1926, p. 5 et 25.

TABLEAU COMPARATIF DES GLYCÉMIES DE DIABÉTIQUES (*suite*).

	Mlle Le... : Diabète avec dénutrition.		Mme Le F... : Diabète *sans* dénutrition.		M. Sq... : Diabète avec dénutrition.		M. Dem... : Diabète avec dénutrition.		M. Mé... : Diabète avec dénutrition.	
Temps.	20 unités d'insuline.	+0,04 Ni.	20 unités d'insuline.	+0,04 Ni.	30 unités d'insuline.	+0,04 Co.	20 unités d'insuline.	+0,06 Co.	20 unités d'insuline.	+0,05 Co.
h m										
[illegible]	2,21	2,75	1,47	1,57	2,51	2,52	2,08	1,85	2,54	1,90
[illegible]			Injection hypoglycémiante + repas type : 500cm³ de lait							
[illegible]	2,27	2,25	1,08	1,28	2,34	2,17	2,08	2,09	2,72	1,85
[illegible]	1,66	1,62	0,81	1,14	2,06	1,69	1,57	1,88	2,02	1,83
[illegible]	1,21	1,30	0,80	1,17	1,23	1,24	1,05	1,27	1,29	0,99
[illegible]	1,00	1,22	0,92	1,16	0,88	0,89	1,01	1,02	1,13	0,93
[illegible]	1,13	1,21	1,00	1,03	0,84	0,88	1,14	0,90	1,01	0,98
[illegible]		1,28								

CHIMIE PATHOLOGIQUE. — *Les altérations de la fonction rénale par hypochloruration.* Note de MM. **Léon Blum** et **P. Grabar**, présentée par M. Widal.

Quand un organisme est appauvri considérablement en chlore et que cette perte n'est pas réparée, des altérations profondes de la fonction rénale pouvant aller jusqu'à l'arrêt complet de la sécrétion urinaire, apparaissent.

L'ingestion de chlorure de sodium agit très rapidement sur ces troubles et les fait disparaître complètement s'ils n'ont pas persisté trop longtemps.

Nous avons pu observer ces faits chez des diabétiques graves arrivés à l'état de coma. Tirés de cette situation dangereuse par l'administration d'insuline, certains malades présentent une albuminurie accompagnée d'une azotémie que nous avons vu s'élever jusqu'à 5g.

La preuve que ces troubles sont déterminés par le manque de chlore, résulte du fait que la suppression de tout sel dans l'alimentation provoque cette altération rénale, qu'inversement l'apport de sel la fait disparaître rapidement.

Voici un exemple qui illustre ces suites de la carence de chlore. Un diabétique en état précomateux a une azotémie de 0g,45; après usage de fortes doses d'insuline, l'intoxication disparaît, et le lendemain l'azotémie tombe à 0g,40 d'urée par litre. Le malade est ensuite mis à un régime hypoazoté sans sel et reçoit en même temps de l'insuline. Progressivement l'azotémie augmente, et après 4 jours elle atteint le taux

39

Compt. Rend. Acad. Sci
186: 181-188

PATHOLOGIE EXPÉRIMENTALE. — *Action des sels de nickel et de cobalt sur le pouvoir hypoglycémiant de l'insuline chez le diabétique.* Note de MM. Marcel Labbé, H. Roubeau et F. Nepveux, présentée par M. Gabriel Bertrand.

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Nous nous sommes demandé si des faits du même ordre pouvaient être observés chez les diabétiques traités par l'insuline associée aux sels de nickel ou de cobalt.

Dans une première série d'expériences, nous avons recherché les effets de l'injection isolée de chlorure de nickel ou de cobalt en solution isotonique sur la glycémie des diabétiques. Notre étude, portant sur des diabétiques avec dénutrition et acidose, basée sur les dosages en série de la glycémie dans les heures qui suivent l'injection, montre que le taux du sucre sanguin n'est pas modifié par les sels de cobalt et de nickel.

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Dans une seconde série d'expériences nous avons recherché si, chez le diabétique comme chez l'animal, l'injection de ces solutions métalliques influençait le pouvoir hypoglycémiant de l'insuline.

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la microméthode de Bang. Les prises étaient faites jusqu'à 16h; nous avions ainsi pu suivre pendant 6 heures l'évolution de la glycémie.

Pour chacun des malades, il fut possible de faire deux séries d'observations : la première avec l'insuline seule; la seconde avec la même quantité d'insuline additionnée de la solution métallique. Nous avons vu ainsi que pour chaque malade les deux courbes de glycémie avec et sans sel de nickel ou de cobalt étaient sensiblement superposables (Tableau). Nous n'avons pas constaté de renforcement de l'action de l'insuline soit dans l'intensité de la réaction, soit dans sa durée.

Conclusion. — 1° L'injection des sels de cobalt et de nickel sans insuline a été, chez l'homme sain ou diabétique, sans effet hypoglycémiant, résultat conforme à ceux qu'avaient obtenus Gabriel Bertrand et Macheboeuf sur l'animal (1).

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TABLEAU COMPARATIF DES GLYCÉMIES DE DIABÉTIQUES
sous l'influence : 1° de l'insuline; 2° de l'insuline additionnée de chlorure de nickel ou de cobalt.

	M. Maz... : Diabète avec dénutrition.		M. Da... : Diabète avec dénutrition.		M. Mou... : Diabète avec dénutrition.		Mme Ar... : Diabète avec dénutrition.	
Temps. h m	20 unités d'insuline.	+0.006 Ni.	30 unités d'insuline.	+0.006 Ni.	30 unités d'insuline.	+0.03 Ni.	20 unités d'insuline.	+0,05 Co.
10.00.....	3,23	3,20	1,98	2,27	2,80	2,99	3,00	3,38
10.15.....	Injection hypoglycémiante + repas type : 500gr de lait.							
11.30.....	4,03	2,93	2,03	2,39	3,14	3,20	2,88	3,19
12.30.....	3,11	2,90	1,98	1,80	2,33	3,05	2,39	2,50
14.00.....	2,79	2,82	1,23	1,32	1,73	2,17	1,55	2,00
15.00.....	2,80	2,24	1,18	0,85	1,40	1,87	1,50	1,95
16.00.....	2,65	2,50	0,95	1,30	1,27	1,73	1,39	1,50
17.00.....								1,55

(1) GABRIEL BERTRAND et MACHEBOEUF, *Comptes rendus*, 182, 1926, p. 1305 et 1501; 183, 1926, p. 5 et 25.

TABLEAU COMPARATIF DES GLYCÉMIES DE DIABÉTIQUES (*suite*).

Temps.	Mme Le... : Diabète avec dénutrition. 20 unités d'insuline.	+0,04 Ni.	Mme Le F... : Diabète *sans* dénutrition. 20 unités d'insuline.	+0,04 Ni.	M. Sq... : Diabète avec dénutrition. 30 unités d'insuline.	+0,04 Co.	M. Dem... : Diabète avec dénutrition. 20 unités d'insuline.	+0,06 Co.	M. Mé... : Diabète avec dénutrition. 20 unités d'insuline.	+0,05 Co.
10.00	2,21	2,75	1,47	1,57	2,51	2,52	2,08	1,85	2,54	1,90
10.15			Injection hypoglycémiante + repas type : 500cm³ de lait							
11.30	3,27	2,25	1,08	1,28	2,34	2,17	2,08	2,09	2,72	1,85
12.30	1,66	1,62	0,81	1,14	2,06	1,69	1,57	1,88	2,02	1,83
14.00	1,21	1,30	0,80	1,17	1,23	1,24	1,05	1,27	1,29	0,99
15.00	1,00	1,22	0,92	1,16	0,88	0,89	1,01	1,02	1,13	0,93
16.00	1,13	1,21	1,00	1,03	0,84	0,88	1,14	0,90	1,01	0,98
17.00		1,28								

CHIMIE PATHOLOGIQUE. — *Les altérations de la fonction rénale par hypochloruration.* Note de MM. **Léon Blum** et **P. Grabar**, présentée par M. Widal.

Quand un organisme est appauvri considérablement en chlore et que cette perte n'est pas réparée, des altérations profondes de la fonction rénale pouvant aller jusqu'à l'arrêt complet de la sécrétion urinaire, apparaissent.

L'ingestion de chlorure de sodium agit très rapidement sur ces troubles et les fait disparaître complètement s'ils n'ont pas persisté trop longtemps.

Nous avons pu observer ces faits chez des diabétiques graves arrivés à l'état de coma. Tirés de cette situation dangereuse par l'administration d'insuline, certains malades présentent une albuminurie accompagnée d'une azotémie que nous avons vu s'élever jusqu'à 5g.

La preuve que ces troubles sont déterminés par le manque de chlore, résulte du fait que la suppression de tout sel dans l'alimentation provoque cette altération rénale, qu'inversement l'apport de sel la fait disparaître rapidement.

Voici un exemple qui illustre ces suites de la carence de chlore. Un diabétique en état précomateux a une azotémie de 0g,45; après usage de fortes doses d'insuline, l'intoxication disparaît, et le lendemain l'azotémie tombe à 0g,40 d'urée par litre. Le malade est ensuite mis à un régime hypoazoté sans sel et reçoit en même temps de l'insuline. Progressivement l'azotémie augmente, et après 4 jours elle atteint le taux

C.R. Acad. Sci. 185:1532-1533, 1927

402

Compt. Rend.

Influence of Nickel and Cobalt on the Hypoglycemic Effect of Insulin in Rabbits

By: Marcel Labbé, Roubeau and F. Nepreux, presented by: Gabriel Bertrand

Today the influence of minerals in trace amounts on the effect of enzymes is known. Gabriel Bertrand has already shown the important role of manganese in the enzymatic reactions of laccase[3]. This same author and Macheboeuf have noted the relative abundance of nickel and cobalt in the liver and especially in the pancreas of certain animals. They verified the even greater abundance of these metals in preparations of insulin and think that these metals, may play a role in the hormonal action of insulin[4]. To verify this hypothesis, they first experimented on rabbits[5], then on dogs, to find the effect of traces of cobalt and nickel on insulin hypoglycemia.

They arrived at the following conclusions:

A minute amount of cobalt or nickel enables the insulin to act more intensely and for a longer time.

Hypoglycemia attained a lower number and was manifested an hour later (4 hours instead of 3 hours).

We undertook these experiments on rabbits of weights averaging 2.40 kg, fasted for 24 hours, and we placed them under conditions so that the amount of injected insulin corresponded to a convulsive amount. The insulin

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[3] GABRIEL BERTRAND, Comptes rendus, 124, 1897, p. 1032 et 1355.

[4] G. BERTRAND et MACHEBOEUF, Comptes rendus, 182, 1926, p. 1305.

[5] G. BERTRAND et MACHEBOEUF, Comptes rendus, 182, 1926, p. 1504.

[6] G. BERTRAND et MACHEBOEUF, Comptes rendus, 183, 1926, p. 5.

employed was liquid Byla insulin. The blood sugar was measured by Bang's micromethod.

We report in the table below, the results obtained with insulin alone and with insulin to which traces of nickel and cobalt salts were added.

Rabbit B (2.40 kg)--3 clinical units of hypodermic insulin.

1 Glycémie à jeun	1.19.	1.33.	1.30.
2 Injection de	Ins. : 3 unités.	3 Ins. : 3 unités + sulf. de Co. 0mg,3.	4 Ins. : 3 unités + chlorure de Ni 0mg,3.
h m			
1. 0	0,84	1,09	0,82 (1)
1.30	0,66	0,99	0,68
2. 0	0,54	0,68	0,57
2.30	0,71	0,46	0,45 (2)
3. 0	0,77	0,44	0,43
3.45	0,94	0,545	0,54
5. 0	1,03	0,80	0,79
6. 0	1,06	0,99	1,01

1--Glycemia upon fasting
2--Injection of 3 units of insulin
3--Insulin: 3 units
+ Co sulfate 0.3 mg
4--Insulin: 3 units
+ Ni chloride 0.3 mg

Observations--(1) No convulsions with insulin alone. (2) Convulsions at 2 hours 30 minutes, 4 hours, 30 minutes.

In the table above, hypoglycemia from insulin alone shows a maximum lowering of 51.3 per 100 at the end of two hours.

With insulin with cobalt, maximum hypoglycemia is observed at the third hour and the lowering is 67/100.

With insulin with nickel, maximum hypoglycemia is observed at the third hour and the lowering is 66/100.

Conclusion--the nickel and cobalt salts clearly favored and prolonged insulin hypoglycemia.

C. R. Acad. Sci.
185: 1532-1533 (1927)

vant la méthode indiquée par Rogers (¹) et étudiée par Smith (²).

155ᵍ de dichlorosaligénol ont été mis en contact, dans 530ᶜᵐ³ d'acétone pure du bisulfite hydratée, avec 8ᵍ de glucose et 5ᵍ d'émulsine. La déviation est passée en quinze jours de +1°6′ à +40′, le ferment ayant été renouvelé une fois.

Cet essai et plusieurs autres semblables montrent que l'émulsine est capable d'exercer son action synthétisante sur un dérivé dihalogéné. Toutefois le glucoside formé n'a pu être isolé jusqu'à présent.

Je poursuis mes recherches sur cette série de glucosides halogénés dérivés du saligénol.

CHIMIE BIOLOGIQUE. — *Influence du nickel et du cobalt sur l'action hypoglycémiante de l'insuline chez le lapin.* Note de MM. **Marcel Labbé, Roubeau** et **F. Nepreux**, présentée par M. Gabriel Bertrand.

On connaît aujourd'hui l'influence des minéraux à l'état de traces sur l'action des diastases. Gabriel Bertrand avait déjà montré le rôle important du manganèse dans les réactions diastasiques de la laccase (³). Ce même auteur, et Macheboeuf, ont remarqué l'abondance relative de nickel et de cobalt dans le foie et surtout dans le pancréas de certains animaux. Ils constatèrent l'abondance encore plus grande de ces métaux dans les préparations d'insuline et pensèrent que ces métaux pouvaient jouer un rôle dans l'action hormonique de l'insuline (⁴). Pour vérifier cette hypothèse, ils ont expérimenté sur le lapin d'abord (⁵), sur le chien ensuite (⁶), l'action des traces de cobalt et de nickel sur l'hypoglycémie insulinienne.

Ils sont arrivés aux conclusions suivantes :

Une dose infime d'un sel de cobalt ou de nickel permet à l'insuline d'agir plus intensément et plus longtemps.

L'hypoglycémie atteint un chiffre plus bas et se manifeste une heure plus tard (4 heures au lieu de 3 heures).

(¹) *Inaugural Dissertation*, Göttingen, 1875.
(²) *Ber. d. Deutsch. chem. Ges.*, 11, 1875, p. 1225.
(³) Gabriel Bertrand, *Comptes rendus*, 124, 1897, p. 1032 et 1355.
(⁴) G. Bertrand et Macheboeuf, *Comptes rendus*, 182, 1926, p. 1305.
(⁵) G. Bertrand et Macheboeuf, *Comptes rendus*, 182, 1926, p. 1504.
(⁶) G. Bertrand et Macheboeuf, *Comptes rendus*, 183, 1926, p. 5.

Nous avons repris ces expériences sur des lapins d'un poids moyen de 2kg,400, à jeun depuis 24 heures et nous nous sommes placés dans des conditions telles que la dose d'insuline injectée corresponde à la dose convulsivante. L'insuline employée a été l'insuline liquide Byla. Le sucre du sang était dosé par la microméthode de Bang.

Nous rapportons, dans le tableau ci-dessous, les résultats obtenus avec l'insuline seule et avec l'insuline additionnée de traces de sels de nickel et de cobalt.

LAPIN B (2kg,400). — 3 unités cliniques insuline hypodermique.

Glycémie à jeun	1.19.	1.33.	1.30.
Injection de	Ins. : 3 unités.	Ins. : 3 unités + sulf. de Co. 0mg,3.	Ins. : 3 unités + chlorure de Ni. 0mg,3.
h m			
1. 0	0,84	1,09	0,82 (1)
1.30	0,66	0,90	0,68
2. 0	0,54	0,68	0,57
2.30	0,71	0,46	0,45 (2)
3. 0	0,77	0,44	0,43
3.45	0,94	0,54	0,54
5. 0	1,03	0,80	0,79
6. 0	1,06	0,99	1,01

Observations. — (1) Pas de convulsions avec insuline seule. — (2) Convulsions de 3h 30m à 4h 30m.

Dans le tableau ci-dessus, l'hypoglycémie à *l'insuline seule* présente un abaissement maximum de 51,3 pour 100 au bout de deux heures.

Avec l'insuline additionnée de cobalt, l'hypoglycémie maxima est observée à la troisième heure et l'abaissement est de 67 pour 100.

Avec l'insuline additionnée de nickel, l'hypoglycémie maxima est observée à la troisième heure et l'abaissement est de 66 pour 100.

Conclusion. — Les sels de nickel et de cobalt ont manifestement favorisé et prolongé l'hypoglycémie insulinienne.

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The Carcinogenicity of Intravenous Nickel Carbonyl in Rats[1]

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SUMMARY

The carcinogenicity of i.v. nickel carbonyl, $Ni(CO)_4$, was tested in Sprague-Dawley rats. In 72 rats (Group A) that survived a single 50% lethal dose injection of $Ni(CO)_4$ (2.2 mg nickel per 100 g), the incidence of malignant tumors (8.3%) was not significantly greater than the incidence in 47 rats in the control group (4.3%). In 121 rats (Group B) that survived six 5% lethal dose injections of $Ni(CO)_4$ (0.9 mg nickel per 100 g) at intervals of 2 or 4 weeks, the incidence of malignant tumors (15.7%) was significantly greater than in the controls ($p < 0.05$). The 19 malignant tumors in Group B included 6 undifferentiated sarcomas (lung, pleura, liver, pancreas, uterus, and abdominal wall), 3 fibrosarcomas (neck, pinna, and orbit), 3 carcinomas (liver, kidney, and breast), 1 s.c. hemangioendothelioma, 1 leukemia, and 5 lymphomas (lung). This study demonstrates that multiple parenteral injections of $Ni(CO)_4$ can induce diverse malignant tumors in varied organs and tissues of the rat.

INTRODUCTION

Nickel carbonyl is a toxic, volatile liquid which is an intermediate product in the Mond process for refining nickel matte and is used as a catalyst in the petroleum, plastic, and rubber industries. Nickel carbonyl has been implicated as a possible respiratory carcinogen in industrial atmospheres (4, 6) and in tobacco smoke (18). In rats that were exposed to inhalation of gaseous nickel carbonyl, Sunderman *et al.* (16, 17) found increased incidence of pulmonary carcinomas, and Sanina (15) observed increased incidence of malignant tumors of the uterus, ovaries, and mammary glands. The present study was performed in order to ascertain whether or not i.v. administration of liquid nickel carbonyl to rats results in increased incidence of malignant tumors. This investigation was stimulated by our findings that the acute pathological (9) and biochemical (12, 19, 26, 28) reactions that develop in rats after i.v. injections of nickel carbonyl are similar to those that occur following exposures to nickel carbonyl by inhalation.

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MATERIALS AND METHODS

The experimental animals were randomly bred albino rats of the Sprague-Dawley strain (Dublin Farms, Dublin, Ga.), maintained on Purina laboratory rat chow. The rats were 8 to 9 weeks old at the time of the initial injection. $Ni(CO)_4$ (Matheson Chemical Co., East Rutherford, N. J.) was administered by i.v. injection into a tail vein by means of a microsyringe, as previously described (10, 11). The purity of the $Ni(CO)_4$ was verified by gas chromatography on 3 different chromatographic columns, as described by Sunderman *et al.* (27). Group A consisted of 72 rats (26 males, 46 females) that survived a single injection of $Ni(CO)_4$ in LD_{50}[3] dosage [2.2 mg nickel per 100 g body weight, equivalent to 5 μl of $Ni(CO)_4$ per 100 g body weight]. Group B consisted of 121 rats (61 males, 60 females) that survived 6 injections of $Ni(CO)_4$ in LD_5 dosage [0.9 mg nickel per 100 g body weight, equivalent to 2 μl of $Ni(CO)_4$ per 100 g body weight] administered at intervals of 2 or 4 weeks. The control group consisted of 47 rats (15 males, 32 females) that received sham injections of 5 μl of 0.9% (w/v) NaCl solution. The rats either died spontaneously or were killed when they became so moribund that they could not move about in their cages and hence could not obtain food or water. The rats were autopsied and the tissues were examined by light microscopy.

RESULTS

As indicated in Table 1, the longevity of rats in the control group and in the 2 treated groups did not differ significantly. There were no significant differences between the incidences of benign tumors in the control group and in the 2 treated groups. In female rats, benign fibroadenomas of the breast were found in 13/46 (28%) of Group A, in 11/60 (18%) of Group B, and in 10/32 (31%) of the control group. In male rats, the only benign tumors were 2 small s.c. plexiform neuromas that were found in 2 rats of the control group. In the control group, the overall incidence of malignant tumors was 4.3%, which is consistent with published statistics for spontaneous malignant tumors in Sprague-Dawley rats (5, 29). There were no significant differences between the incidences of malignant tumors of male and female rats within the control group or within either of the treated groups. In rats of Group A that survived a single LD_{50} injection of nickel carbonyl, the overall incidence of malignant tumors was 8.3%, which was not significantly greater than that in the control

[3]The abbreviations used are: LD_{50}, 50% lethal dose; LD_5, 5% lethal dose.

Table 1

Incidence of malignant tumors in rats after i.v. nickel carbonyl

Sprague-Dawley rats in Group A survived 1 i.v. injection of $Ni(CO)_4$ at LD_{50} dosage (2.2 mg nickel per 100 g). Rats in Group B survived 6 i.v. injections of $Ni(CO)_4$ at LD_5 dosage (0.9 mg nickel per 100 g) at intervals of 2 or 4 weeks. Rats in the control group received sham injections of 5 μl of 0.9% (w/v) NaCl solution.

Experimental group	No. of rats in group	Median age at death	Total incidence of malignant tumors		Pulmonary lymphomas		All other malignant tumors	
			No.	%	No.	%	No.	%
A								
	26 (M)	22 (9–25)[a]	3	11.5	2	7.7	1[b]	3.8
	46 (F)	23 (6–37)	3	6.5	2	4.3	1[c]	2.2
	72 (M, F)	23 (6–37)	6	8.3	4	5.6	2	2.8
B								
	61 (M)	21 (5–30)	10	16.4	3	4.9	7[d]	11.5
	60 (F)	24 (8–36)	9	15.0	2	3.3	7[e]	11.7
	121 (M, F)	23 (5–36)	19	15.7[f]	5	4.1	14	11.6[g]
Control								
	15 (M)	24 (10–30)	0	0	0	0	0	0
	32 (F)	24 (6–33)	2	6.3	2	6.3	0	0
	47 (M, F)	24 (6–33)	2	4.3	2	4.3	0	0

[a] Numbers in parentheses, range.
[b] Fibrosarcoma (orbit).
[c] Cholangiocarcinoma (liver).
[d] Fibrosarcomas (orbit, pinna, neck); undifferentiated sarcoma (lung); hemangioendothelioma (s.c. tissue); undifferentiated leukemia; carcinoma (kidney).
[e] Undifferentiated sarcomas (pleura, liver, pancreas, uterus, abdominal wall); carcinomas (liver, breast).
[f] $p < 0.05$ *versus* control group (χ^2 test).
[g] $p < 0.02$ *versus* control group (χ^2 test).

group. In rats of Group B that received 6 injections of nickel carbonyl in LD_5 dosage, the overall incidence of malignant tumors was 15.7%, which was significantly greater than that in the control group ($p < 0.05$). There were no significant differences between the percentages of pulmonary lymphomas in the control group and in the 2 treated groups. However, there was a significant increase in the percentage of nonlymphomatous malignant tumors among rats of Group B ($p < 0.02$). The histological varieties and sites of origin of the nonlymphomatous malignant tumors that were found in this study are listed in the footnotes to Table 1. These tumors were all unequivocally malignant, and distant metastases were present in the majority of the tumor-bearing rats. The histological appearances of selected tumors are illustrated in Figs. 1 to 6.

Table 2

Resumé of acute biochemical effects of i.v. nickel carbonyl in Sprague-Dawley rats

$Ni(CO)_4$, 2.2 mg/100 g, i.v., 6 to 28 hr before sacrifice.

Experimental system[a]	% of control values	*p vs.* controls
Hepatic tryptophan pyrrolase after cortisone induction	72 ± 7[b]	<0.01
Hepatic benzpyrene hydroxylase after phenothiazine induction	45 ± 8	<0.01
Hepatic cytochrome P-450 after phenobarbital induction	48 ± 5	<0.01
Leucine-^{14}C uptake in hepatic microsomal proteins *in vivo*	82 ± 6	<0.01
^{14}C-Labeled orotic acid uptake in hepatic RNA *in vivo*	25 ± 2	<0.01
RNA polymerase activity in hepatic nuclei	40 ± 7	<0.01
RNA synthesis *in vitro* by hepatic chromatin-RNA polymerase complex	49 ± 6	<0.01
Template activity of hepatic DNA for *M. lysodeikticus* RNA polymerase	98 ± 6	N.S.

[a] References are cited in the text.
[b] Mean ± S.E.

DISCUSSION

In the present investigation, a significant increase in the incidence of malignant tumors was observed in rats that received 6 i.v. injections of nickel carbonyl. The malignant tumors included a variety of histological types, and developed in diverse organs and tissues. Thus, this study provides additional evidence of the carcinogenicity of nickel carbonyl in the rat. Previous investigations which have demonstrated the carcinogenicity of various nickel compounds in experimental animals have been reviewed by Sunderman (24). Nickel carcinogenesis has been documented in several strains of rats, as well as in mice, guinea pigs, hamsters, rabbits, and cats (24). In rats, the carcinogenic potency of nickel compounds appears to be inversely related to the solubilities of the compounds in aqueous media. Thus, the carcinogenic nickel compounds, which include nickel dust, nickel sulfide, nickel carbonate, nickel oxide, nickel carbonyl, and nickelocene, are all sparingly soluble in water at 37° (8, 14, 24). Soluble nickel salts, such as nickel chloride, nickel sulfate, and nickel ammonium sulfate, have not been found to be carcinogenic (8, 14). The biological characteristics of the malignant tumors that are induced in rats by administration of nickel

compounds have been summarized by Sunderman (22) and Maenza *et al.* (13).

The acute biochemical effects that have been reported to occur in hepatocytes of Sprague-Dawley rats following i.v. injection of nickel carbonyl in LD_{50} dosage are listed in Table 2. Nickel carbonyl inhibits enzyme induction in several experimental systems, including cortisone induction of tryptophan pyrrolase (20), phenothiazine induction of benzpyrene hydroxylase (19), and phenobarbital induction of cytochrome P-450 (21). There is mild inhibition of protein synthesis, as measured by *in vivo* incorporation of leucine-^{14}C into hepatic microsomal proteins (23), and there is marked inhibition of RNA synthesis, as measured by *in vivo* incorporation of ^{14}C-labeled orotic acid into hepatic RNA (2). The inhibition of RNA synthesis is attributable to nickel carbonyl inhibition of DNA-dependent RNA polymerase activity, as demonstrated *in vitro* with intact hepatic nuclei (25) and also with chromatin-RNA polymerase complex isolated from hepatic nuclei (3). Nickel carbonyl does not affect the template activity of hepatic DNA or chromatin for transcription *in vitro* by exogenous RNA polymerase from *Micrococcus lysodeikticus* (1). The finding that i.v. nickel carbonyl inhibits DNA-dependent RNA synthesis in rat hepatocytes furnishes a metabolic effect whereby nickel carbonyl can cause acute alterations of gene expression. A possible relationship to the mechanism of nickel carbonyl carcinogenesis is suggested by similarities between the acute biochemical alterations produced by nickel carbonyl and those produced by certain nonmetallic carcinogens, such as aflatoxin B_1 (7). Caution should be exercised in interpreting this analogy, however, since aflatoxin B_1 is a very potent liver carcinogen, whereas nickel carbonyl is a relatively weak liver carcinogen.

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Fig. 1. Malignant lymphoma of lung of a female rat of Group B. *A*, tumor surrounding a small pulmonary artery. × 100. *B*, pleomorphic round cells with prominent nuclei. × 250.

Fig. 2. Fibrosarcoma of orbit of a male rat of Group A. *A*, poorly differentiated primary tumor. × 250. *B*, pulmonary metastasis of the tumor, with infiltration of aveolar septae by neoplastic cells. × 100.

Fig. 3. Undifferentiated sarcoma of abdominal wall of a female rat of Group B. *A*, sarcoma cells surrounding degenerating skeletal muscle fibers. × 250. *B*, pleomorphic cells with hyperchromatic nuclei. × 400.

Fig. 4. Cholangiocarcinoma of liver of a female rat of Group A. *A*, tumor compressing fatty liver tissue (*left corner*). × 250. *B*, neoplastic tubular structures lined by polyhedral cells. × 400.

Fig. 5. Hemangioendothelioma of s.c. tissues of abdomen of a male rat in Group B. *A*, neoplastic endothelial cells lining vascular channels. × 100. *B*, prominent vascular spaces. × 100.

Fig. 6. Undifferentiated leukemia in a male rat of Group B. *A*, focus of small round cells with large hyperchromatic nuclei in the myocardium. × 450. *B*, focus of undifferentiated round cells in the liver. × 450.

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SYMPOSIUM: METAL-CATALYZED LIPID OXIDATION

presented at the ISF-AOCS World Congress, Chicago, Illinois

REINHARD MARCUSE, Program Chairman

Activation Analysis of Trace Elements in Lipids With Emphasis on Marine Oils[1]

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ABSTRACT

A short introduction to the activation analyses is given where some of the main features of the method, especially when applied on biological material, are described. The following trace elements are analyzed in marine and vegetable oils: arsenic, bromine, sodium, copper, manganese, zinc, nickel and iron. Both arsenic and bromine are present as organic compounds. The arsenic is removed in the alkali refining process. The distribution of trace elements in oils has been studied by use of autoradiography and γ-spectroscopy. The results indicate that this distribution is dependent on the phospholipid content in the oil. A high phospholipid content seems to give a more homogeneous distribution of inorganic trace elements.

[1]One of 28 papers presented at the Symposium, "Metal-Catalyzed Lipid Oxidation," ISF-AOCS World Congress, Chicago, September 1970.

INTRODUCTION

The neutron activation analysis combines both high sensitivity and good accuracy for many elements. Today, when high sensitivity is required, the activation is mostly performed by thermal neutrons from a nuclear reactor. The activity is here induced by a (n, γ) reaction. Neutrons are captured by stable isotopes and these are then converted to new isotopes of the same element. At the same time a γ-quant is simultaneously emitted. Some of these isotopes may be unstable, i.e. radioactive, and will disintegrate by emitting characteristic γ and β radiation. The induced activity is measured either by recording the β particles with a Geiger Muller counter or the characteristic γ photons with a multichannel γ-spectrometer. The latter is by far the most used.

The sensitivity of the method will depend on several factors. Among these are the neutron flux (n/cm^2 sec), the irradiation time and the activation cross section, the probability for the isotope to capture a neutron (σ_{act}). In practical analytical work, that is to say, among the elements reported analyzed by this method, the sensitivity has been

INDEX

517-522 ACTIVATION ANALYSIS OF TRACE ELEMENTS IN LIPIDS WITH EMPHASIS ON MARINE OILS, by G. Lunde

523-524 EFFECT OF HEME COMPOUNDS ON LIPID OXIDATION, by Y. Hirano and H.S. Olcott

525-526 COPPER(I) AND COPPER(II) COMPLEXES IN SOLUTION AND THE CRYSTALLINE STATE, by R. Osterberg and B. Sjoberg

527-531 METAL-CATALYZED OXIDATION IN THE PRESENCE OF WATER IN FOODS, by T.P. Labuza, M. Silver, M. Cohn, N.D. Heidelbaugh and M. Karel

532-538 CALCIUM REQUIREMENT FOR LIPOXYGENASE CATALYZED LINOLEATE OXIDATION, by R.B. Koch, B.L. Brumfiel and M.N. Brumfiel

539-543 CONTINUOUS REMOVAL OF METALLIC IONS FROM SOYBEAN OIL, by R.E. Beal and V.E. Sohns

544-546 TRACE METALS AND THE FLAVOR STABILITY OF MARGARINE, by W.G. Mertens, C.E. Swindells and B.F. Teasdale

547-549 LIPID OXIDATION IN MEAT AND MEAT PRODUCTS—A REVIEW, by J.D. Love and A.M. Pearson

550-555 CATALYSTS OF LIPID PEROXIDATION IN MEATS, by T.L. Kwoh

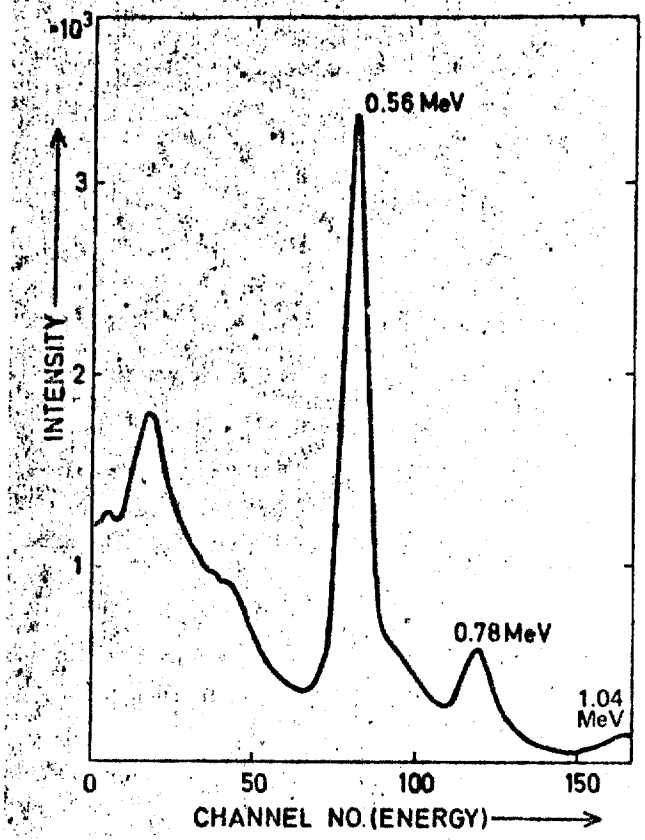

FIG. 1. Gamma-spectrum of neutron activated capelin oil recorded about one day after activation. The main peaks belong to As^{76} with 0.56 MeV and Br^{82} with 0.56 MeV, 0.78 MeV and 1.04 MeV.

from about 10^{-11} to about 10^{-4} g. In addition, the sensitivity will also depend on how the samples are prepared and how they are recorded. Many excellent review articles and books have been published both dealing with the theory for this analytical method and with practical applications (1-3).

In connection with the analysis of trace elements in oils, the following elements are well suited to be analyzed by this method: manganese, iodine, chlorine and nickel, with half lives from 25 min to 2 1/2 hr, copper, sodium, bromine and arsenic, with half lives from 12 to 36 hr, and zinc, cobalt, iron, chromium, with half lives from about 30 days to about 5 years. In addition phosphorus should also be mentioned. The radioactive phosphorus isotope P^{32} has a half life of about 14 days and emits only β-particles.

Some important features of this analytical method relevant to the analysis of oils should be pointed out in more detail.

1. The registration of the activity from an activated element is mostly performed by recording high energy γ-photons. This reduces matrix effects. As a result, it is not necessary to prepare the standards in the same way as the sample to avoid an uneven absorption of γ-photons in the standard and in the sample. Activities in biological materials may, for instance, be compared with standards dissolved in water.

2. Especially when trace elements in the sub-microgram area (1 ppb to 0.1 ppm) are analyzed, the risk of contamination during handling and analyzing the samples is considerable. For the activation analysis this is only a problem before the activation. After activation, the addition of impurities, even foreign sources of the elements to be analyzed, will be of no consequence for the results.

3. In many cases the matrix activity and also the activity induced in trace elements is negligible or does not interfere with the registration of the induced activity in the trace element to be analyzed. In these cases it is therefore possible to perform a nondestructive analysis of one or more elements. Examples of such matrixes are organic and biological materials. This type of nondestructive analysis is an interesting way to study the level of impurities in samples taken for instance from different steps during the processing of the oil, and also to study the relation between two or more impurities and how the different steps in the process may change this relation. This can be done by comparing γ-spectra of activated samples taken from the different steps.

If the γ-radiation (the γ-photons) from the induced activity in the element to be analyzed, is covered or interfered by γ-photons from other activities, it is necessary to carry out a radiochemical separation to purify the element enough to perform a registration with the γ-spectrometer. This is done usually by adding a carrier (milligram amount of the element to be analyzed). When the carrier has exchanged with the activated isotope, ordinary chemical separation methods are used to isolate this. Often only one or a few separation steps are necessary to get the sample pure enough to secure an adequate registration.

4. A distribution pattern of trace elements in flat sections (films, polished or flat surfaces, etc.) can sometimes be obtained by using autoradiographic methods. A uniform exposure of the film reveals a homogeneous distribution whereas areas with a higher exposure indicate an inhomogeneous distribution of inorganic impurities or of particles. Due to high specific activity in these areas the corresponding exposed areas on the film may be rather large and no conclusion can be drawn as to the real size of the area. Also a variation in the background exposure may be observed when the oil is not evenly distributed. A γ-spectrum of the oil will indicate the main radioactive isotope present. This technique is especially useful for solids but also for frozen liquids or liquids with high viscosity.

TABLE I

Nondestructive Activation Analyses of Trace Elements in Unprocessed and Processed Marine Oils

Sample	As (ppm)	Br (ppm)	Na (ppm)
Crude oil	8-14	7-10	~0.1
After alkaline treatment and washing	<0.2	7-10	~0.3
After bleaching	<0.2	6-10	~0.2
After hydrogenation and deodorization	<0.2	4-7	~0.1

TABLE II

Destructive Activatonalyses of Trace Elements in Hydrogenated Marine Oils

Sample		Cu (ppm)	Ni (ppm)	Fe (ppm)
No. 1	10 samples	0.004-0.04	0.03-0.14	<0.5-12
No. 2	6 samples	0.006-0.012	0.10-0.72	5 -12

GENERAL INFORMATION ON ACTIVATION ANALYSIS OF OILS

The main elementary constituents of oils (carbon, hydrogen, oxygen, and in raw oils also phosphorus) will not give any detectable γ-radiation (γ-peaks) when irradiated with thermal neutrons. The β-particles from phosphorus, P^{32}, will be recorded as bremsstrahlung on the γ-spectrometer. It is therefore possible to perform a nondestructive analysis of some of the trace elements present in the oils. Other trace elements, where the induced activities are low or where the γ-spectrum is complicated by γ-photons from

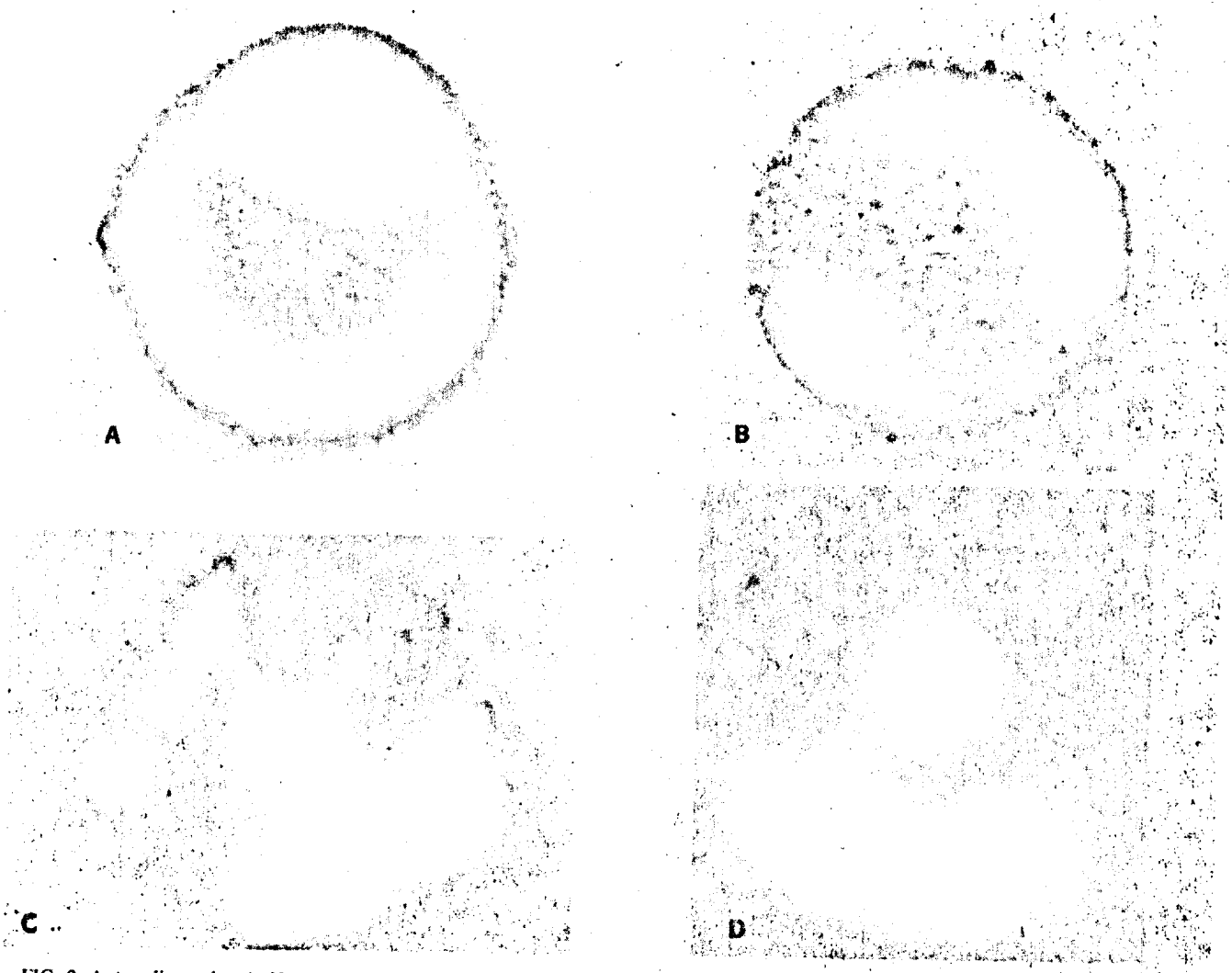

FIG. 2. Autoradiographs: A, Neutron activated refined fish oil; exposure time about 2 hr. B, Inactive fish oil where radioactive arsenic has been introduced from a water phase. C, Neutron activated refined soybean oil. The phospholipid content is low (25-50 ppm). D, Neutron activated refined peanut oil with a low phospholipid content (~25 ppm).

other activities, have to be separated by chemical methods before registration. This can be done either by wet or dry destruction of the oil or by different types of extraction methods. The extraction can be carried out by dissolving the oil after activation in a suitable solvent and extraction with water at an adjusted pH. The elements are then transferred into the water phase and subsequently separated by chemical methods. Saponification is an alternative method for bringing some of the trace elements into the water phase without destruction of the organic matter.

EXPERIMENTAL PROCEDURES

Materials

The oils analyzed in this investigation were either commercially available or produced in our laboratory.

Irradiation

About 1 ml of each oil was sealed off in quartz ampoules and was then ready for neutron activation. P.A. Chemicals (Merck, Darmstadt) of the elements to be analyzed (NaCl, NH_4Br, NaH_2PO_4, As_2O_3, $ZnCl_2$, $FeCl_3$, $MnCl_2$, $NicCl_2$ and $CuCl_2$) were sealed in the same kind of ampoules and irradiated together with the oil samples. Especially at irradiation times up to 24 hr, it is often advisable to dissolve the standard before sealing in the ampoules. The irradiation was performed with a neutron flux of $4 \cdot 10^{12}$ n/cm^2 sec in the nuclear reactor JEEP 2 Kjeller, Norway.

Separation and Registration

After activation the following procedures were used for preparing the different trace elements in the oils for registration. Both principles and detailed separation procedures for the elements to be analyzed have been discussed elsewhere (4) and only some of the main steps of the analysis are outlined here:

Saponification. During conventional saponification copper and nickel ammonium complexes were added as carrier. The carrier will exchange with the activated copper and nickel in the oil samples. After the saponification the nickel is precipitated with dimethylglyoxime and copper as copper sulfide.

Wet Destruction. The oils were treated with concentrated warm sulfuric acid, nitric acid and hydrogen peroxide. Carriers of the elements to be analyzed were added and conventional separation methods were applied after the destruction of the organic matter.

Extraction. The oils are dissolved in hexane and mixed with hydrochloric acid at a pH of about 2. Carriers of the elements to be analyzed are added to the water phase. The activated trace elements are extracted into the water phase and will exchange with the carriers; the different elements

TABLE III

Nondestructive Activation Analyses of Trace Elements in Vegetable Oils

Sample	(ppm)				
	Mn	Zn	Cu	Na	Br
Crude oils	a	<0.05-1.1	<0.005-1.0	0.03-5	<0.01-4
Refined oils	<0.005-0.1		<0.005-0.3	<0.01-2	<0.01-4

[a]not determined.

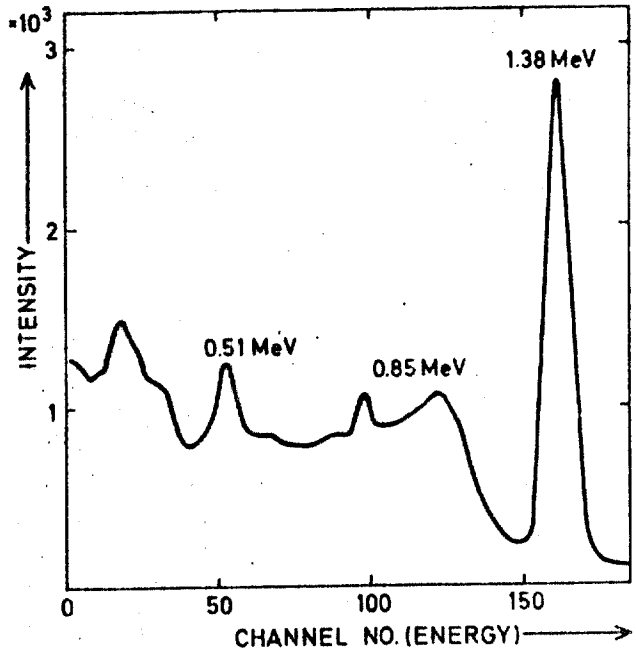

FIG. 3. Gamma spectrum of neutron activated linseed oil. The main peaks belong to Cu^{64} with 0.51 MeV, Mn^{56} with 0.85 MeV and Na^{24} with 1.38 MeV.

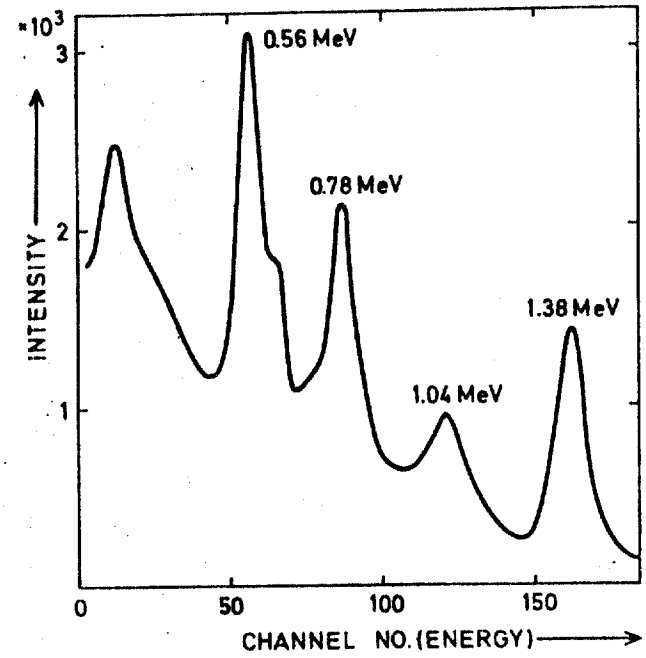

FIG. 4. Gamma spectrum of neutron activated coconut fat. The main photopeaks belong to Br^{82} with 0.56 MeV, 0.78 MeV, 1.04 MeV and Na^{24} with 1.38 MeV.

are then separated to the degree of purity that enable a recording with a γ-spectrometer.

Direct Registration. The oils are poured into inactive glass vials and are recorded directly with a γ-spectrometer.

Distribution Studies. The neutron activated oil is poured onto a thin plastic film and placed in contact with a photographic emulsion. An ordinary x-ray film can be used. After exposure the film is developed and handled as an ordinary x-ray film. The exposure is about 14 hr on a fine grain x-ray emulsion or 2 hr with a medium grained film. Gamma spectra of the oils are recorded after the exposure. The procedure should be repeated after a few days to see how the shortlived isotopes influence the distribution pattern on the film and correlate this with the γ-spectrum.

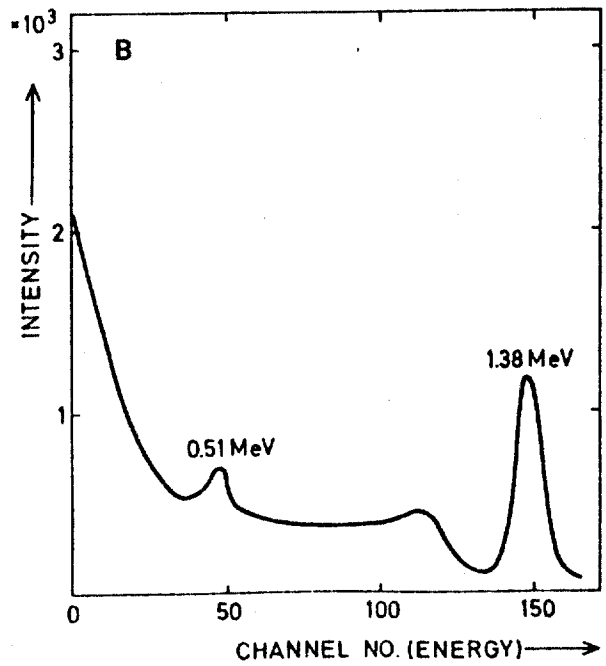

FIG. 5. Neutron activated linseed oil. A, Autoradiograph, exposure time about 2 hr. The background exposure is from P^{32} (phospholipid content ~2500 ppm). B, Gamma-spectrum recorded immediately after the exposure. Copper, Cu^{64} with 0.51 MeV and sodium Na^{24} with 1.38 MeV are the main γ-emitting isotopes. The bremsstrahlung from P^{32} is also recognized.

RESULTS AND DISCUSSION

Marine Oils

Crude oils manufactured from marine fishes (mackerel, herring, capelin and others) by conventional methods are relatively low in inorganics. Nondestructive analysis indicates that these oils contain usually less than 0.2 ppm sodium and less than 0.05 ppm copper. This is probably due to the way these oils are produced. The oil is in contact with relatively large amounts of water at 100 C followed by centrifugation. This insures an effective washing procedure and an efficient removal of the water from the oil.

Marine oils however contain some trace elements present as organic compounds (5,6). Among these arsenic and bromine have been studied in more detail. Figure 1 shows the γ-spectrum of capelin oil recorded about one day after activation and without any chemical treatment after the activation. The γ-spectrum consists mainly of the radioactive arsenic isotope As^{76} and the radioactive bromine isotope Br^{82}. When the oil is treated with alkali in the refining process the arsenic will follow the alkaline phase together with most of the phospholipids left in the oil. The bromine content will be reduced during the hydrogenation to about half of the original value. See Table I.

Due to the high content of bromine and arsenic a nondestructive analysis cannot be performed when the amount of inorganics in marine oils are low. In this case other methods have to be used to carry out an analysis. The contents of copper, nickel and iron have been analyzed in hydrogenated marine oils partly by the saponification and partly by the destruction procedure. Some of the results are shown in Table II. The two series of oil samples, No. 1 and No. 2 are from different productions.

The distribution of trace elements in a refined fish oil with a low phosphorus content is shown in Figure 2A. This oil however has a high background exposure and contains some inhomogeneities. The corresponding γ-spectrum indicates that sodium-24, arsenic-76 and bromine-82 are the main activities. As the sodium-24 with a half life of about 15 hr disappears, the inhomogeneities disappear. This indicates that the main radioactive isotopes left, bromine-82 and arsenic-76 with half-lives of 36 hr and 24 hr respectively, are homogeneously distributed and are therefore probably present as organic compounds soluble in the oil. These results were also confirmed when some experiments were carried out where radioactive arsenic was introduced into inactive oil from a water phase. Figure 2B shows an autoradiograph of radioactive arsenic introduced into a fish oil.

Vegetable Oils

There is no evidence so far that organic bound trace elements are present in vegetable oils. Nondestructive analysis of inorganics present in the oils with a high sensitivity for activation analysis is therefore possible. In vegetable oils one or more of the following trace elements can usually be analyzed without any chemical treatment: copper, manganese, zinc, sodium, chlorine, bromine and iron. Other trace elements have to be analyzed by radiochemical separation methods based on chemical treatment of the oils after the acvitation. Table III shows some results of nondestructive analysis of vegetable oils. Among the oils snalyzed are coconut fat, soybean oil, olive oil, peanut oil and linseed oil. Figure 3 and 4 show the γ-spectra of linseed oil and coconut fat. Copper, manganese and sodium are observed in the linseed oil whereas coconut fat contains mostly bromine and sodium. The content of inorganic ions in the different types of oils depends greatly on the possibility of contamination during the refining and hydrogenation steps and also on how the processed oils are stored afterwards. A simple washing procedure of the finished product may often reduce the level of impurities (parts per million) by a factor of 5-10.

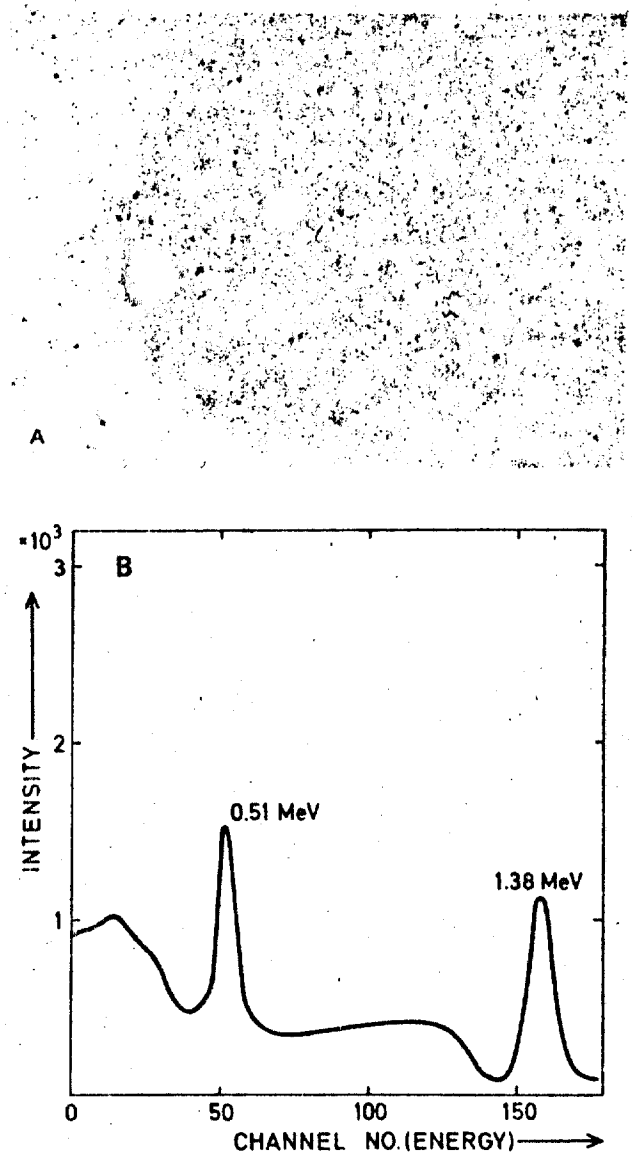

FIG. 6. Neutron activated olive oil. A, autoradiograph, exposure time about 2 hr (phospholipid content ~25 ppm). B, Gamma-spectrum recorded after the exposure. Cu^{64} and Na^{24} are the main radioactive isotopes present in the oil.

Figures 5 and 6 show two autoradiographs of activated linseed and olive oil together with corresponding γ-spectra. The linseed oil contained about 0.25% phospholipids and the olive oil about 0.003%. When the γ-spectra of the oils are compared with the corresponding autoradiographs it is evident that the inhomogeneous areas on the exposed film consist mainly of radioactive copper, sodium and bromine. The inhomogeneities on the autoradiographs disappear as the radioactive isotopes of copper, sodium and bromine disintegrate.

Although too few experiments have been performed, the results seem to indicate that oils relatively high in phospholipids have a more homogeneous distribution of trace elements than oils low in phospholipids. Figure 2C and 2D show two more autoradiographs of vegetable oils with a low phospholipid content. Oils with about the same amount of trace elements and with a high phospholipid content show an approximately homogeneous distribution of trace elements. These results are also confirmed by autoradiographing inactive oils with high and low phospholipid

content where radioactive tracers (Br^{82}, Cu^{64}, and Na^{24}) have been introduced from a water phase.

Besides the content of phospholipids, small amounts of water present in the oils may also be of importance in this connection, providing small areas with a higher water concentration where the trace elements probably can be concentrated.

When studying the effect of trace elements on the autoxidation of oils more emphasis should be concentrated on the distribution of these trace elements, and on how this distribution is influenced by the content of phospholipids and probably also of the content of small amounts of water in the oils.

ACKNOWLEDGMENT

The Central Institute for Industrial Research provided the facilities to perform and to publish this research.

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Compt. Rend. Soc. de biol.
98: 1169-170 (1928)

Effect of the Salts of Nickel and Cobalt on Insulin Hypoglycemia

by M.A. Magenta

Bertrand and his co-workers found that the pancreas is rich in nickel and cobalt, these substances going into insulin or into the blood at the same time as the insulin. This author pointed out some very interesting facts on this subject and we referred to his work for them.

In 1926, we did some experiments on dogs, the results of which coincided with those of Blatherwick and Sayhun (1927) obtained with rabbits. We used animals who were fasted for 24 hours; they received a half unit of insulin per kilogram by the subcutaneous route and nickel or cobalt nitrate or sulfate in the jugular vein, in amounts of 0.01 to 1 mg per kilogram. Glycemia (method of Folin and Wu) was determined before, then, 1, 2, 3, 4, 5, and 7 hours after the injections. Control dogs received separately either insulin or the metal salts. We used a total of 28 dogs.

The metal salts used alone slightly increased the glycemia. In the dogs who received insulin and the metal salts simultaneously, these exerted, in general, a slight preventive effect on hypoglycemia or none at all; they never increased the hypoglycemia producing effect of insulin. (Physiology Institute of the Faculty of Medicine).

461

C. R. Soc. de Biol
98: 169-170 (1928)

Action du fluorure de sodium sur la glycémie,

par M. A. Magenta.

Goldenberg (1*) a observé une glycosurie assez durable chez des agneaux et des chevreaux soumis à l'ingestion prolongée de fluorure de sodium. Nous avons dosé le sucre réducteur sanguin chez des Chiens non anesthésiés, injectés par voie jugulaire avec des doses de 0,5 mgr. à 500 mgr. de fluorure de sodium par kgr. de poids. La glycémie p. 1.000 fut déterminée dans le sang de la jugulaire par la méthode de Folin et Wu, avant, puis 30 min., 1 heure, 1 h. 1/2, 2 heures, 3 heures et 4 heures après l'injection. Avec 0,5 mgr., la glycémie n'augmenta que de 0,056 ; avec 1 mgr., de 0,07 ; avec 5 mgr., de 0,121 et 0,034 ; avec 10 mgr., de 0,306 et 0,100 ; avec 20 mgr., de 0,18. Avec 50 mgr., on observa des symptômes très violents : cris, tremblement, dyspnée, vomissements, abattement, etc. ; deux Chiens moururent après 1 heure et 1 h. 40 : la glycémie augmenta de 0,18-0,128-0,278-0,086. Avec 75 mgr. par kgr., la mort survint en 20 minutes, avec une augmentation de la glycémie de 0,262. Avec 100 mgr. par kgr., la mort survint en 10 minutes et la glycémie monta de 0,284. Avec 200 mgr. par kgr., la mort fut presque instantanée. On n'observa point de glycosurie chez 14 Chiens ; mais on trouva 2 p. 1.000 de glycose dans l'urine, chez un Chien ayant reçu 50 mgr. par kgr.

Chez le Chien, le fluorure de sodium, introduit par voie veineuse, n'a donc qu'une action hyperglycémiante très faible et ne provoque pas la glycosurie.

(*Institut de physiologie de la Faculté de médecine.*)

Action des sels de nickel et de cobalt sur l'hypoglycémie insulinique,

par M. A. Magenta.

Bertrand et ses collaborateurs ont trouvé que le pancréas est riche en nickel et en cobalt, ces corps passant dans l'insuline ou étant rejetés dans le sang en même temps qu'elle. Cet auteur signale à ce sujet des faits très intéressants pour lesquels nous renvoyons à ses travaux.

Nous avons fait en 1926, sur des Chiens, des expériences dont

(1*) 3e Congrès national de médecine, juillet 1926, t. VI, fasc. 2, p. 663.

les résultats coïncident avec ceux que Blatherwick et Sayhun (1927) ont obtenus sur des Lapins. Nous avons employé des animaux à jeun depuis 24 heures ; ils reçurent une demi-unité d'insuline par kgr., par voie sous-cutanée, et du sulfate ou du nitrate de nickel ou de cobalt, par la veine jugulaire, à la dose de 0,01 à 1 mgr. par kgr. La glycémie (méthode de Folin et Wu) fut déterminée avant, puis 1, 2, 3, 4, 5 et 7 heures après les injections. Des Chiens témoins reçurent séparément soit l'insuline, soit les sels métalliques. On employa au total 28 Chiens.

Les sels métalliques employés seuls ont augmenté légèrement la glycémie. Chez les Chiens qui ont reçu simultanément l'insuline et les sels métalliques, ceux-ci ont exercé, en général, sur l'hypoglycémie, une action empêchante légère ou nulle; ils n'ont jamais augmenté l'effet hypoglycéminant de l'insuline.

(Institut de physiologie de la Faculté de médecine.)

Entamoeba coli PARASITÉE PAR UNE *Sphaerita*,

par J. BACIGALUPO.

La *Sphaerita* est connue depuis longtemps comme parasite des Amibes et Protozoaires libres, mais chez les Protozoaires habitant l'intestin de l'Homme, sa découverte date de peu de temps, et sa présence a été constatée chez toutes les Amibes qu'on rencontre dans l'intestin (1). Nous avons observé ce parasite dans 5 cas : deux fois chez une forme végétative d'*Endolimax nana*, une fois chez *Entamoeba histolytica*, et deux fois chez *E. coli*.

La *Sphaerita* est formée par la réunion de petits corps sphériques qui n'atteignent pas 1 μ de diamètre et qui se colorent en noir par l'hématoxyline ferrique. Dans le cas que nous présentons, le nombre des Amibes parasitées a été à peu près de 10 p. 100. Un fait important que nous avons observé est la grosseur des Amibes parasitées. Elles mesuraient entre 32,5 et 37,5 μ, tandis que la dimension des Amibes non parasitées oscillait entre 18 et 25 μ. L'activité de ces Amibes n'était pas influencée par la présence des parasites, leurs pseudopodes avaient la même activité, leur forme et leur contenu, le parasite à part, était identique à ceux des individus non parasités. Dans aucun cas, nous n'avons trouvé de *Sphaerita* parasitant le noyau. A l'état frais, la *Sphaerita* s'observe très facilement, et il est impossible de la

(1) Cragg. *Indian Journ. of med. res.*, 1919. — Dabell. The amoebae living in Man, 1919. — Lwoff. *Bull. de la Soc. de pathol. exot.*, 1925; etc.

Bulletin of Environ. Contamination & Toxicology 5(3) 271-75 (1970)

The Heavy Metal Content of Fruit Juices and Carbonated Beverages by Atomic Absorption Spectrophotometry

by J. C. MERANGER
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Fruit juices and carbonated beverages are consumed on an average by Canadians at the rate of 80 g. per day per person, or approximately 5% of the total diet (1, 2, 3). It is well known that fruit juices contain traces of heavy metals such as manganese, cobalt, nickel, zinc, and copper (4, 5). Therefore, the heavy metal contribution of fruit juices and carbonated beverages to the total diet cannot be neglected.

Atomic absorption spectrophotometry is a rapid and accurate method for the determination of trace elements. Carbonated beverages can be analysed directly after removal of carbon dioxide by evaporation (6, 7). With fruit juices large particles must first be removed by centrifuging, and interference from sugar is compensated for by using the method of additions (8).

Experimental

Standard Solution: A multi-element standard solution of 9 metals was prepared to give the following concentration when diluted a hundred fold:

Cu 1.0 µg/ml, Zn 0.2 µg/ml, Ni 0.2 µg/ml, Cr 0.2 µg/ml, Mn 0.05 µg/ml, Pb 0.5 µg/ml, Cd 0.5 µg/ml, Co 0.2 µg/ml, and Sn 10.0 µg/ml.

Sample preparation for fruit juices: Fruit juices were centrifuged for 15 minutes at 4500 r.p.m. to obtain pulp-free liquid. Aliquots 0, 1, 2 and 3 ml of the multi-element standard solution were added to 100 ml volumetric flasks containing 3, 2, 1 and 0 ml of distilled water respectively and diluted to 100 ml with clear juice.

Sample preparation for carbonated beverages: Four 100 ml aliquots of beverages were pipetted into 250 ml beakers containing 0, 1, 2 and 3 ml of multi-element standard respectively. After heating on a hot plate until the volume was reduced to 75 ml the samples were cooled and diluted to 100 ml.

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Atomic absorption: A Perkin Elmer Model 303 atomic absorption spectrophotometer equipped with a boling burner and a null recorder read-out accessory was used. (The prepared samples were aspirated directly in the spectrophotometer). The operating parameters are given in (table 1).

TABLE 1

Operating Parameters

	Cd	Co	Cr	Cu	Ni	Pb	Zn	Mn	Sn
Wave-length Å	2288	2407	3579	3247	2320	2170	2138	2795	2246
Slit width, mm.	1	0.3	0.3	1	0.3	1	3	1	1
Source, ma.	6	30	20	20	30	12	15	20	25
Air flow	8	9.7	9	9.2	9	9	9.2	9	9
Acetylene flow	9	8.0	9	8	9	9	8	9	9
Gain	6.3	6.1	5.8	5.7	6.2	6.3	5.4	5.4	5.7
Scale expansion	3x	3x	3x	1x	3x	3x	1x	3x	1x

Results and Discussion

The heavy metal content of fruit juices has been the subject of a number of investigations. Levels of lead, cadmium, copper and zinc of <0.05 p.p.m., 0.05 p.p.m., 0.3 - 1.1 p.p.m. and 1.3 - 8.6 p.p.m. respectively have been reported by Westöö (9). Picasso de Micheli (10) found the average zinc content of canned sweetened juices to be 10.6 p.p.m. Values up to 2.1 p.p.m. for manganese and 150 p.p.m. for tin, have been found in citrus juices by Castro (11) and by Oda (12) respectively.

The heavy metal content of ten fruit juices (Table 2) is in general agreement with previously reported data with the exception of manganese in pineapple juice and lead in lemon and lime juice in which higher levels were detected. The presence of cadmium and cobalt was not detected in any of the fruit juices examined.

TABLE 2

The heavy metal content (p.p.m.)[a] of ten fruit juices

Type of juice	Cr p.p.m.	Cu p.p.m.	Ni p.p.m.	Pb p.p.m.	Mn p.p.m.	Zn p.p.m.	Sn p.p.m.
Grape	.08	<.1	<.02	<.08	.84	.38	<2
Prune	.15	.55	<.02	<.08	1.3	1.63	<2
Hawaiian Punch	<.01	<.1	<.02	<.08	1.3	27	<2
Pineapple	<.01	<.1	.28	<.08	16	.93	46
Apple	<.01	.26	<.02	<.08	.53	.17	<2
Orange	<.01	.25	.06	<.08	.45	.19	59
Grapefruit	<.01	<.1	.17	<.08	.18	.27	43
Lime	.24	.85	.40	.30	.26	.97	<2
Tangerine	<.01	.25	.08	<.08	.40	.56	114
Lemon	.05	.69	<.02	.57	.23	.93	<2

[a] In all fruit juices Cd <.01 p.p.m., Co <.03 p.p.m.

Less attention appears to have been given to the analysis of metals in carbonated beverages, although Larkin (13) has found up to .1 p.p.m. and 3.2 p.p.m. for lead and zinc respectively, and Sanz Pedrero (14) up to .65 p.p.m. for lead.

In the present survey, when possible, bottled and canned samples of the same brand were obtained and analysed. Lead, cobalt, copper, chromium, and cadmium could not be detected in any of the samples (Table 3). From these data it can be observed that the zinc content of canned beverages is much higher than that found in bottled samples, and in one cola sample was as high as 6.8 p.p.m.

TABLE 3

The heavy metal content (p.p.m.)[a] of twenty three carbonated beverages

Type of carbonated beverage	Type of container	Ni p.p.m.	Mn p.p.m.	Zn p.p.m.	Sn p.p.m.
Ginger ale	Bottle	<.02	<.01	<.02	<2
	Can	"	"	.08	8
Ginger ale	Bottle	"	"	<.02	<2
	Can	"	"	2.2	"
Lemon flavoured	Bottle	"	"	.07	"
	Can	"	"	3.3	"

TABLE 3 - Continued

Type of carbonated beverage	Type of container	Ni p.p.m.	Mn p.p.m.	Zn p.p.m.	Sn p.p.m.
Lemon flavoured	Bottle	<.02	<.01	.02	<2
	Can	"	"	.02	"
Lemon flavoured	Bottle	"	"	<.02	"
	Can	"	.03	1.9	"
Soda water	Bottle	"	<.01	<.02	"
	Can	"	"	2.0	"
Cola	Bottle	"	"	.05	"
	Can	"	"	.07	"
Cola	Bottle	"	"	<.02	"
	Can	"	"	.07	"
Root beer	Can	"	"	3.2	"
Grape	Can	"	.02	2.3	"
Orange	Can	"	<.01	2.7	"
Lemon flavoured Low Cal.	Bottle	"	"	<.02	"
Lemon flavoured Low Cal.	Can	"	"	5.5	"
Cola Low Cal.	Can	.07	.02	.15	"
Cola Low Cal.	Can	<.02	"	6.8	"

[a] In all carbonated beverages Cd <.01 p.p.m.
Cr <.01 p.p.m.
Cu <.1 p.p.m.
Co <.03 p.p.m.
Pb <.08 p.p.m.

The levels of heavy metals found in fruit juices are generally within the limits of the Canadian Food and Drug regulations. The relatively high concentration of lead in lemon and lime juice and manganese in pineapple juice indicate the need for a more detailed study of these juices. The carbonated beverages on the other hand are relatively heavy metal free except the zinc content of canned beverages and as such offer no specific problem areas.

The average percentage deviation of the calibration curve for 72 additions of each metal ranged from .8% for copper to 4.0% for lead. It is apparent therefore, that atomic absorption spectrophotometry is a reliable method for the direct determination of heavy metals in fruit juices and carbonated beverages.

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JOURNAL OF THE A.O.A.C. (Vol. 51, No. 4, 1968)

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Determination of Heavy Metals in Wines by Atomic Absorption Spectrophotometry

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The effect of alcohol and sugar concentration on the atomic absorption analysis of copper, zinc, nickel, chromium, lead, cadmium, and cobalt has been investigated. Sugar reduced the sensitivity of analysis but could be compensated for by using the method of additions. Interference from alcohol was avoided by introducing an evaporation stage, and the concentration of the above cations was determined by direct aspiration in 24 native and imported wines.

Atomic absorption spectrophotometry is a convenient and sensitive method for the analysis of trace metals in wines. Zeeman and Butler (1) used this technique to determine copper, lead, and zinc in ashed wines, and Strunk and Andreasen (2) recently reported a collaborative study in which copper in wines and spirits was determined by direct aspiration of the samples. It is known, however, that alcohol, as well as many other organic solvents, enhances the sensitivity of atomic absorption, largely by increasing the amount of solution reaching the flame (3), and that high results for metals have been reported when wines (4) and cider (5) were directly aspirated. Also, the presence of sugar in the wines increases their viscosity and so reduces the rate of uptake by the atomizer.

To determine the general applicability of the direct aspiration technique to the analysis of heavy metals in wines we have investigated the separate effects of alcohol and sugar concentration on the atomic absorption of copper, zinc, nickel, chromium, lead, cadmium, and cobalt. A simple method for the determination of these cations is described and applied to the analysis of a range of native and imported wines.

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.* — A Perkin-Elmer Model 303 atomic absorption spectrophotometer equipped with a Boling burner and a null recorder read-out accessory coupled to a Westronics Model S11A/U 11-inch strip-chart recorder was used. The operating parameters for the determination of the metal ions are given in Table 1.

(b) *Standard solutions.* — Stock solutions (1000 ppm) were prepared from high purity Cu, Zn, Ni, and Cd metal and from $K_2Cr_2O_7$, $Pb(NO_3)_2$, and $CoSO_4.7H_2O$. The metals were dissolved in concentrated acid and the salts in distilled water. In all cases the final acidity was adjusted to 1% (v/v) as follows: Cu, Cd, Pb, Ni in HNO_3; Zn and Cr in HCl; Co in H_2SO_4. The above solutions were further diluted to obtain a multi-element standard solution containing the seven metals with the following concentrations in μg/ml: Cu 1.0, Zn 0.2, Ni 0.2, Cr 0.2, Pb 0.5, Cd 0.05, and Co 0.2. The acidity of this standard solution was adjusted to 5% (v/v) with HNO_3.

Procedure

Four 50 ml aliquots from each wine were pipeted into 100 ml beakers. To three of the aliquots, 1, 2, and 3 ml, respectively, of the multi-element standard solution was added. The solutions were heated on a hot plate until the volume was reduced to 25 ml; after cooling, 15 ml distilled water was added and the volume was again reduced to 25 ml. When cool, the samples were diluted to 50 ml and aspirated directly in the spectrophotometer.

For the analyses in sugar and alcohol solutions, AR grade sucrose and absolute ethanol (USP) were used.

Results and Discussion

The sugar content of the wines surveyed (Table 2) varied from 0.5 to 16% (Liquor Control Board of Ontario Index). To determine the effect of sugar alone on atomic absorption analysis, 1 ml of the multi-element standard was added to solutions containing 1 to 20% sugar (total volume 100 ml). Low results (Table 3) were obtained for all the metals, as expected from the increased viscosity of the solutions. However, the presence of these concentrations of sugar did not affect

Table 1. Operating parameters for absorption spectrophotometer

Parameter	Cu	Zn	Ni	Cr	Pb	Cd	Co
Wavelength, Å	3247	2138	2320	3579	2170	2288	2407
Slit width, mm	1	3	0.3	0.3	1	1	0.3
Source, ma	20	15	30	20	12	6	30
Air flow	9.2	9.2	9	9	9	8	8
Fuel (acetylene) flow	8	8	9	9	9	9	9.7
Gain[a]	5.7	5.4	6.2	5.8	6.3	6.3	6.1

[a] The gain control changes the signal output of the photomultiplier detector for a given light input.

Table 2. Trace metal content (ppm) of wines[a]

Type	Country of Origin	Cu	Zn	Ni	Cr	Pb
Vermouth (dry)	France	0.28	0.09	0.08	0.02	0.19
Vermouth (dry)	Canada	1.87	0.40	0.08	0.03	0.12
Vermouth (sweet)	Italy	0.37	0 25	0.08	0.06	0.17
Vermouth (sweet)	Canada	0.73	0.67	0.17	0.03	0.17
White (dry)	Canada	0.46	1.00	0.08	0.02	0.14
White (dry)	France	0.15	0.22	0.08	0.02	0.19
White (dry)	Germany	0.19	0.20	0.05	0.04	0.13
White (dry)	Italy	1.14	0.87	0.06	0.04	0.23
White (dry)	Yugoslavia	0.16	0.07	0.05	0.03	0.16
Retsina	Greece	0.17	3.32	0.04	0.02	0.26
White (sweet)	Canada	0.30	0.77	0.12	0.02	0.09
White (sweet)	Canada	0.66	0.15	0.10	0.03	0.22
Red (dry)	Canada	0.28	0.17	0.05	0.02	0.19
Red (dry)	Canada	0.94	0.95	0.10	0.03	0.27
Red (dry)	France	1.04	0.99	0.06	0.05	0.19
Red (dry)	France	0.11	2.16	0.06	0.05	0.36
Red (dry)	Italy	0.10	1.91	0.04	0.01	0.22
Red (dry)	Spain	0.68	0.84	0.04	0.01	0.15
Red (sweet)	Canada	0.41	0.53	0.05	0.02	0.21
Rosé	Canada	1.10	0.32	0.09	0.01	0.22
Rosé	Canada	0.39	0.58	0.07	0.03	0.21
Rosé	Canada	0.16	0.50	0.10	0.02	<0.08
Rosé	France	0.70	0.37	0.06	0.06	0.22
Rosé	Portugal	0.28	0.91	0.09	0.03	0.13
Sensitivity (ppm/1% absorption)		0.09	0.02	—	—	—
Detection limit (ppm)		—	—	0.02	0.005	0.08

[a] In all wines Cd <0.01 ppm, Co <0.03 ppm.

Table 3. Percentage depression of metal absorbance produced by the addition of sucrose

Metal	ppm	Sucrose Found, %[a]									
		2	4	6	8	10	12	14	16	18	20
Cu	1.0	4	6	7	10	12	13	18	19	21	22
Zn	0.2	5	14	18	18	18	18	23	27	36	36
Ni	0.2	0	5	11	16	16	32	37	43	54	54
Cr	0.2	1	1	1.5	1.5	2.0	—	—	—	—	—
Pb	0.5	2	2	2	20	18	30	30	—	36	32
Cd	0.05	0	0	4	4	7	9	15	—	18	24
Co	0.2	5	15	19	19	29	24	29	38	38	48

[a] Numbers over each column of data represent the per cent sucrose added to the samples.

the background for any of the metals so that this reduction in sensitivity could be compensated for by using the method of additions.

When ethanol, at concentrations up to 50% (v/v), was added to 1 ml of the standard multi-element solution in a total volume of 100 ml, there was a pronounced baseline shift as well as the recognized enhancement in sensitivity. Hartlage (6) has recently reported a similar effect. Examples of the increasing and decreasing baseline shift ob-

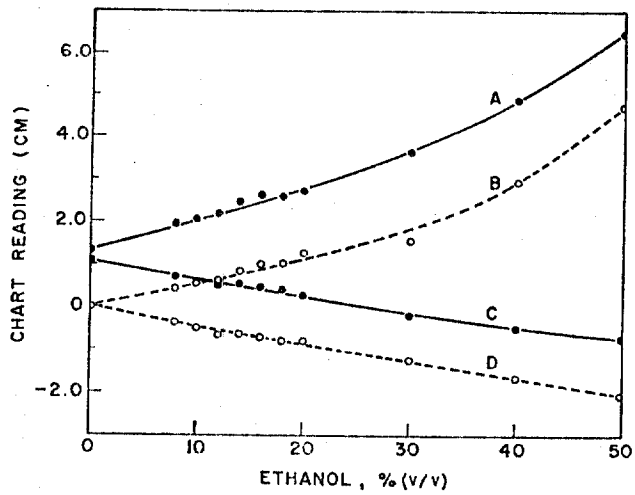

***Fig. 1*—Effect of ethanol concentration on the absorbance of cobalt and lead: A, 0.5 ppm lead; B, background; C, 0.2 ppm cobalt; D, background.**

tained with lead and cobalt, respectively, are shown in Fig. 1. Background alteration of this type will introduce errors into the analysis of metals in wines unless instrument settings for fuel ratio and sample uptake can be optimized with standards and blanks containing the same alcohol concentration as each specific wine. A simpler approach is to remove the alcohol by evaporation in a similar manner to the official procedure (7); we have adopted this technique for the analysis of wines. Of the metals tested, the baseline shift was negligible only for copper at alcohol concentrations below 50%; this explains why it has been possible to determine copper in wines and spirits by direct aspiration of the alcoholic solutions (2, 4).

The heavy metal content of a range of native and imported wines is given in Table 2. The analyses were calculated by the method of additions and it was noted that the slope of the working curve for a particular metal could vary by as much as 60% between the different wines, probably because of the difference in their sugar content. The average percentage deviation of the slope of the calibration curve for 72 additions for each metal was 1.9, 2.2, 1.7, 1.1, and 1.7% for nickel, zinc, lead, copper, and chromium, respectively. For the analysis of copper and iron in a number of wines, Slavin (4) reported that this slope was constant but our experience is that the slope must be determined separately for each wine. The levels of copper, zinc, and lead given in Table 2 are within the official limits of the Canada Food and Drugs Act and of the same order of those in the literature (1, 8). However, lead, which is presumed to be a contaminant (9), can even reach a concentration of 0.36 ppm. The nickel content of the wines examined was at least one hundred times that of wines recently analyzed by Eschnauer (10).

It is apparent that atomic absorption analysis of copper, zinc, nickel, chromium, lead, cadmium, and cobalt ions in wines can be carried out by a direct aspiration technique after alcohol has been removed by evaporation. Sugar, at concentrations up to 20%, does not affect the analysis.

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Effect of Nickel and Cobalt on the Development of *Aspergillus niger*

By: M. Mokragnatz
(Study presented at the meeting of December 16, 1930)

Nickel and cobalt have been found by G. Bertrand and myself first of all in all arable land (1) and then, in all plants (2) which we examined. Following these works, G. Bertrand (3) in collaboration with M. Macheboeuf, also found these metals in animal tissues subjected to analysis, then G. Bertrand (4) and Nokamura studied the effect of these metals on the development of mice; in these studies, they verified that these metals have a favorable effect in producing a prolongation of the life span of mice.

In the work which I now want to describe, I proposed to study the effect these metals exert on the development of *Aspergillus niger*.

This has been studied more or less accessorily by different authors. First of all, Richards (5) studied the effects of different metallic salts on this plant and among the metals were zinc, nickel and cobalt. He found in some cases increases sensitive to the weight of the plant. Then Boutkevitch (6) and Orloff studied the effect of zinc, mercury and cobalt on this mold. These authors found that the salts of cobalt and mercury exerted on cultures of *Aspergillus niger* stimulation of the development of the mycelium, similar to that exerted by zinc sulfate.

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[7] Biochem. Ztschr., 1927, 184, 370.

In 1927, Mrs. Anneliese Niethammer (7) studied the effect exerted on Aspergillus by a certain number of chemical compounds, mineral salts and organic substances considered as aseptic. Among these substances were the following mineral salts: $CuSO_4$, $AgNO_3$, $NiSO_4$, $Pb(NO_3)^2$, $Cr^2(SO^4)^3$, $ZnSO_4$ and a certain number of organic substances. This author studied the effect of these substances with the idea of determining whether the law of Arndt-Schultz, according to which each toxic substance must produce in a certain determined concentration, a favorable stimulating effect, had general application. In her work, A. Niethammer arrived at the conclusion that the substances envisaged may be divided into two categories; the substances which produce at specified concentrations a favorable stimulating effect, and the substances which produce only a negative effect. According to this author, among the latter is nickel.

These various authors only considered the metals studied as stimulants, in other words, as toxic substances which under certain conditions may eventually have a favorable influence on the increase of the weight of the plant. Thus, for example, A. Niethammer denies even for zinc a specific effect and considered it only as a common excitant, without; however, taking into account the numerous studies which have led through experimental facts to the consideration of zinc rather as an active physiological element.

In 1912, M. Javillier (1) tested the effect of a very large number of chemical elements on Aspergillus in order to determine whether zinc could be replaced as the catalytic element in the Aspergillus culture. Nickel and cobalt were among the metals. He arrived at the conclusion that none of the elements could be considered as able to replace zinc.

In summary, in all the works cited, the effect of nickel and of cobalt was only studied secondarily, without there being a special study of this problem.

Experimental Section

I proposed to take up this question and to study it in detail and with as much precision as possible, specifically the effect of nickel and cobalt on *Aspergillus niger*. To begin, I will describe in the present work, the result of my research concerning the effect of these two metals on the increase in weight of *Aspergillus* cultures.

The experiments were done in round glass dish of Iena, 12 centimeters in diameter. Each dish contained 150 cm^3 of Raulin's liquid but without zinc sulfate. The liquid was sterilized in an autoclave at 120° for a quarter of an hour. The exact composition of the nutritive solution was the following:

Water	1000 g
Sugar	46 g
Tartric acid	2.66 g
Potassium carbonate	0.40 g
Ammonium nitrate	2.66 g
Ammonium phosphate	0.40 g
Ammonium sulfate	0.155 g
Magnesium sulfate	0.71 g
Iron sulfate	0.46 g
Sodium silicate	0.046 g

The salts used were Merck *pro analysi* products. Only redistilled water under reduced pressure in a glass apparatus was used.

The solutions contained in the dishes were seeded with *Aspergillus* spores; the crystallizers were collected from watch-glasses which were lifted up with glass rods to allow the aeration of the cultures. Then the dishes were put into a thermostat with temperature maintained at 34°. The duration of each experiment was 4 days and the operation was done in series, each one including two or three controls and as many dishes containing nickel or cobalt in different concentrations. The numbers given in the following tables are the averages of two of three cultures.

After four days, the myceliums are removed, the lower surface is washed with distilled water, they are pressed and dried at 100° and weighed. In the present study, I have adopted the duration of four days because it was established in previous research done by different authors and in particular by M. Javillier, that Aspergillus cultivated on Raulin's liquid reaches a maximum weight after four days--at a temperature of 34°--and I wanted to see first of all what influence nickel and cobalt exerted under the same conditions.

I will first of all give the results obtained with nickel. The nickel salt used was the sulfate--pure, no cobalt, Merck brand. The experiments which I did with nickel included 20 series; I experimented with different concentrations. In the following tables, I give only the results of some series, the others having the same meaning.

Thus, one can see from the numbers shown in the preceding tables that nickel showed itself as favorable to the development of Aspergillus. The concentration from which this favorable effect begins to be clearly felt was $\frac{1}{50,000}$. From this concentration, the favorable effect increases progressively to reach the maximum at a concentration of $\frac{1}{15,000}$. When the concentration of the solution reached $\frac{1}{7,500}$ Ni, we begin to observe a significant decrease of the recovered weight. The concentration of $\frac{1}{3,000}$ almost completely prevents the formation of myceliums under the conditions of these experiments.

If we take the figure for the average weight collected obtained from solutions of concentration $\frac{1}{15,000}$ Ni--optimal concentration--and if this is compared to the figure for the average weights collected in the control liquids, it is discovered that 100 g of the control crop corresponds to 178 g of crop obtained in the presence of nickel in other words, the coefficient of utility was on the average, 1.78.

Table I. Effect of Ni on Growth of *Aspergillus niger*

	Dry weight recovered in grams
Control culture	0.670 g
Culture in presence of $\frac{1}{500,000}$ Ni	0.712 g
Culture in presence of $\frac{1}{100,000}$ Ni	0.776 g
Culture in presence of $\frac{1}{50,000}$ Ni	0.840 g
Culture in presence of $\frac{1}{25,000}$ Ni	0.926 g
Culture in presence of $\frac{1}{15,000}$ Ni	0.997 g
Culture in presence of $\frac{1}{10,000}$ Ni	0.936 g

Coefficient of utility (for the optimal concentration): 1.43

Table II. Effect of Ni on Growth of *Aspergillus niger*

Control culture	0.410 g
Culture in the presence of $\frac{1}{50,000}$ Ni	0.610 g
Culture in the presence of $\frac{1}{20,000}$ Ni	0.687 g
Culture in the presence of $\frac{1}{15,000}$ Ni	0.830 g

Coefficient of utility (for the optimal concentration): 2.02

Table III. Effect of Ni on Growth of *Aspergillus niger*

Control culture	0.700 g
Culture in the presence of $\frac{1}{150,000}$ Ni	0.825 g
Culture in the presence of $\frac{1}{15,000}$ Ni	1.497 g
Culture in the presence of $\frac{1}{3,000}$ Ni	0.08 g

Coefficient of utility (for the optimal concentration): 2.13.

Table IV. Effect of Ni on Growth of *Aspergillus niger*

Control culture	0.77 g
Culture in the presence of $\frac{1}{15,000,000}$ Ni	0.74 g
Culture in the presence of $\frac{1}{5,000,000}$ Ni	0.78 g
Culture in the presence of $\frac{1}{1,500,000}$ Ni	0.74 g
Culture in the presence of $\frac{1}{500,000}$ Ni	0.826 g
Culture in the presence of $\frac{1}{150,000}$ Ni	0.837 g
Culture in the presence of $\frac{1}{50,000}$ Ni	0.860 g

Table V. Effect of Ni on Growth of *Aspergillus niger*

Control culture	0.445 g
Culture in the presence of $\frac{1}{500,000}$ Ni	0.457 g
Culture in the presence of $\frac{1}{25,000}$ Ni	0.530 g
Culture in the presence of $\frac{1}{15,000}$ Ni	0.670 g
Culture in the presence of $\frac{1}{10,000}$ Ni	0.607 g
Culture in the presence of $\frac{1}{7,500}$ Ni	0.40 g
Culture in the presence of $\frac{1}{3,000}$ Ni	mycelium were formed only at a few points

Coefficient of utility (for the optimal concentration): 1.50

I now go on to experiments done with cobalt. The experimental conditions were the same as with nickel. The cobalt salt used was sulfate, with no nickel. The experiments were done on 12 series of cultures. In the following tables, I only give a certain number of results, the others having the same meaning.

(Tables I, II, III on pages 66 and 67 deal only with cobalt and are not translated).

The results shown above indicate that under the conditions in which these experiments were done, no appreciable increase in the weight of the cultures obtained in the presence of different quantities of cobalt could be verified. By contrast, the clearly injurious effect of this metal began to make itself felt at a concentration of $\frac{1}{250,000}$.

In comparing the results of the cultures obtained in the presence of nickel with those obtained in the presence of cobalt, it can further be seen that the injurious effect of cobalt slowly progresses from the concentration of $\frac{1}{250,000}$, and it is only at the concentration of $\frac{1}{2,500}$ that cobalt completely prevents the formation of mycelium--at least in the delay of the duration of our experiments--while nickel whose favorable effect begins to be felt from a concentration of $\frac{1}{500,000}$, to arrive at a maximum at the concentration of $\frac{1}{15,000}$, becomes clearly harmful from a concentration of $\frac{1}{7,500}$ and that of $\frac{1}{3,000}$ almost completely prevents the formation of mycelium.

All the related experiments up to now were done with Raulin's solution, slightly modified and containing no zinc.

The experiments which I will now briefly describe were done with the idea of comparing the effects of nickel and cobalt with that of zinc. These experiments were done under the same conditions as described for the preceding experiments; only each series further included three dishes with nutritive solution containing $\frac{1}{100,000}$ of zinc. The experiments were done on 10 series. In the following table, certain of the results are outlined. Nickel and cobalt were introduced in the nutritive milieux in quantities, which according to the preceding results, may be considered optimal quantities.

1--Concentration in the solution of Ni, Co, or Zn
2--Dry weight gathered in grams
3--Coefficient of utility
4--Average of coefficients of utility
5--Control culture
6--Culture in the presence of Ni
7--Culture in the presence of Co
8--Culture in the presence of Zn

As can be seen, the results obtained in these experiments were overall of the same meaning as those previously related, in other words, nickel showed itself to be clearly favorable while for cobalt, an identical effect could not be verified. Concerning zinc, these results only confirm already old experiments and particularly those of M. Javillier, showing that the effect of zinc on Aspergillus is not replaceable by any other metal.

From these weight differences, appreciable differences were also confirmed in comparing the appearance of cultures obtained on milieux respectively containing nickel, cobalt, and zinc.

The myceliums obtained on the nutritive liquid contained none of the three metals, were thin and showed none of the folds or undulations which are found with cultures on complete Raulin's liquid, in other words, also containing zinc. The spore production was also very advanced: from the third day the whole surface of the mycelium was covered with black cones. The mycelium was grayish-white. With cultures produced on milieux containing cobalt, the mycelium has a similar appearance with this difference that when the concentration of cobalt reaches $\frac{1}{100,000}$, the mycelium becomes brownish-yellow. The coloration becomes more intense with the increase of the quantity of cobalt put at the disposition of the plant.
quantities of nickel are purer white, and the lower part of the mycelium is more or less folded and wavy.

It may be wondered now to what degree the plant fixes the metals which favor its development.

To clarify this point, I did a series of research studies on this subject. Given that with cobalt, I couldn't verify a consistent and clear favorable effect on the development of the mycelium, for the time being I only did research concerning the fixation of nickel by Aspergillus.

Nickel was determined in the plant ashes according to a method devised by G. Bertrand (1) and myself and specially adapted for the study and determination of small quantities of this metal. Consequently, I returned to this work for the experimental details.

I outline in the table below some experimental results:

1--Ni introduced in 100 cm^3 of milieu
2--Dry weight of myceliums
3--Ni fixed
4--Proportion of Ni fixed per 100 g of dry matter
5--Proportion of Ni fixed per 100 g Ni introduced

It can be seen from these results that the plant fixes nickel. It is even likely to fix it in a very appreciable proportion. As in the case of manganese and contrary to what is observed with zinc, Aspergillus in no case, fixes the totality of nickel at its disposal.

The absolute quantity of fixed metal increases with the increase of the quantity introduced, but the value of the relationship between the weight of the nickel fixed and the weight of the metal introduced into the nutritive milieu is decreasing.

Conclusions

The experiments described in this work were able to draw the following conclusions:

1--The nickel in commonly chosen concentrations, has a clearly favorable effect on the development of Aspergillus niger; this effect is seen in a significant increase in the weight of the cultures. The favorable effect begins to make itself felt when the concentration of the nickel solution reaches $\frac{1}{500,000}$. By increasing the concentration, the growth of the weight of the cultures becomes increasingly significant, to arrive at a maximum at the concentration of $\frac{1}{15,000}$; which may be considered as the optimal concentration. For this optimal concentration the average of the coefficients of utility was 1.65. However, in the most favorable experiments, this coefficient reached the figure of 2.1 (Table III).

Table - Fixation of Ni by *Aspergillus niger*

Ni Introduced in 100 cm^3 of Milieu	Dilution	Dry Weight of Myceliums	Ni Fixed	Proportion of Ni fixed per 100 g of dry matter	Proportion of Ni fixed per 100 g Ni introduced
0.0002 g	$\frac{1}{500,000}$	0.705 g	0.00008 g	0.01	40
0.002 g	$\frac{1}{50,000}$	0.84 g	0.00062 g	0.073	31
0.004 g	$\frac{1}{25,000}$	0.986 g	0.00081 g	0.08	26.2
0.007 g	$\frac{1}{14,285}$	1.05 g	0.0013 g	0.12	18.5
0.010 g	$\frac{1}{10,000}$	0.986 g	0.0015 g	0.15	15

2--In the experiments with cobalt, we could not clearly and consistently verify a favorable effect under the given conditions of these experiments.

3--In considering the concentrations of the nickel and cobalt salts which produced a harmful effect, which was shown by a decrease in the weight of the cultures, we found a great difference in the effects of the nickel and cobalt salts.

The harmful effect of cobalt begins to make itself felt when the concentration of the solution reached $\frac{1}{250,000}$. This injurious effect progressively increases with the increase of the concentration. In the given conditions of the experiment, the presence of $\frac{1}{2,500}$ of cobalt completely prevents the formation of mycelium.

However, the injurious effect of nickel didn't begin to make itself clearly felt until a concentration of $\frac{1}{7,500}$ Ni and the presence of $\frac{1}{3,000}$ of nickel prevented almost completely the formation of mycelium under the envisaged experimental conditions.

4--It results from the experiments done on the fixation of nickel by Aspergillus that the plant fixes this metal; the value of the relationship between the weight of the fixed nickel and the weight of the metal introduced into the nutritive liquid decreases with the increase of the quantity introduced. The mycelium does not fix in any concentration the totality of the nickel introduced.

MONIER

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[NICKEL]

NICKEL does not, as far as is known, play any part in human or animal nutrition or in plant growth and no diseases of plants or animals have been attributed to the lack of it. Its interest to the food chemist arises from its frequent use as the pure metal, as nickel plate, or as a constituent of alloys in the construction of plant and vessels in food industries and for cooking, and also as a metallic catalyst in the hydrogenation of edible oils.

NICKEL IN SOILS. Schieckenthal (1927) has given the nickel and iron content of a number of ferruginous soils near Vienna. The nickel content averaged about 0·004 per cent (40 p.p.m.) and the iron content about 4 per cent. Bertrand (1922) found traces of nickel in all soils examined, up to 17 p.p.m., and Russian analyses have also shown amounts ranging from 2 to 14 p.p.m. in different soils. Probably only a small fraction of this is in a condition in which it could be absorbed by plants, as measured by solubility in dilute acetic acid (Scott and Mitchell 1943). Mitchell (1944) has given a review of spectrographic work on the distribution of nickel, cobalt and a number of other trace elements in Scottish soils.

Excess of nickel is toxic to plants. Certain soils on serpentine outcrops in Maryland and Pennsylvania are stated to owe their lack of fertility to the presence of relatively large amounts, over 0·1 per cent, of nickel and chromium salts.

NICKEL IN PLANTS. Very few determinations have been made of nickel naturally occurring in plants. Bertrand and Mokragnatz (1930) found that the mean nickel content

of a number of vegetables, fruits and grains (calculated on the dry material) was about 0·9 p.p.m. as against only 0·04 p.p.m. of cobalt. Lettuce, cabbage, spinach and peas contained from 1·5 to 3·0 p.p.m., wheat 0·35 and fruits 0·15 to 0·25 p.p.m. In potatoes they found 0·25 p.p.m. of nickel and 0·06 p.p.m. of cobalt. Scott and Mitchell found from 0·56 to 3·62 p.p.m., calculated on the dry matter, in various pasture samples from north-east Scotland. According to Ramage (1936) nickel occurs in a number of spices and herbal drugs and is also present in tea. He found as much as 0·014 per cent in *Strychnos Ignatii* beans.

NICKEL IN HUMAN AND ANIMAL ORGANS. Bertrand and Macheboeuf (1925) found that human and animal organs contain as a rule far less nickel than cobalt, in contrast to plants. Fresh human liver contained 0·09 and pancreas 0·04 p.p.m. of nickel, and ox liver and pancreas 0·125 and 0·135 p.p.m. respectively. They found 0·004 p.p.m. in cows' milk and 0·02 p.p.m. in egg-yolk. Fish contained on an average about 0·015 p.p.m. Fresh lobster showed 0·1 p.p.m. of nickel and 2 p.p.m. of cobalt, but in mussels the nickel predominated, being 0·45 p.p.m. as against 0·14 p.p.m. of cobalt.

NICKEL IN FOOD. Small quantities of nickel may be found in various manufactured foods. In edible gelatin, according to Martini (1929) and Maclaurin (1932), it occurs rather frequently owing to its presence in traces as a normal constituent of bones. It also occurs in hardened oils which have been in contact with a nickel catalyst. The amount found in such oils varies considerably, from barely perceptible traces of the order of 0·01 p.p.m. to as much as 1 or 2 p.p.m. Some hardened oils give a strong magenta colour on heating and shaking with dilute hydrochloric acid and adding a small quantity of potassium dithio-oxalate solution (p. 280), but with others this test may fail unless a large sample is extracted and the extract concentrated.

Nickel salts may gain access to food from corrosion of nickel vessels. Cox (1935) states that a beverage brewed in nickel contained 60 p.p.m., equivalent to about 2 grains

of crystallised nickel sulphate per pound, which corresponds closely to the amount of copper sulphate formerly used for greening vegetables. Lehmann (1908) found that food cooked in nickel utensils took up from 10 to 58 p.p.m., and he calculated that if nickel ware was used exclusively in a kitchen the total amount of nickel ingested daily would be about 117 mg. or 1·5 to 2 mg. per kilogram of the consumer's weight. This he considered negligible. Normann and Hugel (1913) found from 19 to 83 p.p.m. in apples and vegetables cooked in nickel vessels and as much as 127 p.p.m. in sauerkraut. According to Ludwig (1898) food cooked in nickel contained from nil to 35 p.p.m. and sauerkraut 129 p.p.m. Dzerzgowsky (1906) found that solutions of various organic acids up to 4 per cent strength, with and without salt, when boiled in nickel for 3 hours and allowed to stand for a further 3 to 11 hours, took up 60 to 320 p.p.m. of nickel. According to Järvinen (1923) a 40 per cent sugar solution containing 1·5 per cent of citric acid, boiled for 3 hours in a nickel vessel, took up 76 p.p.m.; a 5 per cent salt solution under similar conditions took up only 5 p.p.m., but salt in presence of organic acids is more corrosive. Nickel is corroded even at room temperature by citric acid solutions in presence of oxygen. Johnston (1935) records that a 66 per cent sucrose solution containing pineapple chunks, allowed to remain in contact with sheet nickel for six weeks, took up an amount corresponding to about 43 p.p.m. Hagues (1931) found that in nickel-lined fermentation vessels nickel was corroded where it came into contact with the yeast head and the yeast contained up to 140 p.p.m. of nickel. The beer contained only about 2 p.p.m.

Food cooked in nickel vessels may become more or less discoloured and have a metallic taste. 5 to 10 p.p.m. will give a taste to fruit, etc., and 14 p.p.m. of nickel lactate in water has a decidedly metallic taste. Charley (1933) states that nickel vessels do not affect the flavour of cider. Nickel and iron salts have been found to turn tomato products brown.

According to some authorities nickel is eminently suitable

for dairy plant and is more resistant to corrosion than aluminium and tin (Donauer 1922, Searles 1930). Milk pasteurised under commercial conditions in nickel apparatus does not contain more than 0·25 to 1·3 p.p.m. Other authorities, however, assert that milk if pasteurised in nickel vessels may take up as much as 12 to 15 p.p.m., and may acquire a metallic taste from the presence of nickel lactate (McKay and others 1929, Pratt 1930, Burrell 1938). Quam (1928) found that milk agitated with metal strips at 75° for 30 minutes took up 6 to 7 mg. of nickel per dm.^{2} of surface as against 1·5 mg. of copper and 0·85 mg. of zinc. Nickel appears to be more suitable for surface preheaters in continuous pasteurisation than for surface coolers, since a protective film of milk protein is formed on the surface of hot nickel in contact with cold milk, while no such film is formed by cold metal in contact with hot milk. Génin (1935) found that corrosion of nickel in condensed milk evaporators was greater during intermittent than during continuous working. Nickel salts inhibit bacterial action, and it has been stated that 11 p.p.m. delays lactic fermentation in milk, but it seems doubtful whether such a small amount would have any appreciable effect.

According to Hartley (1935) steam-jacketed nickel pans are used for processing pineapples, cranberries, soups, jams, dairy produce, vegetables, etc. Nickel does not appear to act as an oxidation catalyst for vitamin C. It is attacked less easily than aluminium and tin by alkaline detergents and brines.

Of the alloys of nickel, monel metal (Ni 67, Cu 28, Fe and Mn 5 per cent) is more resistant than nickel and is widely used in plant and apparatus in food industries. It resembles pure nickel in its resistance to brine products (Tay 1936), but Génin (1938) finds that it is attacked by salt solutions unless small amounts of calcium hydroxide or sodium chromate are present, which protect the metal from corrosion. In brewery fermentation vessels monel metal is about five times as resistant as nickel. Inconel metal (Ni 80, Cr 14, Fe 6 per cent) is still less liable to corrosion

and tarnishing and is a most suitable metal for dairy plant. Titus and others (1930) have investigated the resistance shown by nickel-chromium-iron alloys to various foods during cooking and storage. The average amounts dissolved in one hour's cooking in contact with an alloy containing 15 per cent of nickel and 18 per cent of chromium were 1 mg. of iron, 0·05 mg. of chromium and 0·06 mg. of nickel from 4 dm.^{2} of surface. White nickel bronze (Cu 75, Ni 25 per cent) is stated to be readily corroded and to be unsuitable for cooking vessels.

TOXICITY OF NICKEL. Nickel compounds are similar in their effects to those of copper and zinc. According to Dzerzgowsky (1906) doses of 10 to 20 mg. of nickel per kilogram cause no symptoms in dogs, but 22 to 44 mg. per kilogram produce vomiting and diarrhoea. 2 to 5 gr. of the sulphate (containing 31 to 67 mg. of nickel) may cause nausea and vomiting in human beings (Kolipinsky 1911), whereas the emetic dose of copper sulphate is given as 5 to 10 gr. (80 to 160 mg. of copper) and of zinc sulphate 10 to 30 gr. (147 to 445 mg. of zinc), from which it would seem that nickel sulphate is a more severe stomach irritant than the sulphates of copper or zinc. One cannot, however, assume that the action of these metals in combination with food acids is comparable with that of irritant salts like the sulphates. Even ferrous sulphate has a medicinal dose corresponding to only 13 to 65 mg. of iron. Organic salts of these metals are as a rule much less irritant. 180 mg. of nickel in the form of acetate has been given to a dog daily for a month with only slight diarrhoea and loss of appetite, and the dog gained in weight. According to Arnold (1939) a daily dose of 80 mg. of nickel in the form of nickel pectinate given to young rats, corresponding to 1·25 g. per kilogram of body weight, did not affect their rate of growth over a period of 8 weeks.

When administered by the mouth in subemetic doses no ill effects have been observed either in animals or man, and Dzerzgowsky (1906) concluded that if the alimentary tract is intact the metal is wholly excreted in the faeces without

absorption. If the mucous membrane is injured nickel may be absorbed. Flinn and Inouye (1929) also found that it was excreted to the extent of 98 to 99 per cent by the intestine and not by the kidneys. Kent and McCance (1941), on the other hand, in an experiment on two normal men with very low intakes of nickel in food (0·3 to 0·5 mg. daily), found that roughly two-thirds of the nickel was excreted in the urine and only one-third in the faeces. Their experiment did not show what would be the proportion at higher rates of intake.

In 1886 the use of nickel for cooking vessels was forbidden in Austria, but the decree was rescinded in 1897. Several investigators have recorded that their families have used nickel vessels exclusively in the kitchen for a number of years without any ill effects whatever, but negative evidence of this kind does not exclude the possibility that they might have been still better without nickel. Dzerzgowsky suggested that continued ingestion of subtoxic doses of nickel salts dulls the nerves of the alimentary tract, making it possible for more metal to be given without irritation, but there is no evidence of this. It is stated that Offerdahl in 1913 took 0·5 g. of powdered metallic nickel daily for a month without ill effects.

Nickel salts appear to be more toxic when injected into the blood-stream. Stuart in 1882 found that rabbits and dogs were particularly susceptible and were killed by 7 to 8 mg. of nickel sodium citrate per kilogram, death being due to cardiac paralysis and toxic action on the central nervous system. Caujolle and Canal (1939) found that 10 to 20 mg. of nickel per kilogram injected into dogs proved fatal, while the corresponding dose of cobalt was 30 mg. per kilogram, indicating that nickel if it gains access to the circulation is the more toxic of the two.

According to Richet (1881) fish are less sensitive to nickel than to copper, zinc, iron or cadmium and can live for more than 48 hours in water containing 125 p.p.m.

A full discussion of the hygienic significance of nickel is given by Drinker and others (1924) with many references

on the subject, and there are also reviews by Schreiber (1934, 1935).

It is difficult to suggest practical limits for nickel in foods. It is rarely found in foods in amounts comparable to those of zinc, since it is far more resistant than zinc to oxidation and corrosion. Moreover, nickel is cathodic to iron, whereas zinc, being anodic to iron, dissolves freely from galvanised iron vessels in contact with acid liquids. There are no records of any ill effects being caused by the ingestion of subemetic doses of nickel, but the same is true of zinc salts and it is by no means certain that irritant substances of this kind even in small doses are wholly without effect on health. On the other hand, there does not seem to be any strong evidence that nickel when ingested is more toxic than iron. The action of nickel salts on animals seems to resemble that of zinc salts. Both metals are excreted mainly in the faeces and the effect, in similar doses, is primarily that of a stomach irritant. It would seem that the doses of each required to produce toxic effects, either as irritants or through absorption from the alimentary tract, may be roughly similar. Pending further knowledge of the absorption and physiological action of nickel, provisional limits might be of the same order as those for zinc, that is about four times the limits appropriate to copper (pp. 35–8). The possibility that some persons may be specially susceptible to nickel poisoning is suggested by the frequent occurrence of nickel dermatitis among factory workers from contact of the skin with nickel salts. It is said that even white-gold spectacle frames containing nickel have been known to cause dermatitis (Cormia 1935). Stewart (1933) records the case of a patient whose skin was sensitive to nickel and cobalt but not to iron.

NICKEL AND INSULIN. Salts of nickel, in common with those of certain other metals, reinforce and sustain the hypoglucaemic action of insulin when added in small amounts (0·05 to 4 mg. per 100 units), but in larger amounts they completely inhibit its action (Sahyun and others 1939, Schwab 1938). A crystalline nickel insulinate can be prepared containing 0·41 per cent of nickel (Fischer and Scott,

p. 110). There is no reason to suppose, however, that the physiological action of insulin is in any way dependent on the presence of nickel.

THE DETERMINATION OF NICKEL IN FOOD

WITH DIMETHYL-GLYOXIME. This reagent was introduced by Tschugaeff in 1905. It has the formula $CH_3.CNOH.CNOH.CH_3$ and its alcoholic solution gives with neutral solutions of nickel salts a red crystalline precipitate, $[CH_3.CNOH.CNOH.CH_3]_2Ni$. This compound is soluble in mineral acids but practically insoluble in neutral or acetic acid solutions. The precipitate can be dried at 100° to 120° and weighed. On being heated to 250° it sublimes without decomposition. With small amounts of nickel the colour of the liquid containing the red precipitate in suspension is compared with that of standards, or the precipitate is filtered through a paper disc and the depth of colour of the deposit compared with that of standards filtered through similar paper discs.

Von Fellenberg (1940) in examining hardened fats for nickel proceeds as follows. 10 g. of the fat are heated in a 100-ml. conical flask with 10 ml. of hydrochloric acid (6 parts of concentrated acid and 4 of water) for 30 minutes, during which a current of air is driven through the liquid to ensure thorough and continuous agitation. Without this air current or alternatively constant shaking, nickel may not be completely extracted from the fat by the acid. The aqueous liquid is then filtered through a wet cotton-wool plug. The filtrate is concentrated to a small bulk, made alkaline with ammonia, again brought to the boil and filtered through a plug of paper pulp in a small filter funnel. This filtration removes any iron hydroxide present. The clear filtrate, which may be yellowish in colour, is treated with one drop of a 1 per cent alcoholic solution of dimethyl-glyoxime, and after being allowed to stand for 2 minutes is filtered through

a small wad of filter-paper pulp. The wad of paper is washed with water and the intensity of the red colour on its surface compared with that of standards. Von Fellenberg claims that the test will detect as little as 0·001 mg. of nickel in 10 g. of fat, but it would seem that this degree of sensitiveness can be reached only if the solution to be tested has been concentrated to a very small volume, since Strafford (1923) gives the sensitiveness of the glyoxime precipitation as not more than 1 in 500,000. Torricelli (1937) gives a somewhat similar method.

Struszynski (1935) gives the following test for nickel in hardened fats. 100 to 200 g. of the fat are melted and filtered clear, an equal volume of concentrated hydrochloric acid is added, together with one drop of nitric acid, and the flask is heated on a steam bath with shaking. The acid layer is separated and evaporated to dryness, and the residue ignited and dissolved in 2 to 3 drops of a mixture of hydrochloric and nitric acids. The solution is diluted to 2 ml., a small quantity of tartaric acid added and aqueous ammonia until the liquid is faintly alkaline, followed by a 1 per cent solution of dimethyl-glyoxime in alcohol. The liquid is extracted with chloroform and the chloroform solution evaporated to dryness. A red spot or ring indicates the presence of nickel. Iron does not interfere. The test is claimed to be extremely sensitive.

Armit and Harden (1905) determined nickel in organic material by dry ashing and removing iron and phosphate with ammonia, and copper with hydrogen sulphide. From the filtrate nickel is precipitated with sodium hydroxide and converted into nickel chloride and the solution made up to a definite volume in a Nessler glass. Nickel is then determined colorimetrically with dimethyl-glyoxime. The method is applicable to rather larger amounts of nickel than those referred to above. Fairhall (1926) criticises this method, with which he was unable to obtain more than a rough approximation. Passamaneck (1945) dissolves the nickel compound of dimethyl-glyoxime in pyridine and compares the colour of the clear yellow to orange-red solution with

that of a standard nickel solution in a Duboscq micro-colorimeter.

Other α-dioximes give similar reactions with nickel, and Atack (1913) recommended α-benzil-dioxime as having decided advantages over dimethyl-glyoxime. It gives with nickel salts an intense red precipitate analogous in composition to that given by dimethyl-glyoxime. The sensitiveness is given as 1 part of nickel in 2,000,000 of water or 0·0025 mg. of nickel in a volume of 5 ml. The test is not interfered with by zinc, whereas large amounts of zinc may prevent the formation of the dimethyl-glyoxime precipitate. Atack used this method for the determination of fairly large quantities, 4 mg. or more, of nickel, the precipitate being weighed on a tared filter-paper. It contains 10·93 per cent of nickel. The method can be adapted to the determination of smaller quantities as with dimethyl-glyoxime. When so used it has the advantage that the colour is not so likely to be affected if cobalt is present.

DETERMINATION WITH POTASSIUM DITHIO-OXALATE. This reagent was introduced by Jones and Tasker (1909). It has the formula SK.CO.CO.SK and is a white powder which becomes brown on keeping. It is readily soluble in water to a yellow solution which gives a deep magenta colour with traces of nickel. Jones and Tasker state that it will detect 1 part of nickel in 40,000,000 parts of water if enough of the solution is used to fill a large beaker, and that 1 part in 8,000,000 can be detected readily in a test-tube. The colour given with cobalt (deep brown) is just as intense as that given with nickel. The nickel colour is slowly destroyed by dilute hydrochloric acid and quickly by potassium cyanide. The cobalt colour, on the other hand, is stable in dilute hydrochloric acid and also in cyanide solution. Cobalt can therefore be detected in presence of nickel by adding cyanide to the solution, when the magenta colour disappears and the brown due to cobalt remains. Copper, even at dilutions of 1 in 1,000,000, gives a yellow or greenish-yellow colour, and iron a purplish colour, but only in solutions stronger than 1 in 100,000.

Potassium dithio-oxalate is a better reagent than dimethyl-glyoxime for the colorimetric determination of nickel in that the coloured compound is soluble, whereas nickel glyoxime is an insoluble solid in suspension and the size of the particles and therefore the depth of colour are liable to be affected by salt concentration and other conditions of precipitation. A further advantage is that the colour is developed in neutral or weakly acid solution, which makes it possible to determine nickel without removal of calcium, magnesium and phosphates. Potassium dithio-oxalate has the disadvantage that it is not so specific as dimethyl-glyoxime. Further, it is not very stable unless kept under absolute alcohol and is not usually obtainable commercially. Its preparation by the following method (Jones and Tasker 1909), although not difficult, is an unpleasant operation owing to the offensive and persistent smell of ethyl mercaptan.

Oxalyl chloride is first prepared by the action of 2 mols. of phosphorus pentachloride upon 1 mol. of anhydrous oxalic acid, the mixture being kept cold with ice. It is allowed to stand for two or three days at room temperature until the whole mass is liquid, and oxalyl chloride, which boils at 64°, separated from phosphorus oxychloride by fractional distillation. To prepare ethyl dithio-oxalate, oxalyl chloride (1 mol.) is added carefully drop by drop at room temperature to ethyl mercaptan (2 mols.). The liquid becomes yellow and hydrogen chloride is evolved. The mixture is heated gently to expel any excess of either of the reacting substances, and on cooling sets to a crystalline mass consisting of almost pure ethyl dithio-oxalate. This is dissolved in alcohol and treated with an equivalent quantity of alcoholic potassium hydrosulphide (prepared by passing hydrogen sulphide into an alcoholic solution of potassium hydroxide until the cold solution is saturated with the gas). On stirring the mixture vigorously and allowing it to stand for about an hour potassium dithio-oxalate is obtained as a white crystalline precipitate. This is filtered off and washed free from potassium hydrosulphide with alcohol. The salt is best kept under absolute alcohol in a well-corked bottle in a dark place:

under these conditions it can be kept for years without appreciable deterioration.

Fairhall (1926) gives the following directions for the colorimetric determination of nickel with potassium dithio-oxalate. The material is ashed in a muffle and the ash dissolved in hydrochloric acid. The solution is neutralised to methyl orange with ammonia solution and made faintly acid with hydrochloric acid. Any heavy metals present are removed with hydrogen sulphide, and the filtrate is boiled and treated with bromine water to oxidise iron. The liquid is cooled, and iron is removed as phosphate by adding ammonium acetate solution followed by a few drops of acetic acid. The filtrate, which should be clear and colourless, is diluted to a known volume, say 500 ml. 50 ml. of this solution are transferred to a Nessler cylinder and a small amount of potassium dithio-oxalate solution added. If nickel is present a clear magenta colour develops at once, which is compared with a series of nickel standards containing 0·005 to 0·05 mg. in 50 ml. In absence of nickel the solution may be slightly yellow from excess of dithio-oxalate. If iron has not been completely removed the colour may be purplish, and the determination must be repeated with precautions to remove all iron.

If appreciable amounts of cobalt are present, the reddish-brown colour of the cobalt complex may interfere with the determination of nickel. Drinker (1924) recommends a preliminary separation of nickel from cobalt by precipitation with α-benzil-dioxime. Iron is first de-ionised with sodium citrate, calcium is removed as oxalate and magnesium as magnesium ammonium phosphate. Nickel is then precipitated in slightly ammoniacal solution with α-benzil-dioxime, the precipitate oxidised with aqua regia and the nickel determined colorimetrically with potassium dithio-oxalate. This method is long and troublesome, and it should be possible to base a colorimetric method on the behaviour of the nickel and cobalt complexes in the presence of cyanide referred to above.

Dithio-oxamide, or rubeanic acid, which also forms a violet

nickel compound, has the formula $S{:}C(NH_2).C(NH_2){:}S$, or in its acid tautomeric form $SH.C(NH).C(NH).SH$. It is therefore closely related in constitution to dithio-oxalic acid, but has the disadvantage that it gives a black colour with extremely small amounts of copper which may mask the colour due to nickel.

SEPARATION OF NICKEL AND COBALT FROM OTHER METALS WITH DITHIZONE. Strafford, Wyatt and Kershaw (1945), using a solution of dithizone in toluene, find that the nickel and cobalt complexes with dithizone are stable in presence of a buffered N/10 solution of hydrochloric acid at pH 1·5, and that these metals can thus be separated from lead and zinc. In the table on page 131 nickel and cobalt are shown as not reacting in acid solution with dithizone in chloroform. The dithizone complexes of these metals appear to be formed with difficulty in presence of acid, so that for practical purposes they cannot easily be extracted from acid solutions with dithizone dissolved in chloroform. When once formed, however, by extraction from neutral or slightly alkaline solutions they are not readily decomposed by shaking the chloroform solution with dilute acid.

Young and others (1946) determine nickel by treating the solution first with dimethyl-glyoxime and extracting the resulting nickel glyoxime with chloroform. This separates nickel from all other metals likely to be present. The chloroform solution of the glyoxime is shaken with 2 per cent hydrochloric acid, and nickel thus transferred to the acid solution. This solution is neutralised and brought to pH 8 with ammonia, and the nickel determined by titrimetric extraction with a solution of dithizone in carbon tetrachloride, which has previously been standardised against a nickel solution. The nickel compound with dithizone is purple in carbon tetrachloride solution.

SPECTROGRAPHIC METHOD. Scott and Mitchell's spectrographic method (1945) for cobalt, nickel and molybdenum in soils and plants is referred to on page 295.

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SOME OBSERVATIONS ON THE INCIDENCE OF RESPIRATORY CANCER IN NICKEL WORKERS

BY

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From the Mond Nickel Company Ltd., Swansea

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By far the greater part of all nickel refined in this country is dealt with by the Mond Nickel Company at their Clydach Refinery, near Swansea in South Wales. An inquiry at Clydach works has revealed the following facts: (*a*) No case of nose cancer has occurred among men who first entered employment there after 1925; (*b*) the incidence of lung cancer among these same men has been no higher than that in the country as a whole.

The purpose of this paper is to show how these facts have been established. The nature of the process used at Clydach is described, and the history of the investigation of the problem is given.

The Process

The Clydach Refinery was started in 1902 for the production of nickel by the carbonyl process. During the 55 years it has operated there have been many changes in raw materials, and improvements in the process and equipment.

Nickel ore is mined in Canada. The composition of the ore varies somewhat throughout the ore body but a typical "rich" ore contains about 2·5% of nickel, 30% of iron, 25% of silica, 2 to 4% of copper, and small amounts of cobalt, selenium, tellurium, silver, and the precious metals.

Preliminary concentrating and refining operations are carried out at the mines, and the material is received at Clydach in the form of a "matte" containing some 80% metals.

The process adopted at Clydach may be divided into three periods:—

Period 1 (1902-1933).—The raw material contained 40 to 45% nickel and 35 to 40% copper. Production of copper sulphate was on a large scale during this period.

Period 2 (1933-1953).—The raw material contained less than 3% copper. The sulphur content was at first high (over 20%) but this was reduced to 7%, and later to about 0·5%. Most of the copper was returned to Canada and very little copper sulphate was made (none for the period 1938-46 inclusive).

Period 3 (1953 to Date).—Raw material contained sinter (3% copper). A new, "direct wet treatment" process at Clydach replaced the Orford process. All copper was converted to copper sulphate for sale.

Period 1902-1933.—The process consisted essentially of the following:—

(*a*) The copper-nickel sulphide ores were smelted to a bessemer matte containing approximately 35% copper, 46% nickel, 0·8% iron, 17% sulphur. This process took place in Canada and the matte was shipped to Britain.

(*b*) At the Clydach refinery the matte was first crushed and ground.

(*c*) This matte was calcined to produce mixed oxides of copper and nickel (calcination).

(*d*) The greater part of the copper was leached out with sulphuric acid (copper extraction).

(*e*) The nickel oxide was reduced to an impure nickel powder (reduction).

(*f*) The reduced nickel was acted upon with carbon monoxide at about 50° C., to form a gas called nickel carbonyl (volatilization).

(*g*) The carbonyl thus formed was heated to temperatures of about 200° C. so that pure nickel is deposited and carbon monoxide re-formed (decomposition).

Crushing and Grinding.—The matte as received was fed at regular intervals into Krupp ball mills and ground to pass through a 60-mesh sieve, approximately 220 microns. Each calciner had its own mill and the ground matte passed continually from mill to calciner by means of conveyors, elevators, and distributors.

First Type Calciner (1900-1911).—The ground matte was subjected to a calcining process in order to convert the sulphides into oxides. It was fed into calciners, consisting of long fireproof brick tunnels

which were heated by producer gas. The matte was agitated and moved along by means of three ploughshares fastened to a bar which was dragged through the heated matte on the calciner bed by means of a chain on either side of the plough.

Second Type of Calciner (1924).—These were of the straight line double-deck type, a preliminary roasting being carried out on the upper hearth and a finishing calcination on the lower. The matte was spread and conveyed by ploughs rotating on their own axes and circulating on endless chains. Eight ploughs were attached to each chain and the chain speed was such that it took about 12 hours for the matte to travel the whole length of the top and bottom hearths.

After calcination the original 17% sulphur in the matte was reduced to 1·5%, this being present as sulphides and sulphates.

Copper Extraction.—The calcine was conveyed mechanically to Krupp ball mills, where any lumps formed in the calciners were ground through 40-mesh screens. It was then treated with 12% sulphuric acid, whereby about 75% of the copper was dissolved.

Reduction and Volatilization.—The dried copper-extracted matte was conveyed to the nickel plants where the remaining operations for extracting the nickel were carried out.

On entering the plant the matte was passed through reducers in which the oxides were reduced. The reduced matte was then distributed between volatilizers, where the nickel was volatilized as carbonyl.

The volatilizers were similar in construction to the reducers, but no heating was necessary as the reaction

$$Ni + 4CO = Ni(CO)_4$$

is exothermic. The matte travelled down the volatilizer where it was brought into intimate contact with an upward stream of carbon monoxide.

Decomposition.—In the decomposing apparatus the nickel carbonyl came in contact with nickel pellets heated to a temperature of about 180° C. This caused the dissociation of the carbonyl, so that nickel was deposited on the pellets and carbon monoxide liberated.

Repetition of Extraction Processes.—The discharge still contained nickel which had not been volatilized as nickel carbonyl. It was therefore passed through the above series of operations again in order to effect further extraction.

The product of the last extracting operation was transferred to a concentrating plant, where it was successively oxidized and leached with sulphuric acid to remove the bulk of the base metals.

Concentrating Plant for Precious Metals.—The purpose of this was to take out copper and nickel from the concentrates, which came from the nickel sheds, in order to raise the percentage of precious metals as high as possible. The matte was calcined and sulphuric acid added to extract copper and nickel.

Period 1933-1953.—During these years, the Clydach plant and process underwent many important changes.

As the matte supplied from Canada had a high nickel and low copper content, the manufacture of copper sulphate was discontinued.

The original calciners with their individual Krupp mills were scrapped, and a large air-swept mill installed of sufficient capacity to handle all matte. Rotary hearth calciners were introduced in 1935 with conveyor feed and discharge; these effected a great improvement in calcination conditions. At the same time mechanical dust catchers and electrostatic precipitators were installed to recover dust previously carried forward to the main stacks.

The use of "soft roast sulphide" (7% sulphur) in the process and later of "sinter" (0·5% sulphur) has done much to improve conditions in the refinery.

In 1939 the old hand-fired water gas and producer gas producers were replaced by a modern gas plant in which the fuel is mechanically charged and most operations are automatically performed.

The substitution of high-nickel-content mattes for the original Bessemer matte increased the capacity of the nickel plants, as the extraction of nickel as carbonyl was more rapid and efficient. The number of reducers and volatilizers required for a given rate of feed was substantially reduced, and the higher concentration of nickel carbonyl in the gases from the volatilizers increased the capacity of the decomposers.

More complete and quicker extraction of nickel is achieved when the process is carried out at pressures above atmosphere. The first pressure process was started at Clydach in 1935. Much of the resulting nickel carbonyl was recovered in the liquid phase, and as such was fed into the atmospheric plants. The effect was not only to produce more pellets, but also to increase the carbon monoxide content of the volatilizer gases, and so improve the degree of extraction.

Period 1953 to Date.—The only fundamental change in process operations since 1953 has been the installation of the direct wet treatment plant, and the closing down of the Orford furnace.

Instead of smelting concentrate 2, discharged from the nickel plant, in the Orford furnace, this material is now calcined at a low temperature, and leached

first with water and then with dilute sulphuric acid. The residue is dried and smelted. By these means some 20% of the nickel, 65% of the cobalt, and 80% of the copper are recovered before smelting.

The smelt matte is crushed, ground, calcined, and fed to the old wet treatment plant for extraction of the base metals and preparation of precious metal concentrate.

All copper, coming into the works in the raw sinter, is now converted to copper sulphate for sale.

Statistical Investigations

After the carbonyl process had been in operation for about 30 years, several deaths from respiratory cancer occurred and Professor Bradford Hill was approached with a view to obtaining statistical information relating to the incidence of carcinoma of the respiratory system at the works. Bradford Hill found that not only was there an apparently abnormal incidence of carcinoma of the lung and nose in the refinery population from 1929 to 1938, but that this excess was confined to the process workers. An abstract of Bradford Hill's report of October, 1939, is as follows:—

(1) The numbers and ages of the workmen employed at the refinery and of pensioners on the books were available for two dates, namely April 28, 1931, and June 30, 1937. The age distribution did not vary appreciably between these dates and the mean of the two figures may therefore be taken as a reasonable representation of the population over the nine years of the inquiry 1929-1938. Annual figures for the population of all ages for these years give an average of 950 men. This figure, with the age distribution shown in Table 1, has been taken as the population exposed to risk of dying between 1929 and 1938.

(2) To this population was applied the age specific death rates of men in all England and Wales in the quinquennium 1931-35 for (*a*) all causes of death, (*b*) cancer of all sites, and (*c*) respiratory cancer. The "expected" deaths on this basis are compared in Table 2 with those actually observed.

(3) The results show that there were 105 observed deaths from all causes (excluding three deaths of office staff) and 84 expected at the national rates. Sub-division of these deaths by cause gives a very striking picture. From all causes less cancer, 72 deaths were expected and 67 were observed, only a very small difference. But from cancer as a whole 12 deaths only were expected and as many as 38 were observed, a very heavy excess.

This excess of cancer mortality lies entirely in two sites. From cancer of the lung only one death would be expected at the recorded rates for England and Wales; 16 were observed. Of nasal cancers, 11 were observed and, in the absence of national figures, certainly not more than one would be expected.

If these cancers of the lung and nose are excluded about 10 deaths from cancer of other sites would be expected; actually 11 were observed, a remarkably close concordance.

(4) The abnormal incidence of cancer of two sites can be further and informatively analysed. The population of the refinery can be broadly divided into two groups, the process and the non-process workers, the former including all the processes and the latter the workers in the power house, on plant repairs, electrical, mechanical, traffic, yard, superintendence, and general. The average numbers of each over the years in question were 438 process

TABLE 1

CALCULATION OF POPULATION AT RISK: 1929 TO 1938

Age	No. of Workmen and Pensioners		% Distribution		Mean % 1931 and 1937	Distribution of Population of 950
	1931	1937	1931	1937		
15–	13	36	1·55	3·42	2·49	24
20–	23	78	2·74	7·41	5·08	48
25–	205	234	24·47	22·22	23·35	222
35–	251	292	29·96	27·73	28·85	274
45–	230	230	27·44	21·85	24·65	234
55–	95	154	11·34	14·62	12·98	123
65–	16	25	1·91	2·37	2·14	20
75–85	5	4	0·60	0·39	0·50	5
All ages	838	1,053	100·01	100·01	100·04	950

TABLE 2

COMPARISON OF DEATHS EXPECTED AT RATES FOR MEN IN ALL ENGLAND AND WALES AND DEATHS OBSERVED IN REFINERY POPULATION

	Estimated Expected Deaths in 8½ Years (1929–1938)	Observed Deaths in Same Period
All causes	84	105
All causes less cancer	72	67
Cancer all sites	12	38
Cancer of lung	1	16
Cancer of nose	0·1	11
Cancer less nasal and lung forms	10·11	11

TABLE 3

COMPARISON OF DEATHS IN PROCESS AND NON-PROCESS WORKERS

	No. of Deaths Recorded (June, 1929–January, 1938)	
	Process Workers	Other Workers
Respiratory causes	13	10
Heart disease and cerebral haemorrhage	15	17
Other causes (except cancer)	7	7
Total less cancer	35	34
Cancer: Nasal	11	–
Lung	15	1
Other sites	7	5
Total cancer	33	6
Total all causes	68	40

workers and 389 non-process workers. There were also rather more pensioners who had been process workers. In total, therefore, this group was rather larger and older than the group of non-process workers (the actual age distributions were unknown) and might be expected to suffer a slightly higher mortality. The deaths actually observed in each group are set out in Table 3. It will be seen that from 1929 to 1938 there were 108 deaths in all (including three deaths of office staff not included in Table 2).

From causes other than cancer there were 35 deaths of process workers and 34 of other workers, a position which perhaps rather favours the process workers if they are slightly the larger and older group.

From cancer of all sites there were 33 deaths of process workers and only six of other workers.

There is no material difference between the two groups in any broad cause group except cancer, and the excess here relates entirely to cancer of the two sites, nose and lung. Other forms of cancer are approximately equal (seven deaths amongst process workers, five amongst other workers).

(5) In short, not only does there appear to have been an abnormal incidence of cancer of the lung in the refinery population as a whole in 1929-1938, but it seems that this abnormality has been practically, probably entirely, confined to the process workers.

Carcinoma of the lung and nose in workers who were occupied in a factory where nickel is produced by decomposition of a gaseous nickel compound were included in the list of prescribed diseases in 1949.

It was thought that further investigation was necessary as it seemed that those entering the works after a certain period were not affected. In order to carry out this investigation, a register of all new employees was prepared.

This register (Appendix I) shows that between 1900 and 1957 there were 9,340 new entrants, and of these 131 (1·4%) developed cancer of the lung, and 61 (0·65%) developed cancer of the nose.

The minimum duration of service for a known case was one and a quarter years for carcinoma of the lung and six years for carcinoma of the nose. A truer picture of incidence is therefore given by excluding those new entrants who remained in employment less than one year. Between 1900 and 1929 there were 2,094 entrants who remained over one year; 131 (6·3%) of these developed cancer of the lung and 61 (2·9%) developed cancer of the nose.

From Appendix I it is evident that the incidence varied from year to year, and it was thought pertinent to enquire how this variation affected different groups of entrants.

The percentage of entrants, excluding those who remained in employment less than one year, who developed cancer of the lung or nose was therefore calculated. The results are given in Table 4 and Fig. 1.

The highest incidence of cancer of the lung and nose occurred in those workers who began work between 1905 and 1914. Owing to the very long lag period between exposure and development of cancer, there are probably a few deaths still to occur. There was a progressive fall until 1920, when the incidence rose, but fell again after 1924 (Appendix I). The lung cases were more numerous than the nose cases.

No cases of cancer of the nose and only six cases of cancer of the lung have been recorded in persons who entered the works for the first time after 1924. The expected number of cancer of the lung cases since 1924, calculated at the England and Wales rate for the various age groups, was four.

Fig. 2 shows the actual number of carcinoma cases, both nose and lung, according to year of entry.

In Appendix II an analysis is given of the occupations of men who were known to have suffered from nickel carbonyl poisoning and who later developed cancer of the lung or nose. The detailed occupations of those who entered in 1922-24 and who later developed cancer of the lung or nose are also given.

General data on the occurrence of cancer of the lung and nose are as follows:—

	Lung	Nose
Year of first reported cases	1927	1923
Number of cases up to December, 1957	131	61
Cases still alive	3	—
Youngest age at death	43	36
Oldest age at death	73	78
Shortest service before development of cancer (years)	1¼	6
Longest service before development of cancer (years)	43	42

TABLE 4

PERCENTAGE OF ENTRANTS WITH MORE THAN ONE YEAR'S SERVICE LATER DEVELOPING CARCINOMA OF LUNG OR NOSE

Year of Entry	Carcinoma of Lung (%)	Carcinoma of Nose (%)	Year of Entry	Carcinoma of Lung (%)	Carcinoma of Nose (%)	Year of Entry	Carcinoma of Lung (%)	Carcinoma of Nose (%)
1900–	15·1	1·9	1920–	6·6	1·3	1940–	0·6	—
1905–	15·8	9·6	1925–	0·9	—	1945–	0·1	—
1910–	13·0	10·8	1930–	0·4	—	1950–	—	—
1915–	1·7	0·9	1935–	—	—	1955	—	—

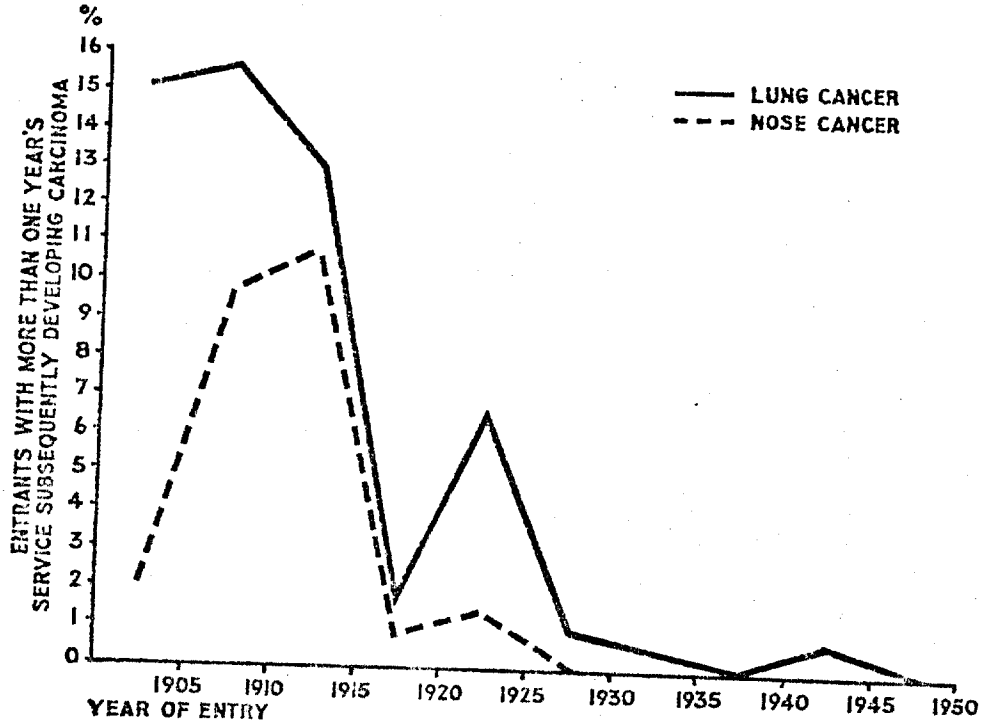

FIG. 1.—The incidence of carcinoma of the lung and nose according to year of entry in entrants with more than one year of service.

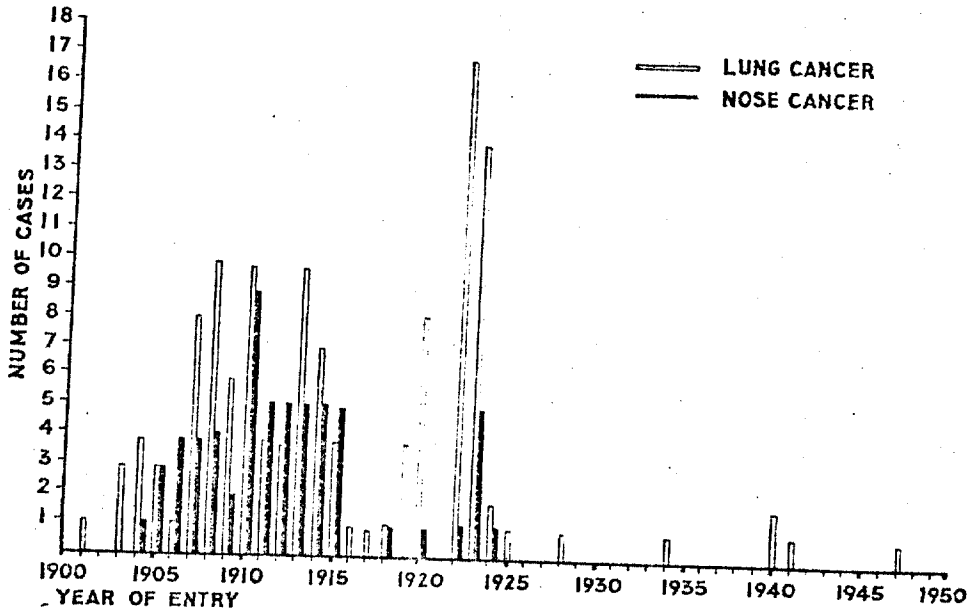

FIG. 2.—Number of cases of carcinoma of lung and nose according to year of entry.

The occurrence of cancer of the lung and nose in nickel workers is likely to be affected by the following factors:—(*a*) Length of service and "lag" period; (*b*) occupations followed; (*c*) materials involved; (*d*) preventive measures.

(a) Length of Service.—It has long been realized that a considerable time elapses between the exposure of an individual to risk and the occurrence of industrial cancer. This interval, for convenience called the "lag" period, may be as long as 25 years and perhaps even longer. It is this factor which accounts for the deaths that have occurred at Clydach in recent years.

The "lag" period is influenced by several factors such as age at entry into the occupation, length of service, variations in concentrations of carcinogen, and individual susceptibility.

In practice difficulty was experienced in establishing the exposure period in many cases. The term "labouring" appears in most men's history, and at first this occupation was not considered hazardous, but further investigation showed that labourers might be called upon to work in any part of the plant. Again, the date of onset of the disease was sometimes difficult to establish with certainty. The length of service from entry to the works until death was therefore recorded. The results are given in Table 5 and Fig. 3.

It is interesting to note that the time from first exposure to death is similar to the time lag of bladder cancer in certain chemical workers, of lung cancer in chrome workers, and in miners at Schneeberg.

TABLE 5

NUMBER OF YEARS BETWEEN ENTRY TO WORKS AND DEATH (ALL CASES DYING UP TO DECEMBER 31, 1957, ARE INCLUDED)

No. of Years' Service	Cancer of Lung	Cancer of Nose
	No. of Deaths	No. of Deaths
0–	6 (4·6%)	—
5–	3 (2·3%)	4 (6·5%)
10–	3 (2·3%)	5 (8·1%)
15–	11 (8·4%)	9 (14·7%)
20–	27 (20·6%)	23 (39·2%)
25–	31 (23·6%)	11 (18·0%)
30–	25 (19·1%)	6 (9·6%)
35–	16 (12·2%)	2 (3·2%)
40–	9 (6·9%)	1 (1·6%)
	131 (100·0%)	61 (100·9%)
Mean 26·9 S.D. ± 6·15		Mean 22·65 S.D. ± 7·0

(b) Occupations Followed.—Table 6 gives the number of patients who worked at a single job at Clydach during the period 15 to 25 years before death, and Table 7 the patients who worked at more than one job. These tables suggest that the occupations meriting special attention are calcination, the production of copper sulphate and nickel sulphate, and furnace work.

Table 6 indicates that the majority of both lung and nose cancers occurred in those engaged in what were formerly dusty processes. The highest

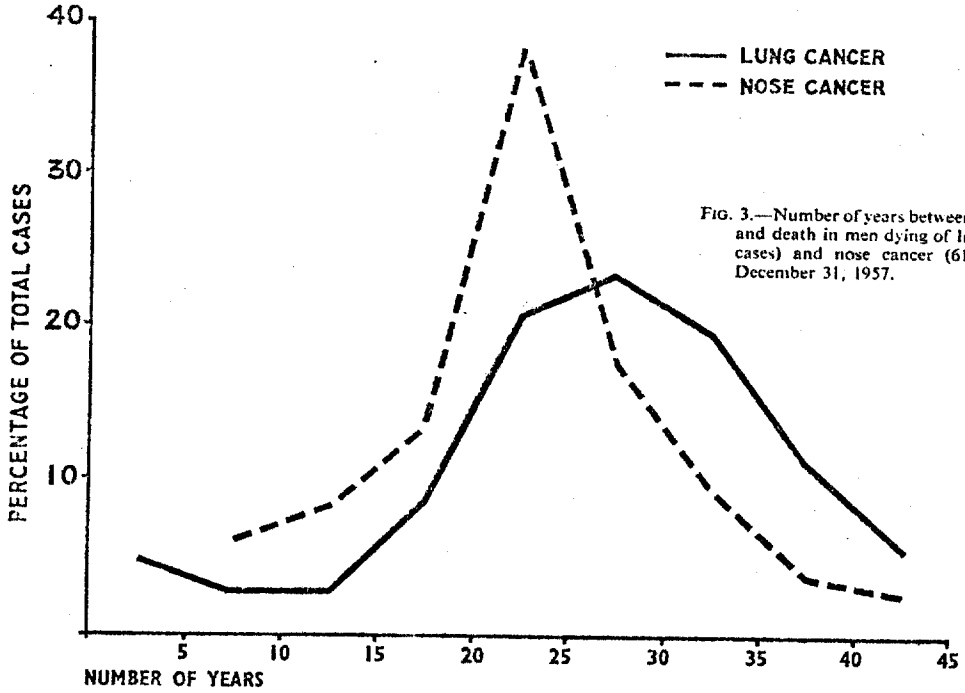

FIG. 3.—Number of years between entry to works and death in men dying of lung cancer (131 cases) and nose cancer (61 cases) up to December 31, 1957.

TABLE 6

OCCUPATIONAL INCIDENCE IN VARIOUS PROCESSES IN MEN WORKING IN ONE DEPARTMENT ONLY DURING 15 TO 25 YEARS BEFORE DEATH

Process or Department	Average Annual Population	No. of Cases of Carcinoma of Lung	No. of Cases of Carcinoma of Nose
Gas producers	—	1	—
Calcination	58	14	14
Copper sulphate	87	20	8
Nickel sulphate	57	4	3
Furnaces	36	2	5
Concentrate	26	2	1
Nickel sheds	163	5	1
Fitters	80	9	1
Totals	507	57	33

TABLE 7

MEN WORKING IN MORE THAN ONE DEPARTMENT DURING 15 TO 25 YEARS BEFORE DEATH

Department	No. of Cases of Carcinoma of Lung	No. of Cases of Carcinoma of Nose
Calciners + nickel sheds	4	1
+ copper sheds	7	9
+ nickel sulphate	1	—
+ furnaces	1	—
Copper sheds + nickel sulphate	4	4
+ furnaces	1	—
Nickel sheds + copper sheds	9	3
+ furnaces	2	—
Non-process workers	7	—
Miscellaneous	36	11
Gas producers + furnaces	1	—
+ copper sheds	1	—
Totals	74	28

frequencies were in the calciners, copper sulphate plant workers, furnace workers, crushing and grinding department workers, and in those transporting matte from the calciners to the nickel sheds.

The dust in the calcination department was of a very fine and pyrophoric nature. In the copper sheds there was a fine copper sulphate powder which had a local corrosive effect; it also contained certain arsenical compounds.

(c) Materials Involved.—Much has been written to indicate the nature, number, and great variety of the chemical carcinogens. Those that might have been present in Clydach are: (i) Radioactive substances, (ii) arsenic, and (iii) nickel carbonyl.

(i) *Radioactive Substances.*—Tests carried out by means of a sensitive electroscope at the works and also by photographic, electroscopic, and emanation methods in the laboratory yielded no evidence of radioactivity of the nickel matte at any stage in the process.

(ii) *Arsenic.*—Arsenic was present both in the matte and in the sulphuric acid used for extraction of copper sulphate. Before 1921 the sulphur smelt matte contained a considerable quantity of arsenic derived from sulphuric acid which had been used for the extraction of copper. The arsenic remained in the matte throughout the process until it came to the melting furnaces and calciners as a copper-nickel arsenite. A record was maintained of the amount of arsenic present in the sulphuric acid used up to 1927, and is presented in Table 8.

TABLE 8

AMOUNTS OF ARSENIC IN SULPHURIC ACID

Year	Amount of Arsenic (tons)	Year	Amount of Arsenic (tons)	Year	Amount of Arsenic (tons)	Year	Amount of Arsenic (tons)
1903	3·0	1910	27·0	1916	68·8	1922	1·1
1904	6·7	1911	30·0	1917	102·5	1923	0·63
1905	7·2	1912	34·0	1918	89·5	1924	0·64
1906	12·2	1913	40·0	1919	108·7	1925	0·35
1907	18·4	1914	50·0	1920	13·6	1926	0·25
1908	25·6	1915	60·0	1921	9·4	1927	Nil
1909	22·0	—	—	—	—	—	—

From 1921 onwards the quantity of arsenic contained in the acid fell, but it was not until 1924 that all the old stock of acid was used. Since 1926 the acid used has been practically free of arsenic.

Amor (1939) found that slight cases of arsenic poisoning did occur in nickel workers, together with characteristic skin rashes and oedema of the scrotum. From an examination of sickness records before 1934 he noted that several cases of cholaemia were reported and that after 1920 they almost completely ceased; the relationship of this to arsenic poisoning, if any, is unknown.

(iii) *Nickel Carbonyl.*—The suggestion that respiratory cancer is in some way linked with nickel carbonyl is not supported for the following reasons:

(1) There has been no material change in the process in the nickel sheds where nickel carbonyl gas has been produced over the last 50 years, to which could be attributed the improvement in the incidence of respiratory cancer which has occurred since 1925.

(2) In the nickel sheds, in addition to the carbonyl hazard, there was also a dust hazard where the matte was brought in and discharged from the plant. Before 1925 the sulphuric acid used contained considerable amounts of arsenic. No case of carcinoma of the nose has occurred in men who entered the works after 1924 and the incidence of cancer of the lung in these men is not above the national average.

(3) Of 131 lung and 61 nose carcinoma cases, only 15 had suffered from the effects of nickel carbonyl inhalation, and of these 10 were lung cases and five were nose cases. Most of these cases had been engaged in various occupations in the works. In five of the cases, gassing occurred less than four years before death.

(4) Løken (1950) has reported carcinoma of the lung in a Norwegian works where nickel is refined other than by the carbonyl process.

(d) Preventive Measures.—As a result of the suggestion that arsenic might be the carcinogenic agent

responsible for the high incidence of respiratory cancer, measures were taken to exclude metallic arsenides from the process, to provide arsenic-free sulphuric acid, and to suppress dust at every stage of the process. Details of the various improvements made are given below:—

In 1910, a plough type of calciner, which operated from 1902 to 1911, was changed to double-deckers with rotary rakes which, although very dusty, were an improvement on the first type.

In 1922, arsenic-free sulphuric acid was used. Nose and mouth respirator pads were issued.

In 1924, calciners were shortened and improved.

In 1929, the copper sulphate plant was closed down.

In 1934, the mattes were changed and now contained only 2% copper instead of 35%, and only 2% sulphur instead of 20%.

In 1935, Lodge Cottrell electrostatic precipitators diminished the amount of dust emitted from the stacks. Also from that date the whole of the crushing and grinding of the material is now done in a central grinding plant which is virtually dust free. Formerly the crushing and grinding was done separately in each plant by means of Krupp crushers, which were very dusty.

In 1936, new calciners were installed. The old type led to underground flues which had to be cleaned very frequently, and it is significant that most of the nose cases occurred amongst the men who were engaged in cleaning these flues. The new calciners are in a well-ventilated room and the flue gasses from these calciners are taken to two Lodge Cottrell electrostatic precipitators.

In 1937, a new type of closed bogie was used instead of the open trucks, which has an automatic emptying device fitted to it with a new suction feed apparatus which makes it possible to feed this matte into the nickel sheds without making the slightest dust if used properly. Also new feed elevators were introduced to the nickel sheds.

In 1944, the matte was free from sulphur.

Medical Control

Comprehensive pre-employment medical examinations, including a chest radiograph, are now carried out. Routine examinations are undertaken annually and particular attention is paid to any possible manifestation of industrial disease; the annual re-examination includes a chest radiograph. All employees are medically examined before return to work if they have been absent on sick leave for more than one month. Regular dust counts are undertaken in all dusty processes; the plant superintendents are given the results of these tests and they are encouraged to keep the counts at a low level.

Summary

(1) At Clydach Works, the incidence of cancer of the lung and nose among workers engaged in the refining of nickel and the preparation of nickel and copper salts was found to be greater than that of the general population. One hundred and thirty-one cases of lung cancer and 61 of cancer of the nose are reported. A description is given of the nickel carbonyl process.

(2) The increased incidence of cancer of the lung and nose, which has not been noted amongst workers engaged in processes involving the use of metallic nickel, appeared to be related to: (*a*) Dusty occupations rather than to occupations where nickel is dealt with as a gaseous compound, and/or (*b*) drying and powdering of copper sulphate, and/or (*c*) the sulphuric acid used before 1921 which was far from pure and contained arsenic.

(3) As an index of the action of any carcinogenic material which had been present at the Clydach Works, the incidence of nasal cancer was far more significant than that of lung cancer. No deaths from nasal cancer have been recorded from workers engaged after 1924, at which date increased precautions were taken against dust.

(4) It is considered that dust, possibly the heated calcined dusts, contained the carcinogen. Before 1924 this dust probably contained arsenic from the sulphuric acid used. After taking into account (i) the period of exposure to the hazard (minimum one year); (ii) the long lag period between exposure and the appearance of the first signs and symptoms; and (iii) the survival period after diagnosis (on the average between 18 and 24 months), it is not unreasonable to draw the inference that carcinogenic material may possibly have been present in the heated calcined dusts—possibly in their arsenic content.

(5) The average period from entry to the works to death was 27 years in lung cancer cases and 22 years in nose cancer cases.

(6) Deaths from carcinoma of the lung and nose which occurred in men not employed in the calcination process may be attributable to the fact that they came into contact with calcined dusts during the course of their employment.

It is a pleasure to express my grateful thanks to my staff for their great help in the preparation of this paper.

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APPENDIX I

Register of Employees

A register was prepared of the new entrants each year; no re-engaged person was included. The following particulars were recorded in the register:—

(1) The number of entrants.

(2) The number employed annually, *i.e.* the establishment.

(3) Numbers employed by years-of-service groups: under one year, one-10 years, 11-20 years, and those who remained over 20 years.

(4) Number of cases of carcinoma of the lung and nose.

(5) Percentage of the entrants who developed (*a*) carcinoma of the lung, (*b*) carcinoma of the nose.

(6) The number of deaths due to other causes.

Table 9 gives the figures obtained from this register.

In calculating the percentages of new entrants who developed carcinoma of the lung or nose, those with less than one year's service have been omitted, as the minimum duration of service for a known case was one and a quarter years for carcinoma of the lung and six years for carcinoma of the nose.

The total number of new entrants from 1900 to December 31, 1957, was 9,340. The Company has a pension scheme which enables it to keep records of all pensioners, wherever they may live; they are visited annually and thus the Company is able to keep in touch with them. When pensioners die a death certificate must be furnished in order that their dependants may obtain a death benefit and the cause of death is, therefore, recorded in every case.

The labour turnover is equal to the average for the area and only a few men change their employment and leave the district. A scrutiny of the local death register

TABLE 9

CASES OF CARCINOMA OF LUNG AND NOSE BY YEARS OF-SERVICE GROUPS AND EXPRESSED AS A PERCENTAGE OF ENTRANTS WITH MORE THAN ONE YEAR'S SERVICE

Year of Entry	New Entrants	No. of Men Employed (Establishment)	Years of Service and No. of Cases												No. of Cases		Cases % of Entrants with More than One Year's Service		No. of Deaths from Other Causes
			Under 1 Year			1–10 Years			11–20 Years			Over 20 Years							
			No.	Cancer Lung	Cancer Nose	No.	Cancer Lung	Cancer Nose	No.	Cancer Lung	Cancer Nose	No.	Cancer Lung	Cancer Nose	Carcinoma of Lung	Carcinoma of Nose	Carcinoma of Lung	Carcinoma of Nose	
1900	13	13	10	—	—	—	—	—	1	—	—	2	—	—	—	—	—	—	—
1901	89	102	83	—	—	4	1	—	2	—	—	—	—	—	1	—	(16·7)	—	—
1902	185	125	171	—	—	—	—	—	4	—	—	10	—	—	—	—	—	—	3
1903	109	190	98	—	—	1	—	—	—	1	—	10	2	—	3	—	(27·3)	—	2
1904	27	230	8	—	—	—	—	—	—	—	—	19	4	1	4	1	(21·0)	(5·2)	—
	423		370			5			7			41			8	1	15·1	1·9	
1905	32	370	14	—	—	5	—	—	2	—	1	11	3	2	3	3	(16·7)	(16·7)	—
1906	69	395	32	—	—	3	—	—	1	—	1	33	1	3	1	4	(2·7)	(10·8)	6
1907	85	475	34	—	—	19	—	—	3	—	3	29	8	1	8	4	15·7	7·8	3
1908	78	470	31	—	—	21	—	—	—	—	—	26	10	4	10	4	21·3	8·6	—
1909	53	545	29	—	—	7	—	—	—	1	1	17	5	1	6	2	(25·0)	(8·3)	—
	317		140			55			6			116			28	17	15·8	9·6	
1910	143	590	52	—	—	45	—	1	2	—	1	44	10	7	10	9	11·0	9·9	1
1911	116	600	69	—	—	24	—	—	2	—	2	21	4	3	4	5	8·5	10·6	4
1912	91	670	56	—	—	12	—	—	3	—	2	20	4	3	4	5	(11·4)	(14·3)	2
1913	221	825	168	—	—	16	—	1	2	3	—	35	7	4	10	5	18·9	9·4	4
1914	495	990	452	—	—	6	—	—	9	1	—	28	6	5	7	5	16·3	11·6	7
	1,066		797			103			18			148			35	29	13·0	10·8	
1915	481	1,200	289	—	—	159	—	—	5	—	1	28	4	4	4	5	2·1	2·5	3
1916	440	1,360	155	—	—	273	1	—	3	—	—	9	—	—	1	—	0·4	—	10
1917	286	1,350	180	—	—	78	—	—	—	—	—	28	1	—	1	—	0·9	—	9
1918	290	920	276	—	—	2	—	—	—	1	1	12	—	—	1	1	(7·1)	(7·1)	12
1919	295	775	225	—	—	29	—	—	7	2	—	34	2	—	4	—	5·7	—	15
	1,792		1,125			541			15			111			11	6	1·7	0·9	
1920	173	415	145	—	—	1	2	—	6	1	—	21	4	1	7	1	(25·0)	(3·6)	8
1921	152	600	59	—	—	55	—	—	1	—	—	37	—	—	—	—	—	—	4
1922	346	1,050	129	—	—	113	—	—	4	1	—	100	16	1	17	1	7·8	0·46	5
1923	415	1,090	236	—	—	120	2	1	5	2	—	54	10	4	14	5	7·8	2·8	3
1924	137	1,085	52	—	—	53	—	—	3	—	1	29	2	—	2	1	2·4	1·2	5
	1,223		621			342			19			241			40	8	6·6	1·3	
1925	33	970	14	—	—	1	—	—	3	—	—	15	1	—	1	—	(5·3)	—	3
1926	79	1,100	20	—	—	17	—	—	3	—	—	39	—	—	—	—	—	—	7
1927	48	1,210	8	—	—	18	—	—	3	—	—	19	—	—	—	—	—	—	11
1928	280	1,414	93	—	—	153	1	—	4	—	—	30	1	—	2	—	1·1	—	12
1929	140	1,185	119	—	—	5	—	—	1	—	—	15	—	—	—	—	—	—	10
	580		254			194			14			118			3	—	0·9	—	

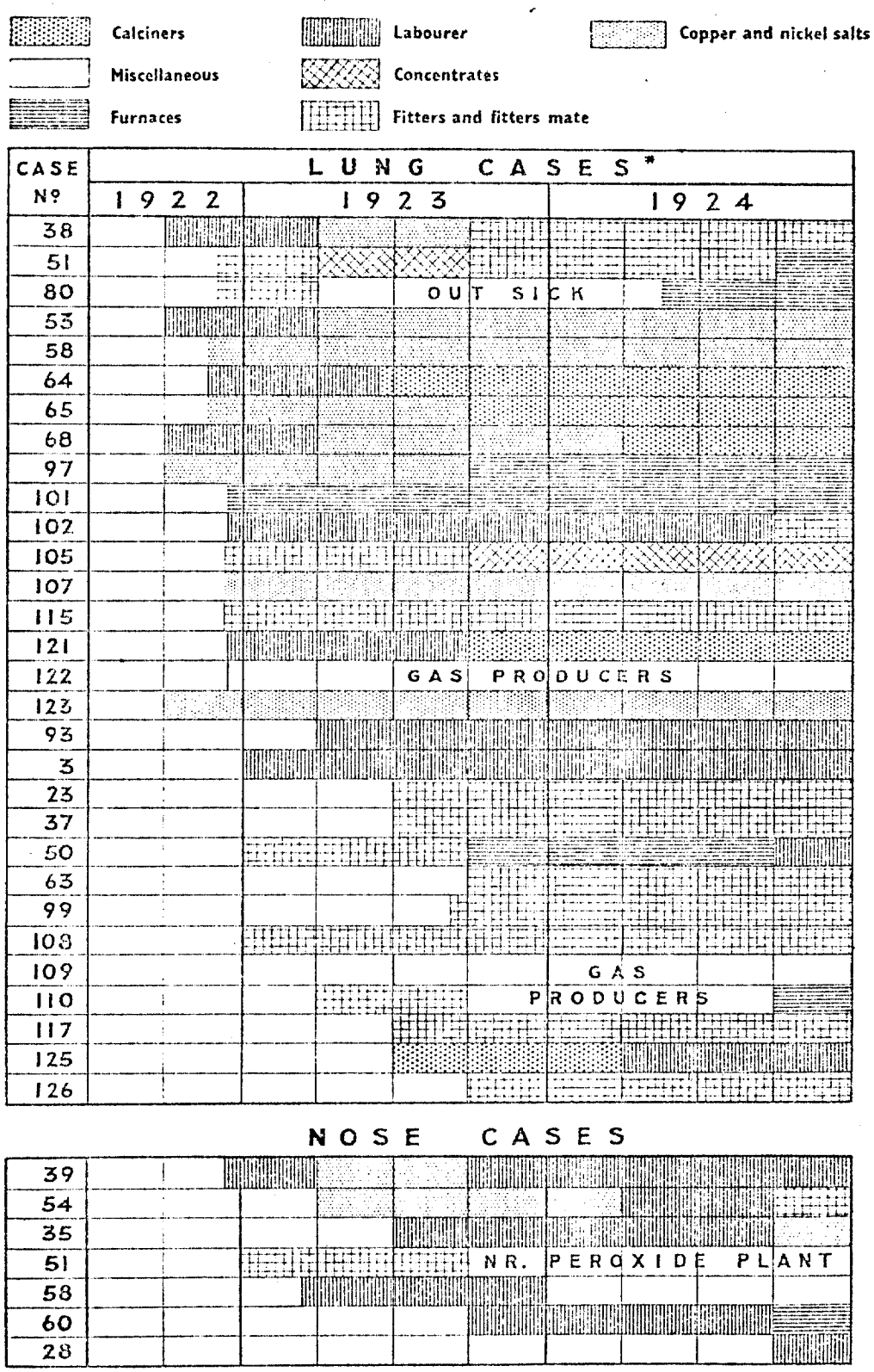

FIG. 4.—Occupations of those who entered the industry in 1922, 1923, and 1924 and later developed carcinoma of the lung or nose.

*The detailed occupations of three lung cases could not be traced.

has yielded a few names in addition to those of pensioners. Two additional cases were thus discovered by W. R. S. Doll (personal communication), during his investigation of death certificates for nasal cancer, but one of these men had had only one year's service with the Company.

APPENDIX II

ANALYSIS OF OCCUPATIONS OF CASES OF CARCINOMA OF LUNG AND NOSE KNOWN TO HAVE SUFFERED FROM NICKEL CARBONYL POISONING

Code No.	Date of Entry	Occupational History	Date of Nickel Carbonyl Poisoning	Date of Death	Years between Gassing and Death
(a) Cancer of Lung					
L.1	1904	Fitter	1922	1927	5
L.33	1904	C½.L2.FM1.NS32	1922	1940	18
L.71	1907	L½.C½.L½.C6.NS2½. FM1.F½.CS1.NS25.	1929	1948	19
L.111	1909	L2.CS3.FM4.NS4.FM19. F6.FM1½.M2.FM1½	1931	1953	22
L.47	1910	L⅔.C2.L½.C6½.L½.FM½. C½.FM1.F½.CS½. FM1½.C2.CS½.C½.CS2. FM8	1940	1944	4
L.95	1911	L2½.C½.L½.FM6.M½. NS26.CS½.M4	1925	1952	27
L.40	1913	L1½.CS5.L½.CS½.L1. CS6½.F½.L½.CS6½. NS½.FM3½	1941	1942	1
L.62	1915	CS4.L4.F2.C1.NS1.CS3. FM½.C½.NS½.F1½.C½. NS7½	1946	1947	1
L.80	1922	FM½. Out sick 1 yr. due to effects of gas. F1½.L1.CS5½.CS5. FM1½.CS9	1923	1949	26
L.37	1923	FM5.NS13	1940	1944	4
(b) Cancer of Nose					
N.31	1910	L6.CS3.L1.CS4½.F½. CS5.NS1.CS6½.FM2½	1931	1941	10
N.52	1910	Fitter—partly days but mostly shift	1944	1951	7
N.24	1911	L2.F5½.CS1.F3.L1.C1½. M1.CS8½.FM½.L1	1936	1939	3
N.59	1911	L1½.C2.FM5.M1.CS10. NS½.CS4½.NS19	1943	1956	13
N.34	1912	L1½.CS1.FM2.M1½. NS3.L½.CS1.NS8.CS½. M½.C½.L1.M1.CS5½. L½.M1.L1	1940	1943	3

C = Calciners; CS = Nickel sulphate and copper sulphate; NS = Ni sheds; L = Labourer; M = Miscellaneous; F = Furnaces; FM = Fitter's mate. (Figures denote years or part years.)

There were 10 men with lung cancer and five with nose cancer who had at some time actually suffered from nickel carbonyl poisoning. If the occupational histories of these cases are closely scrutinized it will be seen that only four were mostly engaged in nickel sheds, whilst the others had a varied occupational history.

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Nickel: a new essential trace element[1,2]

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ABSTRACT

Much indirect evidence exists which indicates that nickel has an essential physiological function. The data have been obtained from pharmacologic, toxicologic, and in vitro biochemical studies. Other evidence has been obtained from chemical analysis of biosubstances which indicates that nickel is consistently present and that nickel concentrations in tissue change in some pathological conditions. Recently, nickel deficiency has been produced in chicks and rats. Chicks raised in a controlled environment free of trace metal contamination and fed a dried skim milk-ground corn diet containing 3-14 ppb nickel exhibited impaired liver metabolism and morphology. This included a reduced ability to oxidize α-glycerophosphate, an increase in the lipid fraction, a decrease in the phospholipid fraction, and an ultrastructural degeneration characterized by dilation of the cisternae of the rough endoplasmic reticulum and by the swelling of the mitochondria. The swelling was in the compartment of the matrix and was associated with fragmentation of the cristae. Dilation of the perinuclear space, condensation of peripheral nuclear chromatin and pyknotic nuclei were also observed. Rats raised under similar conditions also showed abnormalities when deprived of nickel. These included a reduced oxidation of α-glycerophosphate by liver homogenates and abnormalities in the sucrose density gradient polysome profile.—NIELSEN, F. H., AND D. A. OLLERICH. Nickel: a new essential trace element. *Federation Proc.* 33: 1767–1772, 1974.

Examination of the periodic table reveals that of the elements in the transition series from V (23) to Zn (30), nickel is the only one not generally accepted as being essential for animals. This is in spite of the fact that nickel has been suspected of having some physiological role since the 1920's, when it was found to be present in animal tissue. Part of the reason for nickel not being found essential is that nickel-deficient diets have proved difficult to prepare, and appropriate environments for raising animals have not, until recently, been available. Therefore, most evidence presented to date which suggests that nickel has a physio- al role has been of the indirect type. data have been obtained from pharmacologic, toxicologic, and in vitro biochemical studies. Other evidence has been obtained from analyses which indicate that nickel is consistently present in some biological materials, and which show changes in tissue nickel distribution or concentrations in some pathological conditions.

PHARMACOLOGICAL ACTIONS

The earliest animal experiments involving nickel were primarily concerned with its pharmacological effects. In 1926, it was found that parenteral nickel intensifies and prolongs the hypoglycemic effect of insulin in the rabbit and dog (4, 5). Ten years later, it was found that nickel added to posterior pituitary extract prolongs its antidiuretic effect in rats (9, 30). Subsequently, in 1954, it was shown that nickel has an adverse effect on the hypertensive action of epinephrine (20).

Pharmacologic doses of nickel will also apparently alter lipid metabolism. Liver chondria from rats fed large amounts ckel exhibit an in vitro enhancement cholesterol oxidation (52). In addition, the injection of nickel chloride, or nickel amino acid complexes, into rabbits will increase plasma lipids (11, 12).

The effects of nickel on isolated tissues probably should also be considered pharmacologic. Nickel can substitute for calcium in certain steps of the excitation-contraction coupling of isolated skeletal muscle (13, 14). It is thought that this substitution may occur during the spread of excitation in the sarcolemma and transverse tubular systems. Other investigators have found that nickel can also substitute for calcium in the excitation process of the isolated nerve cell (6, 18). It has been postulated that nickel can substitute for calcium in its binding with a membrane ligand such as the phosphate groups of a phospholipid. Through this substitution, nickel may participate in a manner similar to calcium in the process of nerve transmission and muscle excitation and contraction.

Though none of the aforementioned pharmacological actions are specific for nickel, as other ions can have similar effects, they do show that nickel can act in biological systems. They also suggest that its possible physiological function may involve hormone, membrane and/or lipid metabolism.

TOXIC ACTIONS

Nickel toxicity has been studied in mice (50), chicks (49), and cattle (33). The major findings include depression of the activity of certain enzymes in mice and reduction of nitrogen retention in chicks and dairy calves. Both chicks and calves also exhibited reduced growth. The physiological significance of these studies is unclear.

ALTERATIONS IN BLOOD CONCENTRATIONS OF NICKEL

The concentration of nickel in blood is usually maintained within a characteristic range in man and other species of animals (39). In some pathological conditions, the levels may be altered. For example, serum nickel levels may be

[1] From the American Institute of Nutrition Symposium on *Newer Candidates for Essential Trace Elements* presented at the 57th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J., April 20, 1973.

[2] Mention of proprietary product does not necessarily imply endorsement by the USDA.

significantly increased in patients with acute myocardial infarction (8, 25, 32, 39, 41, 42) and high concentrations have been found in the serum of patients with acute stroke and acute burns (>25% body surface) (25, 39). Low levels may occur in patients with hepatic cirrhosis and chronic uremia. In contrast to infarction, serum nickel levels are not increased in patients with myocardial ischemia. Acute trauma with fractured bones, acute delirium tremens, and muscular dystrophy apparently do not cause serum nickel to change. Other studies have shown that blood nickel concentrations increase in women with toxemia of pregnancy (23) or with uterine cancer (3). The apparent specificity of the changes in blood nickel concentrations with disease suggests that nickel has some physiological function.

IN VITRO ACTIONS

Nickel is not an indifferent ion in in vitro studies. For example, it has been shown to activate numerous enzymes including arginase (19), tyrosinase (24), deoxyribonuclease (26), acetyl coenzyme A synthetase (51), and phosphoglucomutase (34). However, these studies have not shown nickel to be a specific activator of an enzyme. Another in vitro effect of nickel is the enhancement of the adhesiveness of polymorphonuclear leukocytes (1, 2, 17). Nickel will also stabilize RNA (16) and DNA (10) against thermal denaturation and is extraordinarily effective in the preservation of tobacco mosaic virus RNA infectivity (7, 47). Finally, it has been reported that nickel may have a role in the preservation of the compact structure of ribosomes against thermal denaturation (43–45) and that nickel will restore the sedimentation characteristics of *Escherichia coli* ribosomes which have been subjected to EDTA denaturation.

It is unknown whether these in vitro phenomena relate to an in vivo function of nickel. They do, however, show that nickel can participate in biochemical reactions or systems.

NICKEL CONTENT OF BIOLOGICAL MATERIAL

Significant concentrations of nickel are present in DNA (10, 48) and RNA (38, 47, 48) from phylogenetically diverse sources. It has been suggested that nickel and the other metals which are present may contribute to the stabilization of the structure of nucleic acids. In addition to nucleic acids, it has been found that a metalloprotein in human serum is rich in nickel (21). This protein which is an α-1 macroglobulin and has been isolated from rabbit serum has been designated "nickeloplasmin" (31). It has an estimated molecular weight of 7.0×10^5 and contains 0.9 g atoms of nickel per mole.

These findings suggest that nickel has an apparent structural role in some biosubstances. It is uncertain, however, whether these structural roles are essential for living organisms.

NICKEL IN ANIMAL NUTRITION

The indirect evidence discussed above implies that nickel has a physiological role in living organisms. However, prior to recent research, it had not been conclusively demonstrated that a deficiency of nickel has adverse effects in the intact animal, and thus, its essentiality had not been proved. Direct evidence for the essentiality of nickel will be presented in the remainder of this report.

Nielsen et al. (27–29) found that feeding a diet containing less than 40 ppb nickel resulted in an apparent nickel-deficiency syndrome in chicks. When compared with controls given a supplement of 3–5 ppm nickel, the deficient chicks showed: *a*) pigmentation changes in the shank skin, *b*) thicker legs with slightly swollen hocks, *c*) dermatitis of the shank skin, *d*) a less friable liver which may have been related to the fat content, and *e*) an enhanced accumulation of a tracer dose of ^{63}Ni in liver, bone, and aorta. These findings were observed under conditions which produced suboptimal growth, and the abnormalities in leg structure and shank skin dermatitis were inconsistent.

Sunderman et al. (40) attempted to confirm Nielsen's findings by feeding a diet containing 44 ppb nickel to chicks raised in a slightly different environment. While they found no gross effects, they did observe ultrastructural changes in the liver. These included dilation of the perimitochondrial rough endoplasmic reticulum in 15–20% of the hepatocytes.

To clarify and extend the above observations, improvements were made in the experimental environment used to produce nickel deficiency and a diet was formulated with a nickel content of 3–4 ppb. With this diet and environment it was consistently possible to produce the findings reported herein.

MATERIALS AND METHODS

A major difficulty in the production of nickel deficiency in animals is the preparation of a diet low in nickel. Nickel is ubiquitous. Therefore, the conventional method of diet preparation using purified proteins, or amino acids, carbohydrates, vitamins, and minerals is unsatisfactory. For example, reagent grade minerals are not suitable because some contain as much as 20,000 ppb nickel. To circumvent this problem, the diet must be prepared from natural feedstuffs low in nickel which contain most of the amino acids, vitamins, and minerals essential for the experimental animal.

The diet formulation for the production of nickel deficiency in the chick is presented in Tables 1–3. It was based on dried skim milk, ground corn, and corn oil. The added vitamins included A, D, E, K, niacin, folate, and biotin because skim milk and corn were calculated to provide insufficient amounts. The mineral additions were only 12.8 g/kg of diet. The major portion was $CaCO_3$ (12.5 g) which was relatively low in nickel (approximately 20 ppb). To assure adequate concentrations, Mn, Fe, Zn, Cu, and I

TABLE 1. Composition of basal diet[a]

Ingredient	g/kg Diet
Skimmed milk powder[b]	645.00
Ground corn[b, c]	142.50
Non-nutritive fiber[d]	40.00
Corn oil[b]	100.00
Glycine[e]	5.00
Arginine[e]	25.00
Choline chloride[b]	0.30
Vitamins, fat-soluble[f]	0.11
Vitamin mix (See Table 2)	4.59
Mineral mix (See Table 3)	37.50
	1,000.00

[a] Diet analyzed 3–14 ppb nickel on an air dried basis. [b] Nutritional Biochemicals Corp., Cleveland, Ohio. [c] Corn was acid washed in *experiment 2*. [d] Solka Floc, SW-40, Brown Co., Boston, Mass. [e] General Biochemicals Co., Chagrin Falls, Ohio. [f] The fat-soluble vitamins were: D,L-α-tocopherol acetate (see footnote[a]), 0.01 g and vitamin D_3 (see footnote[e]) (250 IU/drop), 2 drops or approximately 0.1 g. These were added to corn oil prior to mixing it into diet.

TABLE 2. Vitamin mixture for basal diet

Ingredient	g/kg Diet
Niacin[a]	0.0200
Folic acid[a]	0.0012
Menadione[a]	0.0006
Biotin[a]	0.0001
β-Carotene[a]	0.0120
Vitamin A palmitate[b] (250,000 IU/g)	0.0040
Glucose[c]	4.5521
	4.5900

[a] General Biochemicals Co., Chagrin Falls, Ohio. [b] Nutritional Biochemicals Corp., Cleveland, Ohio. [c] Calbiochem Co., Los Angeles, Calif.

TABLE 3. Mineral mixture for basal diet

Ingredient	g/kg Diet
$^{[illegible]}CO_3$[a]	12.5000
$SO_4 \cdot 5\ H_2O$[b]	0.2000
[illegible]on sponge[h] (dissolved in HCl[c] before use)	0.0550[d]
$CuSO_4$[a]	0.0150
ZnO[e]	0.0250
KI[b]	0.0005
$(NH_4)_6Mo_7O_{24}$[e, f]	0.0035
Na_2SeO_3[f, g]	0.0006
Ground corn[h]	24.7004
	37.5000

[a] "Reagent" grade, J. T. Baker Chemical Co., Phillipsburg, N.J. [b] "Specpure" grade, Johnson, Matthey Chemicals, Ltd., London, England. [c] "Ultrex" grade, J. T. Baker Chemical Co., Phillipsburg, N.J. [d] In first experiment, 0.0350 g iron sponge was used. [e] "Ultrapure" grade, Alfa Inorganics Co., Beverly, Mass. [f] Added to diets only in experiments in which all dietary ground corn was acid washed (*exp 2*). [g] Alfa Inorganics Co., Beverly, Mass. [h] Nutritional Biochemicals, Corp., Cleveland, Ohio.

TABLE 4. Liver analysis of nickel deficient and supplemented chicks

Group	No. of chicks	O_2 Uptake,[a] µl/hour per mg protein	Total lipid,[b] %	Lipid P,[b] mg/g	Cholesterol,[b] mg/g
		Experiment 1			
Ni def (3 ppb)	12	4.7[c] ± 0.2[d]	6.21[e] ± 0.10		6.29 ± 0.07
+ 3 ppm Ni	12	5.5 ± 0.2	5.78 ± 0.11		6.34 ± 0.04
		Experiment 2			
Ni def (4 ppb)	11	5.4[f] ± 0.2	6.27[e] ± 0.18	1.327[e] ± 0.016	
+ 3 ppm Ni	11	6.0 ± 0.2	5.87 ± 0.05	1.379 ± 0.016	
		Experiment 3			
Ni def (14 ppb)	12	5.5[f] ± 0.1	5.71 ± 0.09	1.318 ± 0.016	5.57 ± 0.07
+ 3 ppm	12	5.9 ± 0.1	5.55 ± 0.10	1.335 ± 0.015	5.67 ± 0.08

[a] Using liver homogenates and with α-glycerophosphate as the substrate. [b] Fresh weight basis. [c] Significantly different ($P < 0.025$) from +3 ppm Ni group [d] ± SEM. [e] Significantly different ($P < 0.05$) from +3 ppm Ni group. [f] Significantly different ($P < 0.10$) from +3 ppm Ni group.

were added. The basal diet contained 3–14 ppb nickel on an air-dried basis. The variation in nickel content was due to the amount of nickel in individual batches of ground corn. To consistently obtain a diet containing 3–4 ppb, the corn must be acid washed. Because acid washing [illegible] remove essential amounts of sele- [illegible] and molybdenum, they were added to the diet.

In the following experiments, the control chicks were fed the basal diet, supplemented with 3 ppm nickel as $NiCl_2 \cdot 6H_2O$.

A second impediment to the production of nickel deficiency, and also for the production of deficiencies of other trace elements required in very minute amounts, is the environment in which the animals are raised. Significant amounts of trace elements may be present in such sources as caging, feed cups, water bottles, dust in the air, and the skin of the investigator's hands. To prevent such contamination it was necessary to employ techniques developed by Smith and Schwarz (36). The experimental animals were raised in a controlled environmental system[3] in which most, or all, components were made of plastic and the animals were isolated from environmental contamination. Air entering the isolator was passed through a nonmetallic filter to remove dust. The isolators were lo- [illegible]d in a room maintained at 27 C-29 C, [illegible] plastic covered heating pads were [illegible]ed to maintain the temperature at approximately 34 C at one end of each inside cage. When condensation appeared excessive, humidity was decreased by removal of the end caps of the isolators to improve the flow of air out of the isolator.

In the experiments reported here, chicks were used as the initial experimental animal. They often have a higher requirement for specific minerals than other commonly used experimental animals. In addition, they often show more visible gross deficiency signs in studies of mineral requirements. Golden Giant cockerel chicks[4] were distributed at random, six to each inside plastic cage. The diets, as described above, and high purity distilled, deionized water[5] were given ad libitum. Feed cups were not replaced throughout the experiment, but the water cups were replaced every other day. Droppings were removed daily.

The chicks were weighed and examined for abnormalities after being fed their respective diets for 3.5 weeks. They were then exsanguinated by cardiac puncture and decapitated. Portions of fresh liver were taken for measurement of oxygen utilization and electron-microscopic study. The remainder of the liver was frozen at −16 C for subsequent analysis. The oxygen uptake of liver homogenates was measured by the method of Lee and Hsu (22) with α-glycerophosphate as the substrate. Protein was determined by the biuret method after samples (0.1 ml) were dispersed with 0.4 ml of 1% NaCl and 0.5 ml of 10% sodium deoxycholate.

Tissue for electron microscopy was fixed by immersion in a paraformaldehyde-glutaraldehyde mixture buffered at pH 7.4 with sodium cacodylate. Tissue blocks were subsequently post-osmicated in 2% OsO_4 buffered with sodium cacodylate for 1 hour, dehydrated in ethanol, passed through propylene oxide, and embedded in Epon.[6] Thin sections were stained with lead citrate and uranyl acetate.

The lipids were extracted from the liver by the method of Friedman et al. (15). Lipid phosphorus was determined by the method of Taussky and Shorr (46). Liver cholesterol was determined by the method of Searcy and Bergquist (35) on 1 ml of dried extract which had been dissolved in chloroform.

Experiments were also initiated to evaluate the role of nickel in the nutrition of the rat. The diet and conditions used were similar to those used in raising chicks. Slight modification of the diet was made to meet the requirements of the rat (less Ca, Fe, Zn, glycine and choline; more vitamin E). In addition, laminar flow racks[7] were used to hold the plastic rat cages. To bring out the effects in rats, successive generations were raised. Thus, the animals were exposed to deficiency throughout fetal, neonatal, and adult life. Control rats were treated the same except they were fed the basal diet supplemented with 3 ppm nickel. The rats were observed for possible gross abnormalities and impaired reproductive ability. Also, oxygen utilization of liver homogenates of first generation adult rats was examined by the method described previously.

Statistical analysis was performed by the *t* test (37).

RESULTS AND DISCUSSION

After 3.5 weeks, all chicks weighed 350–400 g. Grossly, the appearances of

[3] Germ Free Laboratories, Inc., Miami, Florida. 33100.

[4] Jack Frost Chicks, Inc., St. Cloud, Minn. 56301.

[5] Produced by a "Super Q High Purity Water System," Millipore Corp., Bedford, Mass. 01730.

[6] Epon 812, Warum Chemical, St. Paul, Minn. 55100.

[7] Carworth, Division of Becton, Dickinson and Co., New City, N. Y. 10956.

the deficient and control chicks were similar except for the difference in the pigmentation of their shank skin as described previously (27–29).

In earlier studies (27–29) abnormalities in leg structure and a dermatitis were present in the deficient chicks. In the experiments reported here, the abnormalities were diminished or inconsistent. The decreased incidence or inconsistency of these abnormalities may have been due to modification of the diet and improved environmental conditions. The other gross sign observed in our earlier studies—a decrease in friability of the liver in deficient chicks—was observed in chicks raised under the conditions described above.

Thus, it appears that the change in shank skin color and the effect on liver consistency are related to nickel status but that the other gross signs originally described may be less characteristic of nickel deficiency.

In contrast to the gross signs, abnormalities in biochemical indices of metabolism were more consistently found in the nickel-deficient chicks. These included a decreased oxygen uptake by liver homogenates in the presence of α-glycerophosphate, an increase in liver lipids, a decrease in the liver phospholipids and no apparent change in liver cholesterol (Table 4).

Ultrastructural abnormalities in the hepatocytes (Figs. 1 and 2) were also a consistent finding. These findings were similar to, though more extensive than, those described by Sunderman et al. (40). They included dilation of the cisternae of the rough endoplasmic reticulum and the swelling of the mitochondria. This swelling was in the compartment of the matrix and was associated with fragmentation of the cristae. Other ultrastructural changes included a dilation of the perinuclear space, condensation of peripheral nuclear chromatin and pyknotic nuclei. These ultrastructural abnormalities coupled with the biochemical evidence of deranged metabolism are considered sufficient evidence to indicate that nickel is essential for the chick.

The results of the rat studies are more preliminary in nature because the experiments are still in progress. The results of measurements of the oxidative ability of rat liver homogenates were similar to those found in the chick (Table 5). In addition, sucrose density gradients of liver postmitochondrial supernatants were consistent with a decrease in polysomes and an increase in monosomes in the nickel-deficient rat liver (Fig. 3).

Seven first generation nickel-deficient female rats which were mated had a significant fetal loss at birth (15%) compared with no perinatal mortality in the newborn pups of the six controls.

These preliminary findings suggest that nickel is also essential for the rat.

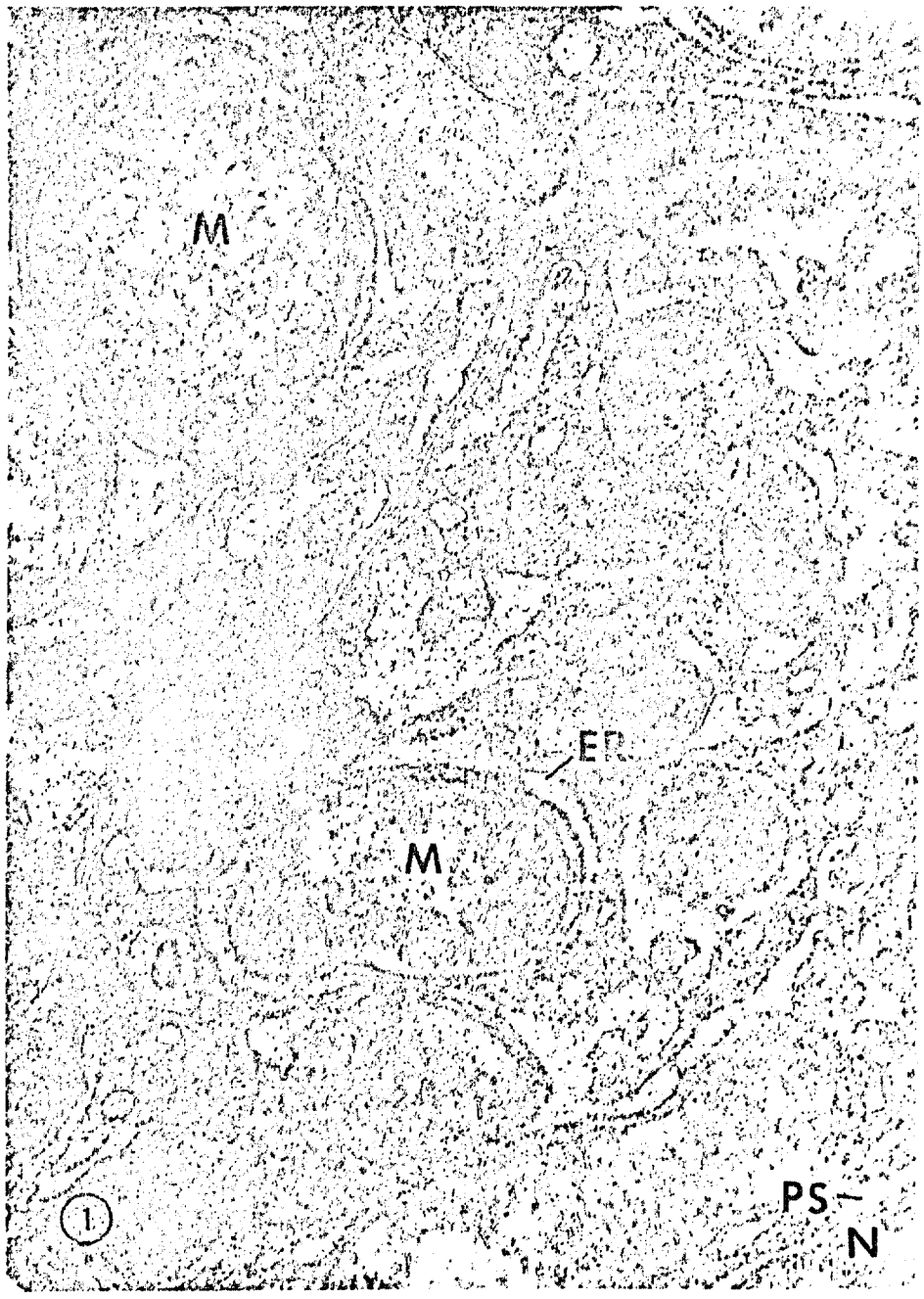
While the above findings indicate that

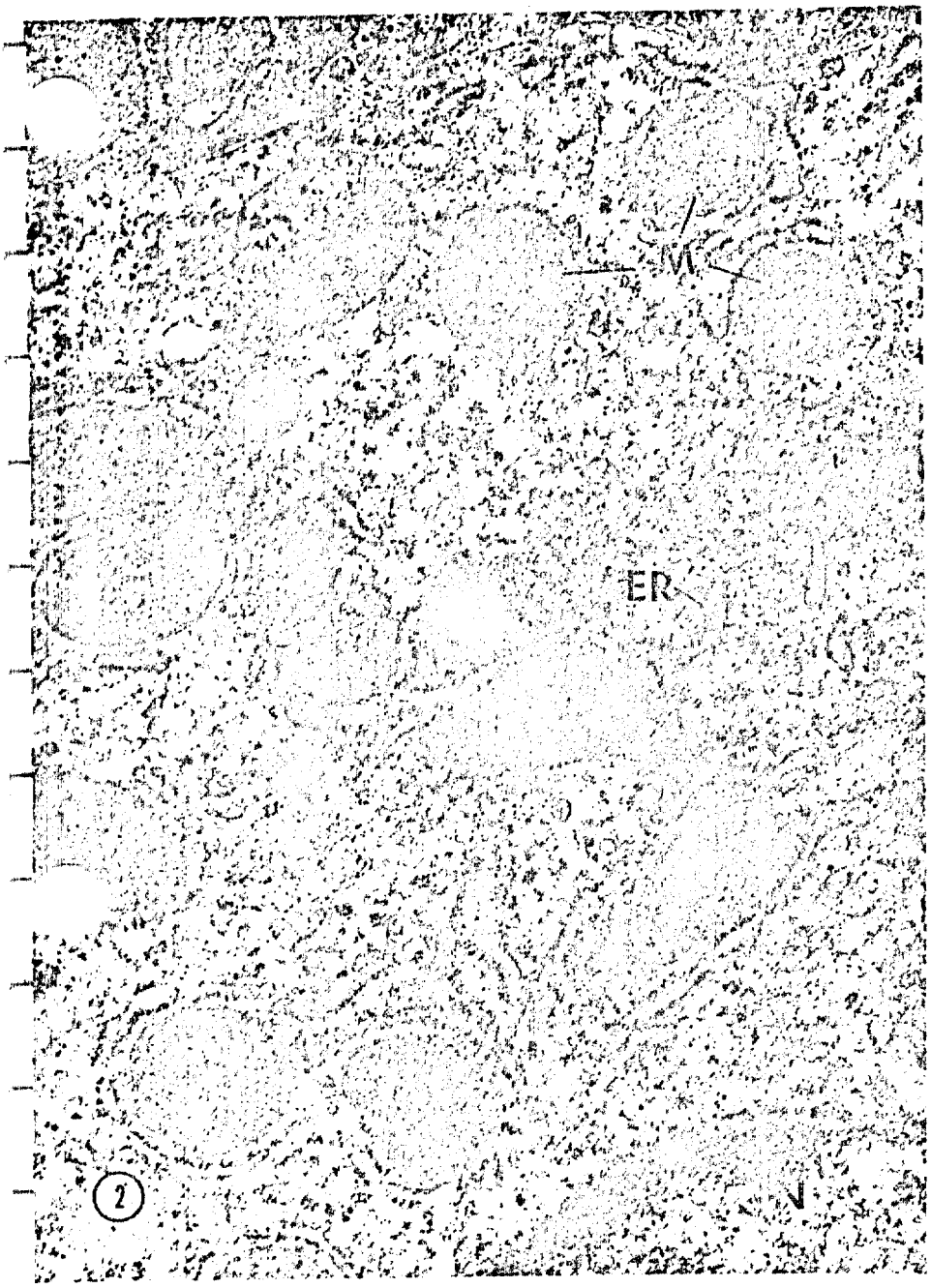
TABLE 5. Oxygen uptake of rat liver homogenates[a]

Group	No. of rats	O_2 Uptake μl hr per mg Protein
Ni def (4 ppb)	13	3.20[b] ± 0.08[c]
+ 3 ppm	12	4.17 ± 0.21

[a] α-Glycerophosphate as substrate. [b] Significantly different ($P < 0.001$) from +3 ppm Ni group. [c] ± SEM.

Figure 1. Hepatic cell from a nickel-deficient chick (4 ppb nickel). Swelling of mitochondria (M) was evident in numerous hepatic cells. The swelling was in the compartment of the matrix and appeared to cause fragmentation of cristae. Note also the dilated cisternae of the rough endoplasmic reticulum (ER) and dilated perinuclear space (PS). Nucleus (N). Uranyl acetate and lead citrate. ×30,000.





igure 2. Hepatic cell from nickel-supplemented chicks (3 ppm nickel). Compare mitochondria (M) with those in Fig. 1. Endoplasmic reticulum (ER). Nucleus (N). Uranyl acetate and lead citrate. ×30,000.

nickel is essential, they provide only meager insights as to its metabolic function. An attractive hypothesis is that nickel has a role in the metabolism or structure of membranes. The morphologic abnormalities, impaired oxidation and changes in phospholipid level in the liver are consistent with this suggestion. In addition, in vitro evidence and our preliminary findings on liver polysomes suggest that nickel may have a structural role in nucleic acids.

SUMMARY

Nickel deficiency in chicks results in suboptimal liver function as evidenced by an ultrastructural degeneration, reduced oxidative ability, increased lipid, and a decreased phospholipid fraction. Rats deprived of nickel also show a reduced oxidative ability in liver and abnormalities in the polysome profile. These findings are consistent with nickel being an essential nutrient for chicks and rats.

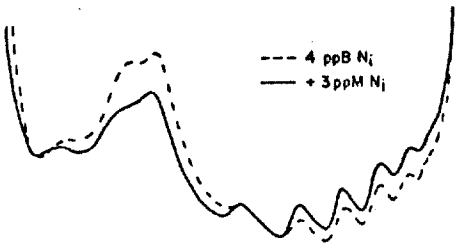


Figure 3. Representative sucrose density gradients of liver postmitochondrial supernatants obtained from 3 nickel-deficient (4 ppb Ni) and 3 supplemented (+3 ppm Ni) rats. Livers were homogenized after being diluted 1:3 (w/v) with medium containing 0.25 M sucrose, 5 mM Tris HCl, 25 mM KCl, and 5 mM $MgCl_2$ at pH 7.4. Supernatants obtained after centrifugation at 4,400 × *g*, at 4 C, were made 0.5% with respect to deoxycholate and centrifuged at 4,687 × *g*, at 4 C. The supernatants were then layered over a linear 15.5–35.5% sucrose density gradient and centrifuged for 160 min at 284,100 × *g* max, at 4 C. The gradients were monitored at 260 nm.

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Evidence of a Possible Requirement for Nickel by the Chick[1] (34896)

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Nickel has been shown to have a ubiquitous distribution in plant and animal tissues (1-3). Schroeder *et al.* (1) have discussed the possibility that nickel may be an essential element, suggesting that it may perform some physiological role in the animal body. Their reasoning is supported by several observations: (1) nickel shows biological activity *in vitro*, affecting certain enzymes; (2) it is present in plants and animals, including the newborn; (3) intestinal and hepatic barriers to nickel transport are implied; (4) there is little tendency for nickel to accumulate in tissues during a lifetime of exposure; and (5) evidence has been presented which indicates that nickel may have a role in pigmentation. Attempts have been made to show essentiality for nickel using methods of special diets (2, 4) and isotopic techniques (5). Definitive data for an *in vivo* role for nickel have not been obtained. The purpose of the studies to be reported here was to determine whether or not nickel performs a physiological role in the chick.

Materials and Methods. Day-old White Rock chicks[3] were used in each of two experiments. The birds were randomly distributed, without segregation according to sex, into groups of three each in experiment 1 and groups of four each in experiment 2. Each group was placed in a plastic cage fabricated in a manner similar to that described by Smith and Schwarz (6). Three of these cages containing the chicks were placed into each of two all-plastic isolators. The isolators were maintained at a temperature of 29°–32°.

The chicks in one isolator, 9 in experiment 1 and 12 in experiment 2, were fed a diet which contained <0.08 ppm nickel on an air-dried basis (determined by atomic absorption spectrophotometry[4]). This represented the low-nickel treatment group. The control group of chicks was housed in the other isolator in each experiment and was fed the same basal diet as the treatment group except that the control diet was supplemented with 5 ppm nickel as $NiCl_2 \cdot 6H_2O$. The compositions of the diets used in experiments 1 and 2 are presented in Table I. Compared to the diet for experiment 1, the following alterations were made in experiment 2: (1) corn oil was added to make the diet less hygroscopic; (2) additional arginine was provided since it has been shown that high levels of dietary arginine can augment a zinc deficiency (7); and (3) vitamin D_3 was added to the vitamin mix. The diets and glass-distilled water were provided *ad libitum* in polypropylene cups made from Erlenmeyer flasks.

At 3 weeks of age, the chicks were given a dose of radioactive nickel[5] as $^{63}NiCl_2$ by gavage. In experiment 1, five chicks from each isolator were given 20 μCi of ^{63}Ni; in experiment 2, nine chicks from each isolator were given 25 μCi of ^{63}Ni. The specific activity of the isotope used was approximately 5 Ci/g; thus, the doses contained approximately 4 and 5 μg of nickel in experiments 1 and 2, respectively.

In experiment 1, three chicks from each

[1] The principles of Laboratory Animal Care as promulgated by the National Society for Medical Research were observed.

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[3] Pollard Hatchery and Feed, Denver, Colorado.

[4] Model 303, Perkin-Elmer Corporation, Norwalk, Connecticut.

[5] Cambridge Nuclear Corp., Cambridge, Massachusetts.

TABLE 1. Composition of the Diets Employed.

Ingredients	Experiment 1	Experiment 2
	g	g
Skim milk powder[a]	500.0	500.0
Corn meal, degerminated, enriched, yellow[b]	475.1	419.6
L-Arginine	3.5	30.0
Glycine	10.0	10.0
Corn oil	—	20.0
Mineral mix[c]	10.4	10.4
Vitamin mix	1.0[d]	10.0[e]

[a] Nutritional Biochemicals Corp., Cleveland, Ohio.

[b] The Quaker Oats Co., Chicago, Illinois.

[c] Mineral mix contained: (in g) $Ca_3(PO_4)_2$, 8.75; NaCl, 1.5; $MnCO_3$, 0.125; ZnO, 0.025; KI, 0.0005.

[d] Vitamin mix contained: (in mg) vitamin A palmitate (250,000 IU/g), 4.0; DL-alpha tocopherol powder (250 IU/g), 4.0; menadione, 0.5; pyridoxine HCl, 1.0; folic acid, 0.9; and corn meal to 1.0 g.

[e] Vitamin mix contained: (in mg) vitamin A palmitate (250,000 IU/g), 4.0; DL-alpha tocopherol powder (250 IU/g), 4.0; menadione, 0.5; pyridoxine HCl, 1.0; folic acid, 0.9; vitamin D_3 (40,000,000 IU/g), 0.01; and corn meal to 10 g.

treatment group (low-nickel and control) were sacrificed 6 hr after isotope administration; and two chicks, 24 hr after isotope administration. In experiment 2, three chicks from each treatment group were sacrificed at each of three time intervals after isotope administration: 6, 24, and 48 hr. Immediately prior to sacrifice by decapitation, the birds were weighed and observed for gross abnormalities. The blood was collected in a centrifuge tube containing 0.1 ml of heparin (100 units). A portion of the blood was centrifuged to facilitate separation of red blood cells from the plasma. Other tissues removed at sacrifice were: tibia, kidney, spleen, liver, duodenum, gizzard, lung, muscle, skin, heart, aorta, and feather. All tissues were frozen until ^{63}Ni analysis could be made. For the analysis, a bone sample was a 100-mg portion including the marrow from the center of the tibia shaft. The tibias were measured for length and widths with calipers, using the largest length and the smallest diameter. To facilitate the measurements, the flesh was removed from the bone by rubbing the shaft with cheesecloth.

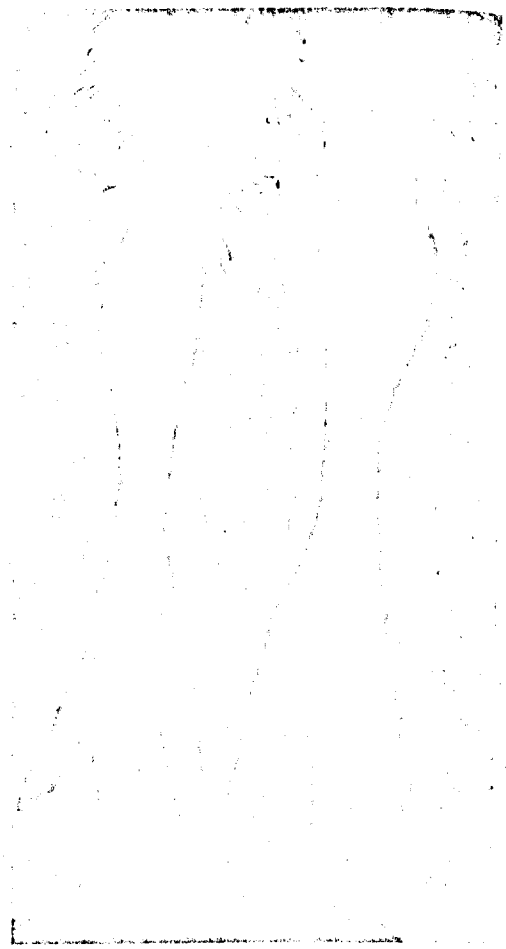

FIG. 1. Effect of dietary nickel upon the leg development of chicks. The leg on the left is from a control chick fed a diet supplemented with 5 ppm nickel. The leg on the right shows the thickened leg bone and swollen hock typical of those chicks fed a diet containing <0.08 ppm nickel. Both chicks were approximately the same weight.

Sample preparation and counting procedure were similar to the method of Smith and Hackley (5) with the counting being done in a liquid scintillation counter[6]. Statistical analysis was by the *t* test (8).

Results. The gross appearance of the chicks was affected by the amount of nickel

[6] Model Mark 1, Nuclear Chicago Corp., Des Plaines, Illinois.

in the diet in both experiments. One of the influences of nickel was upon the pigmentation of the legs. Chicks fed the low-nickel diet (<0.08 ppm) showed a bright orange-yellow color in their legs. By comparison, the chicks fed the nickel-supplemented diet had a pale brown-yellow color in their legs. Another difference noted between the low-nickel and control chicks concerned leg development. Figure 1 shows that the chicks fed the low-nickel diet had slightly thickened legs compared to the control birds. Also, the low-nickel chicks appeared to have somewhat swollen hock joints. Compared to the control chicks, the low-nickel chicks appeared to walk with a slightly abnormal clumsy gait.

Table II shows the results of the measurements of tibia bone size, expressed as the ratio of the length to width. This length: width ratio was significantly reduced in the chicks fed the low-nickel diet compared to the chicks fed supplemental nickel. Also presented in Table II are the average final body weights of the chicks. There was no significant effect of dietary nickel upon body weight.

In experiment 1, the administered ^{63}Ni appeared to be retained to a greater extent in the tissues of the chicks fed the low-nickel diet than in those of the control chicks. This appeared to be evident for bone, kidney, liver, heart, and aorta at both the 6-hr and 24-hr interval after dosage. As a result of these observations, a more detailed study was made on the distribution of ^{63}Ni in tissues in experiment 2 (Table III). In this experiment, the low-nickel chicks retained a significantly greater amount of ^{63}Ni in the liver, spleen, aorta, and, possibly, bone than did the control birds at the 6-hr interval after isotope administration. At the 48-hr time period, the low-nickel chicks showed a significantly higher isotope concentration in the bone.

TABLE II. Body Weights and Tibia Length: Width Ratios of Chicks Fed Different Levels of Nickel.

Experiment[a]	Dietary nickel (ppm)	Body[b] weights (g)	Length:width ratio of tibia
1	<0.08[c]	130	—
1	5.00	128	—
2	<0.08[c]	121	17.62[d]
2	5.00	109	18.38[d]

[a] Mean of 5 chicks in experiment 1; 10 chicks in experiment 2.
[b] Body weights at 3 weeks of age.
[c] Nickel content by analysis of diet with no supplemental nickel.
[d] Significant difference at the 0.05 level.

Several tissues took up relatively large amounts of ^{63}Ni during the 6 hr after dosage, including bone, kidney, liver, and aorta. A large amount of isotope was still retained by the bone, kidney, and liver 48 hr after administration. Conversely, very little ^{63}Ni was found in muscle and blood (red blood cells and plasma) after the 6-hr time period.

Discussion. The data presented herein appear to support the contention that nickel may have a physiological role in chicks with respect to pigmentation and leg structure. Kitkawa *et al.* (9) proposed that color was dependent upon the presence of specific metals. They showed that the yellow and white pigments from rabbit hair contained nickel. Their *in vitro* evidence led to the conclusion that nickel is especially important in white pigmentation. The data reported in this paper provide *in vivo* evidence that dietary nickel affects the yellow color in the legs of the chick.

Other investigators have attempted to find abnormalities in rats and mice fed low-nickel diets (2, 4). Growth rate was monitored in those studies. With chicks, in the present study, dietary nickel had no significant effect upon growth rate. However, nickel did appear to influence bone formation in chicks. Not only did the low-nickel chicks appear to have thickened legs and swollen hocks, the length:width ratios of the tibias from the low-nickel chicks were significantly lower than those from chicks fed the nickel-supplemented diet. The fact that leg abnormalities have not been noted by previous workers in either rats or mice fed low-nickel diets is not surprising. Leg abnormalities normally occur in chicks fed either zinc-deficient or manganese-deficient diets, whereas rats

TABLE III. Distribution of ^{63}Ni in Selected Tissues at Time Intervals After a Single Dose (Experiment 2).[a]

Tissue	Hours after dose					
	6		24		48	
	Ni Low[b]	Ni High[c]	Ni Low	Ni High	Ni Low	Ni High
	(% dose/g fresh tissue)					
Bone	0.296[d]	0.128[e]	0.101	0.076	0.098[j]	0.041[k]
Epiphyseal plate	0.102	0.070	0.023	0.024	0.015	0.017
Primary spongiosa	0.134	0.072	0.034	0.023	0.029	0.024
Hyaline cartilage	0.096	0.043	0.017	0.014	0.016	0.008
Blood	0.041	0.035	0.004	0.005	0.002	0.002
Red blood cells	0.021	0.015	0.002	0.002	0.002	0.001
Plasma	0.054	0.044	0.004	0.006	0.003	0.002
Duodenum	0.035	0.014	0.008	0.008	0.006	0.005
Kidney	0.069	0.292	0.291	0.073	0.193	0.141
Spleen	0.044[f]	0.023[m]	0.021	0.020	0.017	0.010
Liver	0.103[g]	0.042[n]	0.062	0.039	0.069	0.035
Lung	0.052	0.030	0.012	0.011	0.019	0.010
Muscle	0.018	0.017	0.003	0.004	0.003	0.003
Skin	0.051	0.048	0.019	0.022	0.023	0.023
Aorta	0.157[h]	0.053[r]	0.026	0.016	0.021	0.025
Heart muscle	0.020	0.029	0.016	0.023	0.009[s]	0.052[t]
Feather	0.032	0.035	0.040	0.049	0.064	0.046
Gizzard lining	0.102[i]	0.196[o]	0.008[k]	0.016[t]	0.006	0.005

[a] Each value represents the mean of three chicks.

[b] Ni Low indicates those chicks fed the basal diet which contained <0.08 ppm nickel on an air-dried basis.

[c] Ni High indicates those chicks fed the basal diet supplemented with 5 ppm nickel.

[d,e,f,g,h,i] Significant at the 0.1 level.

[j,k,l,m,n,o,p,q,r,s,t] Significant at the 0.5 level.

fed either of these types of diets usually show no gross leg defects. Perhaps a similar phenomenon occurs in the case of nickel.

Smith and Hackley (5) found an apparent selective uptake of ^{63}Ni in their studies with rats fed a diet containing 3.3 ppm nickel. They suggested, however, that the selective uptake may have been due to a difference in blood volume of the various tissues. This could have contributed to the differences as their isotope was administered iv and, thus, the blood was relatively high in ^{63}Ni even 16 hr after isotope administration. In the present experiments, after an oral dose, a relatively small amount of ^{63}Ni was found in the blood, especially for periods of time greater than 6 hr after dosage. Still, relatively large amounts of ^{63}Ni were found in kidney, bone, and liver at all time intervals studied; whereas, muscle retained very little of the isotope. Therefore, it apears that other factors than just blood volume or blood level of nickel are affecting the apparent selective uptake of nickel by the tissues. Furthermore, the low-nickel chicks retained significantly greater amounts of ^{63}Ni in the liver, aorta, and, possibly, bone at the 6-hr time period than did the control chicks. Several other tissues from the low nickel chicks appeared to retain greater amounts of ^{63}Ni than similar tissues from the control birds, but these differences were not significant. These data could be interpreted as indications that homeostatic mechanisms are trying to maintain nickel in sufficient quantities in various tissues in which it may have an important physiological role.

Due to the limitations of the analytical

method, the level of nickel in the low-nickel diet is reported as <0.08 ppm. The data would seem to suggest that this dietary concentration of nickel is low enough to demonstrate that nickel does perform a physiological function in the growing chick. Efforts are being made to determine the exact concentration of nickel in the diet and to formulate a diet of lower nickel concentration than the one presently used. Only then can it be fully determined whether nickel is an essential element and, if so, the exact level of nickel required to prevent changes from normal physiological function.

Summary. After 3 weeks on a dried skim milk-corn meal-based low-nickel diet (<0.08 ppm Ni), chicks which were maintained in an all-plastic controlled environment system had the following symptoms: (1) a change in leg pigmentation; (2) thickening of the long bones; (3) swelling of the hock joint; and (4) a significantly reduced length:width ratio of the tibia. All symptoms were prevented by the supplementation of 5 ppm nickel to the diet. Also, chicks fed the low-nickel diet accumulated a significantly greater amount of ^{63}Ni in the liver, spleen, aorta, and possibly bone during the 6 hr after oral dosage than did control birds. This evidence supports the hypothesis that nickel has a physiological role in the chick.

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Atomic Absorption Spectrometry of Nickel in Serum, Urine, and Other Biological Materials

Shozo Nomoto and F. William Sunderman, Jr.

An atomic absorption technique is described for analysis of nickel in serum, whole blood, urine, and other biological materials. After the sample has been subjected to protein precipitation or acid digestion, nickel is extracted into methylisobutylketone as a pyrrolidine dithiocarbamate complex, and is measured by atomic absorption spectrometry. The following values (mean ±SD) were obtained for nickel in specimens from healthy subjects living in central Connecticut: serum, 0.26 ± 0.08 μg/100 ml ($N = 40$); whole blood, 0.48 ± 0.13 μg/100 ml ($N = 17$); and urine, 0.23 ± 0.14 μg/100 ml, = 2.4 ± 1.1 μg/day ($N = 26$). In sera obtained from 36 patients within 24 h after myocardial infarction, the mean nickel concentration was 0.51 ± 0.27 μg/100 ml (p *vs.* controls = 0.001). In 132 consecutive adult patients, admitted to the hospital for all diagnoses except myocardial infarction, the mean concentration of serum nickel was 0.22 ± 0.11 μg/100 ml. Measurements of serum nickel may prove to be useful in diagnosis of acute myocardial infarction.

Additional Keyphrases *normal values • nickel pyrrolidine dithiocarbamate complex • methylisobutyl ketone extractant • chelation of nickel • acid digestion vs. TCA precipitation • industrial hazard • myocardial infarction*

Schaller, Kühner, and Lehnert (*1*) developed an atomic absorption procedure for measurements of nickel in human blood and plasma. The method of Schaller *et al.* (*1*), used in our laboratory for over a year, was found to be more convenient than the spectrophotometric (*2*) or the atomic absorption (*3*) procedures that we had previously reported. The technique of Schaller *et al.* (*1*) has undergone modifications in our laboratory that have improved its sensitivity and have extended its application to analyses of nickel in urine and tissues.

Our modification of the procedure has been used for determinations of nickel in serum from healthy subjects and from patients with myocardial infarction. These measurements have confirmed d'Alonzo and Pell's finding that the concentration of serum nickel is significantly increased during the 24 h after an acute myocardial infarction (*4*).

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Method and Materials

Principle

Addition of trichloroacetic acid to serum dissociates nickel from serum proteins and simultaneously precipitates the proteins. Nickel in the protein-free supernatant fraction is converted to a pyrrolidine dithiocarbamate complex and is extracted into methylisobutylketone. The concentration of nickel in the methylisobutylketone extract is measured by atomic absorption spectrometry. The same technique is applicable to analyses of nickel in whole blood, urine, and tissues, except that organic constituents are destroyed by preliminary acid digestion.

Reagents for Analysis of Serum Nickel

All concentrated acid and base reagents are of "ultra-pure" grade (E. Merck Co., Darmstadt,

West Germany, purchased from Brinkmann Instrument Co., Westbury, N.Y.). All water is deionized, and distilled in an all-glass still.

Nickel stock standard, 50 μg of Ni per ml. Powdered nickel, 50 mg, is placed in a 50-ml beaker and dissolved with heating in a mixture of 5 ml of concentrated nitric acid and 5 ml of water. The solution is quantitatively transferred to a 1-liter volumetric flask and diluted to volume with water.

Nickel working standard, 0.05 and 0.10 μg of Ni per ml. One-milliliter portions of the nickel stock standard solution are transferred to 1-liter and 500-ml volumetric flasks, and diluted to volume with water.

TCA,[1] *15 g/100 ml.* One-hundred-fifty grams of "metal-free" trichloroacetic acid (Distillation Products Div., Eastman Chemical Co., Rochester, N.Y., Catalog no. X-259) is dissolved in 1 liter of water. This solution is stored refrigerated and is prepared every two weeks.

MIBK. "Certified" grade solvent (Fisher Scientific Co., Pittsburgh, Pa.).

MIBK *saturated with* TCA. This is used in establishing the spectrometer baseline. Into a 1-liter separatory funnel are placed 400 ml of MIBK and 100 ml of TCA solution. The mixture is shaken and allowed to stand in a refrigerator for 1 h. The MIBK phase is removed and centrifuged to eliminate traces of aqueous phase. This solution is prepared each week.

APDC, *2 g/100 ml.* One gram of ammonium pyrrolidine dithiocarbamate (K & K Laboratories, Plainview, N.Y.) is dissolved in 50 ml of water, and the solution is extracted twice with 5 ml of MIBK. This solution is prepared each day.

Phthalate buffer, pH 2.5. To approximately 400 ml of water in a 1-liter volumetric flask are added 10.2 g of potassium hydrogen phthalate and 39 ml of 1.0N hydrochloric acid. The contents of the flask are dissolved, diluted to volume with water, and transferred to a 2-liter separatory funnel. The pH of the solution is tested with a pH meter, and adjusted, if necessary, to pH 2.5 by adding potassium hydrogen phthalate or hydrochloric acid. Ten milliliters of the APDC solution is added, and the mixture is shaken vigorously. The buffer is extracted at least 5 times with 25 ml of MIBK. The nickel content of the final MIBK extract, as measured by atomic absorption, should not differ from that of pure MIBK.

Concentrated ammonium hydroxide, 25 g/100 ml.

Ammonium hydroxide, 2.5 g/100 ml. Twenty-five milliliters of the concentrated reagent is diluted to 250 ml with water.

[1] Abbreviations used: TCA, trichloroacetic acid; MIBK, methylisobutylketone; APDC, ammonium pyrrolidine dithiocarbamate; Ni-PDC, nickel pyrrolidine dithiocarbamate.

Additional Reagents for Analyses of Whole Blood, Urine, or Tissues

Sulfuric–nitric acid. One part of concentrated sulfuric acid (96 g/100 ml) is mixed with 5 parts of concentrated nitric acid (65 g/100 ml).

Perchloric acid, 70 g/100 ml.

Hydrochloric acid. Three-hundred milliliters of concentrated hydrochloric acid (30 g/100 ml) is diluted to 1 liter with water.

Special Apparatus

Atomic absorption spectrometer (Perkin-Elmer Corp., Norwalk, Conn., Model 403). This is fitted with a nickel hollow cathode lamp, a "Boling" three-slot acetylene–air burner, and a 10-in. strip-chart recorder. The settings are: wavelength, 232 nm; nickel lamp current, 16 mA; acetylene flow, 4.2 liters/min; air flow, 23 liters/min; range, *UV*; filter setting, *OUT*; entrance slit, position *3*; recorder response, position *2*; recorder setting, *0.25 A* full scale; concentration dial setting, *75*; and curvature correction, *0*.

Apparatus for collection of blood. Glass syringes fitted with platinum–ruthenium needles (Hamilton Co., Whittier, Calif.), or Vacutainer tubes ("lead-free") with unsoldered steel needles (Becton-Dickinson Co., Rutherford, N.J.).

Procedure for Serum Nickel Analysis

1. Ten-milliliter samples of serum are transferred in duplicate to 50-ml centrifuge tubes. One-milliliter of each of the two nickel working standard solutions and 9 ml of water are transferred in duplicate to additional 50-ml tubes. These standard samples are equivalent to 0.5 and 1.0 μg of Ni per 100 ml of serum. Ten-milliliter samples of water are placed in duplicate 50-ml tubes to serve as the "reagent blanks."

2. Twenty milliliters of TCA solution is added slowly to all of the tubes, with constant mixing. The glass-stoppered tubes are agitated for 30 min by a mechanical apparatus and then centrifuged at 900 *g* for 15 min.

3. Twenty-milliliter aliquots of the protein-free supernatant phases are transferred into clean 50-ml centrifuge tubes, and 2 ml of phthalate buffer added.

4. Concentrated ammonium hydroxide solution is added dropwise with constant mixing until the pH reaches approximately 2.0, as monitored with a pH meter. Then the pH is adjusted to pH 2.5 (2.4 to 2.6) by dropwise addition of dilute ammonium hydroxide solution.

5. Two milliliters of APDC solution is added and the contents are mixed. Three milliliters of MIBK is added, and each tube is mixed with a Vortex mixer for 20 s, and placed in an ice-water bath for

10 min. The tubes are then centrifuged at 900 *g* for 5 min.

6. With Pasteur pipets, each MIBK extract is transferred to a small, stoppered test tube, with care to avoid any transfer of aqueous phase.

7. Reagent-grade MIBK is aspirated into the burner of the atomic absorption spectrometer. The flame is adjusted to dark blue, with a bright-blue segment (5-mm high) immediately above the burner. The reagent-grade MIBK is allowed to aspirate for 20 to 30 min, to stabilize flame conditions. MIBK saturated with TCA is then aspirated for 20 min with the strip-chart recorder on, to verify absolute stability of the recorder baseline.

8. MIBK extracts are aspirated into the burner, and the absorbances of the samples are recorded. Heights of the absorbance peaks are measured, and the concentrations of nickel in the serums are calculated by proportionality with the standard samples.

Procedure for Nickel Analysis in Urine, Whole Blood, and Tissues

1. The unknown samples (50 ml of urine, 10 ml of heparinized whole blood, or 10 ml of a 20 g/100 ml tissue homogenate) are transferred to duplicate 125-ml Erlenmeyer flasks. Standard and blank samples are treated similarly. Ten milliliters of sulfuric–nitric acid mixture is added to each flask. The flasks are heated gently on a hot plate with intermittent swirling until the contents are clear. Digestion is continued with increased heat until charring occurs and white fumes of sulfur trioxide are generated.

2. The flasks are cooled and 2 ml of sulfuric–nitric acid mixture and 0.5 ml of perchloric acid are added. Digestion is continued until white fumes of sulfur trioxide appear.

3. The flasks are cooled, and 1 ml of sulfuric–nitric acid mixture and 0.25 ml of perchloric acid are added. The digestion is continued for 20 min after the samples have become practically colorless. The final volume of the digestion mixture should be less than 2 ml.

4a. In analysis of *urine samples*, the flasks are cooled and the contents are transferred quantitatively with four washes of water into 50-ml centrifuge tubes. The contents are adjusted to approximately 20 ml by addition of water.

4b. In analysis of *whole blood* or samples of *tissues* (such as muscle or liver) that contain appreciable amounts of iron, the flasks are cooled and 5 ml of dilute hydrochloric acid is added. The flasks are then heated to boiling and again cooled. Six milliliters of MIBK is added to extract iron, and the flasks are shaken. After the phases have separated, the MIBK layer is aspirated and discarded. The extraction is repeated with 3 ml of MIBK. The residual aqueous phase is transferred quantitatively, with four washes of water, to 50-ml centrifuge tubes. The contents are adjusted to approximately 15 ml by adding water. The tubes are centrifuged at 900 *g* for 5 min, and remaining traces of MIBK are aspirated and discarded.

5. Two milliliters of phthalate buffer is added, and the procedure is continued exactly as described for serum nickel, beginning at Step 4.

Results

Sensitivity

Under the described operating conditions, the gram atom absorptivity of nickel in MIBK was 1.2×10^4. The limit of detection of nickel was 0.005 μg of Ni per 3 ml of MIBK, the concentration of nickel that yielded a recorder signal equivalent to twice the baseline noise level. With 10-ml samples of serum or whole blood, the detection limit therefore was 0.05 mg/100 ml. of sample. With 50-ml samples of urine, the detection limit was approximately 0.01 mg/100 ml of urine. A typical recorder graph obtained with blank, standard, recovery, and urine samples is shown in Figure 1.

Flame Characteristics

Careful adjustment of the ratio of acetylene and air was essential to optimum sensitivity with minimum baseline noise. The sensitivity achieved under the described conditions (with use of the "Boling" three-slot burner with acetylene–air fuel) was one and a half times that obtained with the Perkin-Elmer single-slot burner with acetylene–air fuel, and six times that obtained with the Perkin-Elmer N_2O burner with nitrous oxide–acetylene fuel.

Calibration Curves and Computation Techniques

Under the described conditions, peak height was linearly related to nickel concentration from 0.01 to

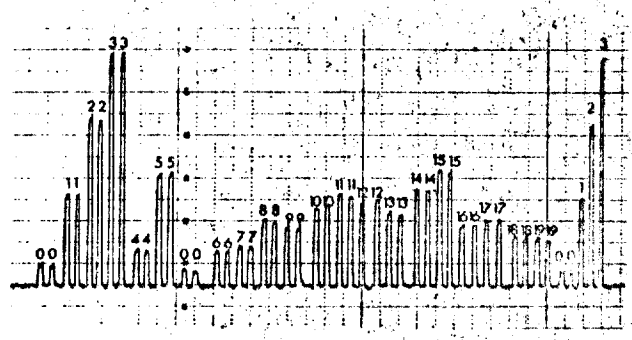

Fig. 1. Typical recorder graph for atomic absorption analyses of urine nickel

0, reagent blanks; 1, 2, and 3 are standards, equivalent to 0.5, 1.0, and 1.5 μg Ni per 100 ml of urine, respectively; 4, urine "control" specimen; 5, urine "control" specimen plus added Ni standard equivalent to 0.5 μg Ni per 100 ml; 6 to 19, urine specimens from patients

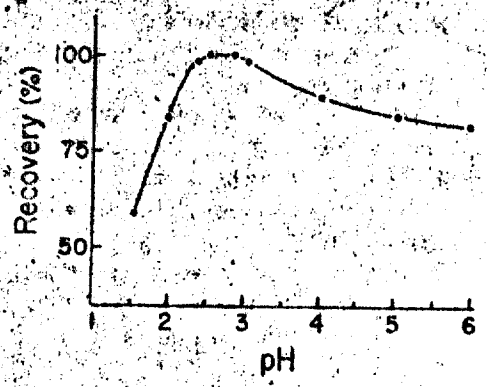

Fig. 2. Influence of pH on extraction of Ni-PDC into MIBK

1.0 μg Ni per sample. When computations were based on measurements of peak height, the coefficient of variation of 20 pairs of duplicate analyses of nickel in serum samples was 8.8%. In comparison, the coefficient of variation of the same pairs of duplicate analyses was 12.4% when computations were based on measurements of the areas under recorder peaks, as described by Rodgerson and Helfer (*5*). A mechanical "ball and disc" integrating device (Disc Instruments, Inc., Santa Anna, Calif.) was attached to the strip-chart recorder to perform these measurements. The technique of Sprague and Slavin (*6*) for computation of nickel concentrations, based upon the different absorbencies of nickel at 232.0 nm and at 231.6 nm, was also investigated: so computated, identical values were found for nickel concentrations in respective samples of serum, whole blood, or urine as when computations were done as described here.

Chelation and Extraction of Nickel

As shown in Figure 2, pH 2.5 was optimal for the extraction of the Ni–PDC complex into MIBK. Extraction of Ni–PDC into MIBK was not affected by exposure of the samples to intense sunlight for 15 min after the addition of APDC. Likewise, if 10 mg of ascorbic acid was added to standard, serum, and urine samples before adding APDC, it did not affect the extraction of Ni–PDC into MIBK. The duration and technique of shaking for the extraction of Ni–PDC into MIBK were not critical. It was necessary to cool and centrifuge the MIBK extracts to remove dissolved water, which was otherwise a source of recorder fluctuations.

In analyses of whole blood and tissue homogenates, it was necessary to remove iron before APDC was added. For this purpose, preliminary extraction of iron into MIBK was used, as suggested by Takeuchi (*7*). If iron was extracted into MIBK before APDC was added, no detectable loss of nickel resulted.

Precision

As a measure of the day-to-day variability of the method, the coefficient of variation of 17 consecutive daily measurements of nickel concentration in a pooled specimen of serum was 9.9%. As measures of within-run variability, the coefficient of variation of duplicate analyses of nickel in 91 sera was 8.7%, and the coefficient of variation of duplicate analyses of nickel in 32 specimens of whole blood was 11.3%. The coefficient of variation for duplicate analyses of nickel in 50 urine specimens was 10.3%.

Recovery

Nickel was added to 17 specimens of serum in concentrations ranging from 1.2 to 2.5 μg/100 ml (Table 1). Based on all of the 17 measurements, the mean recovery of nickel was 101% ,with a range of 97 to 107%. As shown in Table 1, essentially the same recovery values were also obtained when nickel was added to samples of whole blood, urine, or tissues.

Tests for Interference

To a series of standard samples containing 0.5 μg of nickel were added soluble salts containing elements and ions that might interfere. The elements and ions were tested for interference by addition-

Table 1. Recovery of Nickel Added to Biological Materials

Biological sample	No. specimens	Ni added (μg/100 ml or μg/100 g)	Ni recovered (μg/100 ml or μg/100 g)	% Recovery, mean and range
Serum	4	1.20	1.21	101 (100–102)
Serum	3	1.80	1.87	103 (100–105)
Serum	3	2.40	2.44	102 (98–104)
Serum	7	2.50	2.52	101 (97–107)
Whole blood	4	2.50	2.54	102 (97–105)
Urine	5	2.50	2.50	100 (98–102)
Tissues[a]	8	2.50	2.46	98 (90–109)

[a] Lung (4), liver (2), and cardiac muscle (2).

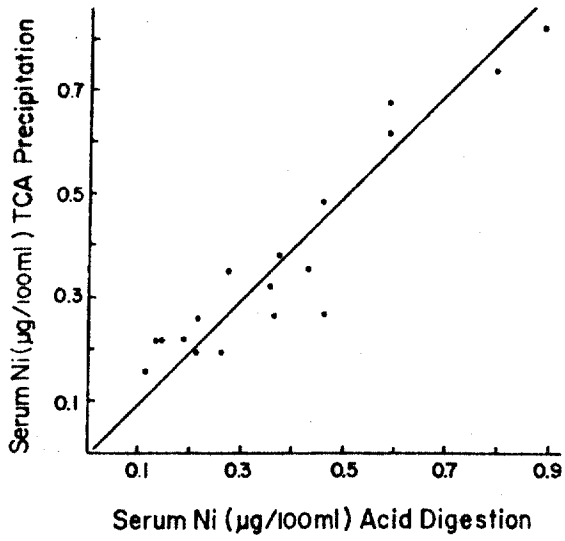

Fig. 3. Serum nickel measurement by atomic absorption

Values obtained after precipitation of serum proteins with trichloroacetic acid compared with those obtained after destruction of organic constituents by acid digestion

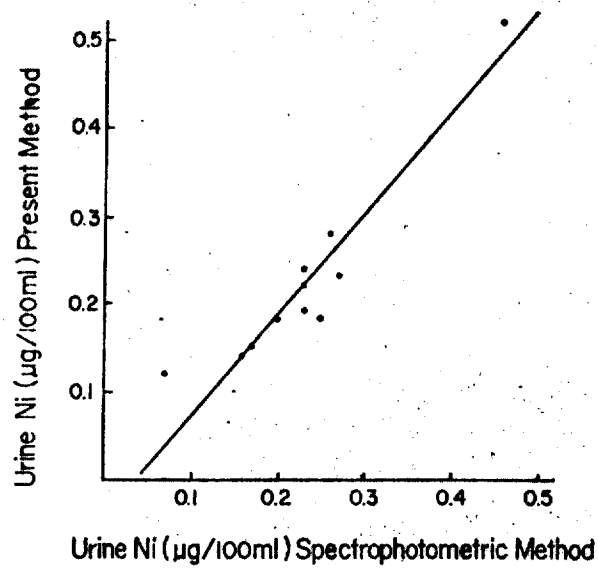

Fig. 4. Urine nickel as measured by the present atomic absorption method and by the ultraviolet spectrophotometric method (*25*)

of 10-ml volumes of solutions containing the following concentrations: sodium, potassium, and chloride, 200 mEq/liter; calcium, magnesium, ammonium, bicarbonate, carbonate, phosphate, and sulfate, 10 mEq/liter; aluminum, arsenic, bismuth, chromium, cobalt, iron, lead, lithium, manganese, mercury, molybdenum, silver, strontium, uranium, vanadium, zinc, borate, bromide, citrate, cyanide, fluoride, nitrate, nitrite, oxalate, persulfate, sulfite, and thiosulfate, 1 mg/100 ml; and copper, 0.5 mg/100 ml.

The samples were carried through the entire procedure for serum nickel, including chelation with APDC and extraction into MIBK. None of these elements or ions changed the absorbance of the nickel standards by more than 1%. Cadmium and gold proved to be the only potential sources of interference. Solutions of cadmium salts (10 µg of of Cd per 100 ml) and gold salts (25 µg of Au per 100 ml) caused 5% inhibition in the atomic absorption of nickel standards. At a concentration of 100 µg of Cd per 100 ml, the inhibition of atomic absorption of nickel averaged 25%. The interference was apparently caused by adsorption of Ni-PDC to insoluble complexes of cadmium or gold with pyrrolidine dithiocarbamate. No interference was observed when cadmium or gold solutions were added directly to nickel standard solutions, immediately prior to atomic absorption spectrometry.

Comparisons with Other Techniques

To determine whether nickel was completely released from serum protein during TCA precipitation, 20 sera were analyzed by atomic absorption after acid digestion and after trichloroacetic acid treatment (Figure 3). The correlation coefficient of the two sets of analyses was 0.93; the standard error of estimate was ±0.078, and the equation of the regression line was $x = 0.997y + 0.003$. Based upon a paired sample "t" test, there was no significant difference between the two sets of measurements. Nickel in one of these specimens of serum was also analyzed by the spectrophotometric method of Sunderman (*2*); the mean of triplicate analyses was 0.52 µg/100 ml (range, 0.44 to 0.55). The mean of six analyses by the present method was 0.50 µg/100 ml (range, 0.40 to 0.57).

Nickel was measured in the urines of 12 healthy subjects by the present method using the spectrophotometric method of Sunderman (*2*), and by the earlier atomic absorption technique of Sunderman (*3*). The older procedure extracted nickel dimethylglyoximate into chloroform, and recovered nickel by re-extraction into hydrochloric acid.

The correlation coefficient between analyses by the present method and the spectrophotometric technique was 0.93; the standard error of the estimate was ±0.032, and the equation of the regression line was $x = 0.82y + 0.048$ (Figure 4). The correlation coefficient for analyses by the present method and the dimethylglyoxime extraction technique was 0.94; the standard error of the estimate was ±0.031, and the equation of the regression line was $x = 0.85y + 0.038$ (Figure 5). From the results of paired-sample "t" tests, we conclude that there were no significant differences between the three sets of measurements.

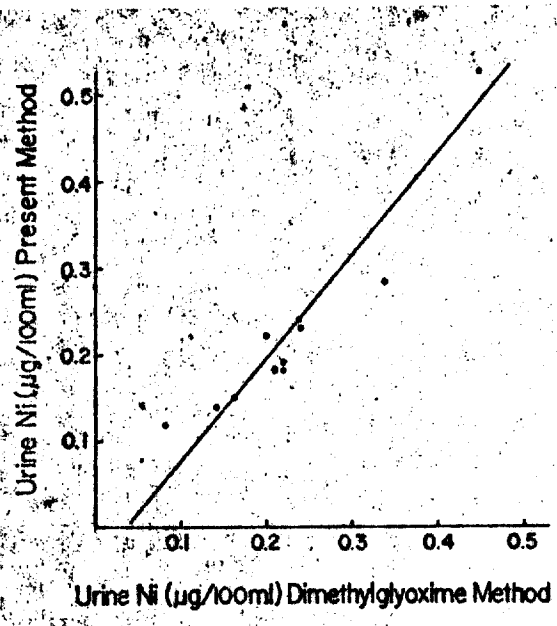

Fig. 5. Urine nickel, as measured by the present atomic absorption method and by the former dimethylglyoxime method (24)

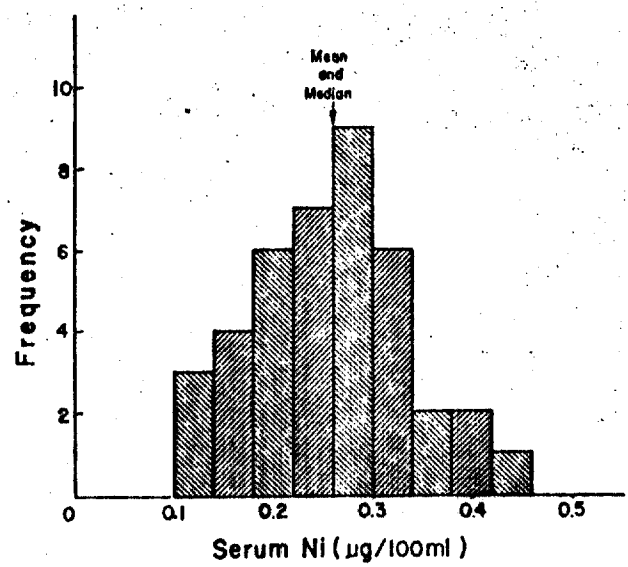

Fig. 6. Frequency distribution graph for nickel concentrations in sera of 40 healthy subjects

Normal Values for Serum Nickel

A frequency-distribution graph of nickel concentrations in sera of 40 healthy persons is shown in Figure 6. The subjects were 23 men and 17 women, residents of central Connecticut. The mean concentration of serum nickel was 0.26 µg/100 ml, (SD ±0.08; range, 0.11 to 0.46). The median concentration of serum nickel was 0.26 µg/100 ml. The mean concentration of serum nickel in the men (0.26 ± 0.08) did not differ significantly from that in the women (0.27 ± 0.07).

Normal Values for Nickel in Whole Blood

Nickel was measured in specimens of whole blood from 17 healthy persons (10 men, 7 women); we found a mean concentration of 0.48 µg/100 ml (SD ±0.13; range, 0.29 to 0.70), and a median concentration of 0.50 µg/100 ml. The mean concentration of blood nickel in the men (0.45 ± 0.14) did not differ significantly from that in the women (0.52 ± 0.11).

Normal Values for Urine Nickel

A frequency-distribution graph for nickel excretion in 24-hour collections of urine from 26 healthy persons is shown in Figure 7. Again, the subjects were 14 men and 12 women living in central Connecticut. The mean concentration of nickel in urine was 0.23 µg/100 ml (SD ±0.14; range, 0.10 to 0.52); the mean urinary excretion of nickel was 2.4 µg/day (SD ±1.1; range, 1.0 to 5.6). The median concentration was 0.20 µg/100 ml, the median excretion 2.1 µg/day. Nickel concentration and excretion values for the men (0.24 ± 0.12 µg/100 ml; 2.6 ± 1.3 µg/day) did not differ significantly from corresponding values for the women (0.23 ± 0.17 µg/100 ml; 2.2 ± 0.8 µg/day).

Serum Nickel Concentrations in Patients

Nickel concentrations were measured in sera collected from 36 patients within 24 h after onset of acute myocardial infarction (Figure 8). The mean concentration of serum nickel in the patients with myocardial infarction was 0.51 µg/100 ml (SD ±0.27; range, 0.09 to 1.27; p *vs.* controls = <0.001). The mean concentration of serum nickel in 132 consecutive patients who were admitted to the medical and surgical services at the University of Connecticut Hospital for any diagnosis except acute myocardial infarction was 0.22 µg/100 ml (SD ±0.11; range, 0.02 to 0.84) (Figure 8).

Measurements of Nickel in Serum and Urine from Nickel Carbonyl Workers

Nickel has been measured in specimens of ser and urines from six men who work in industrial an medical research laboratories in which there i potential hazard of exposure to inhalation of th toxic vapors of nickel carbonyl. To date, only on mild accidental exposure to nickel carbonyl ha been encountered. On the first and second days after the exposure, urinary excretions of nickel (µg/day) by the three workmen (*A*, *B*, and *C*) were: *A*, 16.7 and 15.5; *B*, 16.6 and 12.0; and *C*, 15.3 and 13.3, respectively. The concentrations of serum nickel in *A* and *C* at 24 h after the exposure were 0.48 and 0.43 µg/100 ml, respectively. No

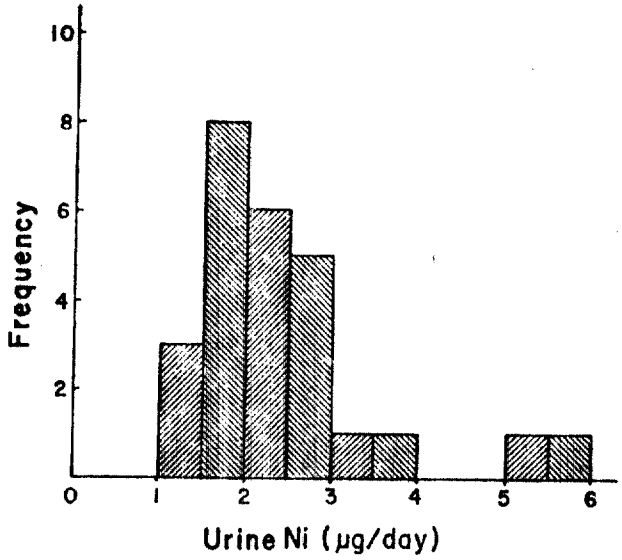

Fig. 7. Frequency distribution graph for nickel excretion in 24-h urines from 36 healthy subjects

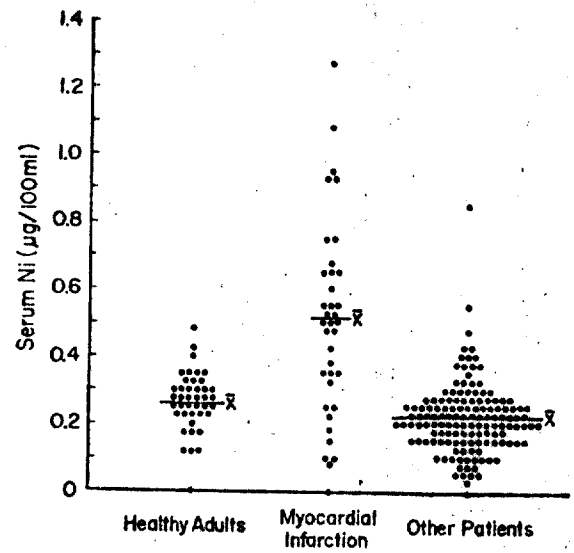

Fig. 8. Comparisons of nickel concentrations in sera of 40 healthy subjects, 36 patients with acute myocardial infarction, and 132 patients with other diagnoses. ($\bar{x}$ = mean concentrations)

clinical symptoms or signs developed in any of these men, and chelation therapy was not administered.

Discussion

D'Alonzo and Pell (*4*) used a spectrographic method for semiquantitative estimations of 12 trace metals in sera from 20 control subjects and in sera collected from 20 patients within 24 h after myocardial infarction. They found that the concentrations of serum nickel were significantly increased in 19 of the 20 patients with myocardial infarction. Some of the patients with infarctions also had less striking increases in serum molybdenum and boron concentrations. There were no significant differences between the concentrations of zinc, silver, lead, manganese, iron, magnesium, silicon, titanium, or aluminum in sera from the control subjects and the infarction patients. D'Alonzo and Pell (*4*) speculated that nickel may either be involved in the etiology of myocardial infarction, or that an enzyme containing nickel may be released into the serum after myocardial infarction. Their data, although fragmentary, suggest that high concentrations of serum nickel are associated only with infarction, not with other manifestations of coronary heart disease.

Ryabova (*8–10*) measured concentrations of nickel in serum and myocardium from 36 dogs after provoking acute myocardial ischemia by ligating the left coronary artery for 10 to 60 min. In Ryabova's experiments, the concentrations of nickel in the myocardium became significantly increased after myocardial ischemia (*9, 10*), but there were no significant alterations in the concentrations of serum nickel (*8*). Donskikh (*11*) reported that nickel ion increases the permeability of frog myocardium to potassium ion, and Kleinfeld and Stein (*12*) reported that nickel ion decreases the contractile tension of the rat atrium. Insofar as the authors can ascertain, these are the only published studies of the role of nickel in cardiac physiology and pathology. This is surprising in view of the widespread interest in the relationship of trace metals to cardiac diseases, which has been stimulated by the implication of cobalt toxicity in the development of "beer-drinkers' cardiomyopathy" (*13, 14*).

Our present finding of a significantly increased mean concentration of nickel in sera collected from patients within 24 h of onset of acute myocardial infarction is consistent with the report of d'Alonza and Pell (*4*). The clinical findings in our patients with myocardial infarction, and the correlations of serum nickel concentrations with serum enzyme activities and electrocardiographic patterns will be described separately (Sunderman, F. W., Jr., Nomoto, S., Pradhan, A., Levine, H., and Bernstein, S., in preparation). It may be noted that the concentrations of serum nickel generally fell within or below the normal range in specimens collected at 48 and 72 h after myocardial infarction. Moreover, in certain cases, the excretion of nickel in urine was significantly supranormal on the day after myocardial infarction. Our observations suggest that measurements of serum nickel may be useful in the diagnosis of myocardial infarction and that investigations of nickel metabolism may contribute to understanding the pathogenesis of myocardial infarction.

As indicated in Tables 2, 3, and 4, the concentrations of nickel that we found in serum, whole blood, and urine samples from healthy subjects were significantly lower than the normal values reported by previous investigators, including measurements by one of us (*2*, *3*). These lower concentrations of nickel cannot be attributed to increased sensitivity and specificity of the present method, since this method correlates closely with nickel analyses by the atomic absorption (*2*) and spectrophotometric (*3*) methods we formerly used. The most likely explanation is that the population of central Connecticut, sampled in this study, has significantly lower levels of nickel in blood and

Table 2. Normal Values for Nickel in Human Serum or Plasma

Reference	Method[a]	Population	No. subjects	Serum (S) or plasma (P)	Nickel, μg/100 ml Mean	Nickel, μg/100 ml Range
15, 16	S	Va.	1	P	1,2	...
17	ES	N. Y.	?	P	3.0 ± 1.9	1.0–8.5
18	ES	Va.	12	P	4.0	1.0–6.0
19	ES	Va.	39	P	2.3	0.0–18.0
20	ES	Va.	109	P	6.0	0.0–27.0
21	ES	Calif.	39	S		0.0–18.0
22	ES	Calif.	48	S	5.3–6.2[b]	
23	ES	Russia	154	S	5.5 ± 2.8♂	
					5.2 ± 3.2♀	
2	S	Fla.	23	S	2.2 ± 1.8	0.1–7.3
1	AA	Germany	26	P	2.1 ± 0.9	0.6–3.7
24	ES	Germany	59[c]	S	0.78	0.06–4.60
Here	AA	Conn.	40	S	0.26 ± 0.08	0.11–0.46

[a] S, spectrophotometry; ES, emission spectrography; AA, atomic absorption.
[b] Range of mean value; nickel not detected in 18 sera.
[c] Includes 25 healthy subjects and 34 patients.

Table 3. Normal Values for Nickel in Human Whole Blood

Reference	Method	Population	No. subjects	Nickel, μg/100 ml Mean	Nickel, μg/100 ml Range
15, 16	S	Va.	8	4.1	2.5–6.7
19	ES	Va.	40	3.6[a]	...
25	ES	[b]	153	4.2	0.9–9.8[c]
22	ES	Calif.	47	32.7	...
1	AA	Germany	63	2.7 ± 1.6	0.6–7.0
Here	AA	Conn.	17	0.48 ± 0.13	0.29–0.70

[a] Calculated from values for plasma and erythrocytes.
[b] Industrial workers from Ohio, New York, Florida, Colorado, and Oregon.
[c] Central 95th percentile limits.

Table 4. Normal Values for Nickel in Human Urine

Reference	Method	Population	No. subjects	Nickel, μg/100 ml Mean	Nickel, μg/100 ml Range
26	S	England	12	2.9	0.0–5.5
27	S	Pa.	69	1.1 ± 0.9	0.0–3.0
28	ES	Mo.	24	2.0 ± 2.6	1.0–7.0
29	S	Wales		4.0 ± 0.2	
25	ES	[a]	154	1.0 ± 0.7	0.1–2.5[b]
3	AA	Pa.	17	1.8 ± 0.8	0.4–3.1
Here	AA	Conn.	26	0.23 ± 0.14	0.10–0.52
				(μg/day)	
3	AA	Pa.	17	19.8 ± 10.0	7.2–37.6
Here	AA	Conn.	26	2.4 ± 1.1	1.0–5.6

[a] Industrial workers from Ohio, New York, Florida, Colorado, and Oregon.
[b] Central 95th percentile limits.

urine than do populations previously studied.

We are now starting to compare normal levels of nickel in serum and urine from healthy subjects who live in several areas of North America.

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Application of the Line-Width Method to the Spectrochemical Analysis of Oils, Fats, and Related Substances

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IN a recent paper (3) the present authors described a method for the preparation of the ash of oils and fats together with a method of spectrochemical analysis of the ash for traces of certain metallic elements having sensitivity of about 2 to 4 parts of individual metals per million parts of the fat or oil. However, a sensitivity of about 1 part of a metallic trace element in 10 million parts of a sample is essential in studies on the stability of oils and fats. The present communication describes a shortened and improved procedure for the preparation of ash and the application of the newly proposed line-width method (2) for the evaluation of the spectrograms. Data are presented from critical studies of the sensitivity, precision, and accuracy of the procedure for the quantitative determination of copper, iron, manganese, nickel, and tin in various types of vegetable oils and fats with a sensitivity of about 1 part in 10 million.

Preparation of Ash

Wet washing methods, involving the use of mineral acids and chemical glassware, were investigated and proved unsatisfactory. The recently adopted tentative method of the American Oil Chemists' Society for the determination of ash in oils and fats (1), in which the oil is heated and then ignited on the surface, was studied in some detail. As shown in Table I, use of this method for ashing and subsequent determination of the trace metals by spectrochemical analysis showed consistently low results in comparison with a more adequate ashing procedure.

The ashing procedure previously proposed (3) was reinvestigated for the purpose of simplification and increasing the sensitivity. Through the use of the line-width method (2) of evaluation of spectrograms the incorporation of internal standards into the ash is not required. By the elimination of this step the over-all time of ashing can be reduced from about 72 to about 48 hours with the need of less personal attention.

The most obvious means of increasing the sensitivity is to increase the size of the sample and at the same time reduce the amount of the magnesium nitrate used to perform the triple function of ashing-aid, carrier, and spectroscopic buffer. The ratio of 2.5 grams of oil to 1.25 grams of magnesium nitrate has been changed to 16.67 grams of oil and 0.50 gram of magnesium nitrate, providing approximately a twenty-fold increase in this ratio, and permitting the quantitative determination to a sensitivity of about 1 part of metal in 10 million parts of sample.

The abbreviated ashing procedure follows. A sample of exactly 16.67 grams of the oil or fat is accurately weighed into a 100-ml. Vycor dish (90 mm. across the top) and 0.50 gram of magnesium nitrate in alcoholic solution [2 ml. of a solution of 250 grams $Mg(NO_3)_2 \cdot 6H_2O$ per liter of 95% C_2H_5OH] is added. The dish is covered with an inverted short-stemmed Pyrex funnel, whose maximum diameter is less than the maximum diameter of the dish. The sample is heated on a hot plate and the temperature gradually and cautiously raised until the maximum temperature of the hot plate, approximately 300°C., is attained, and the heating is continued overnight. The charred sample is then ashed in a muffle furnace, with an initial temperature of 225° and increasing in increments of 25° at 30-minute intervals until a temperature of 450° is reached. The samples are held at this temperature in the muffle furnace overnight and then cooled and quantitatively transferred to a small mortar with the aid of a camel's hair brush. The ash is finely ground and very thoroughly mixed and is then ready for the spectrochemical analysis.

Spectrochemical Analysis by the Line-Width Method

The spectrograms were prepared exactly as described in the earlier paper (3) except that a step-sector was no longer required. All the precautions previously discussed in detail—such as complete absence even of traces of the elements to be determined from all reagents, from glassware and from the carbon rods after their preparation and handling, and very careful mixing of the prepared ash—must be maintained if accurate results are to be obtained. Fig. 1 shows the spectrograms of a number of vegetable oils photographed on a single plate for evaluation by the line-width method. As the step-sector,

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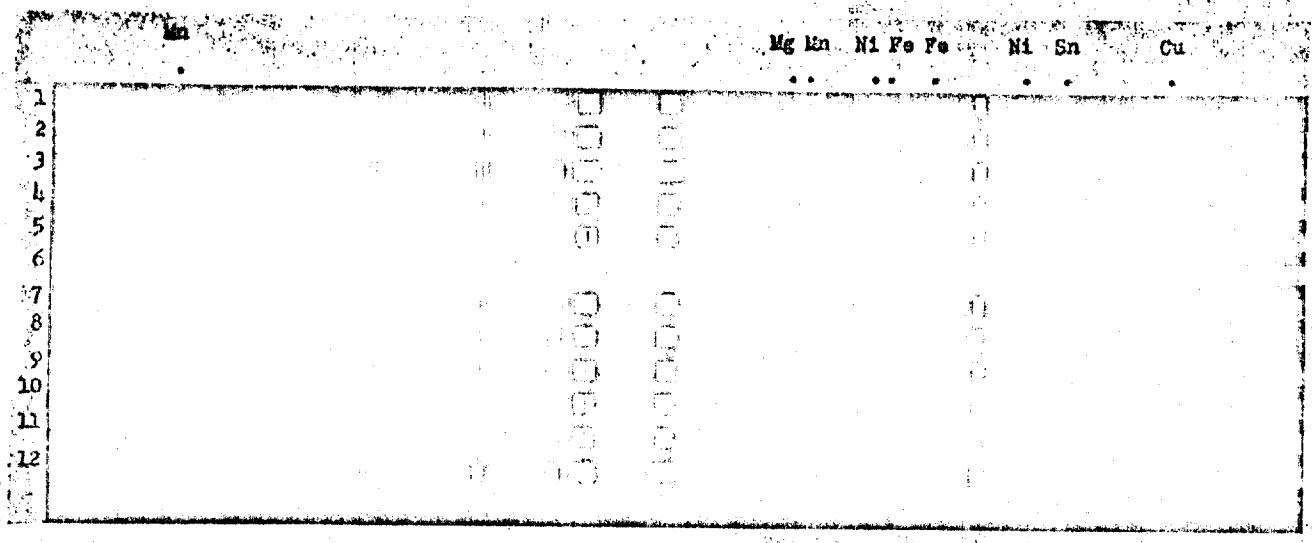


FIG. 1. Spectrochemical—Vegetable Oil Analysis. A spectrographic Plate Showing Spectrograms of Typical Vegetable Oils.

1. Calabash seed oil (crude)
2. Cottonseed oil (crude)
3. Cottonseed oil (refined)
4. Okra seed oil (crude)
5. Peanut oil (crude)
6. Pure carbon rod
7. Rice bran oil (crude)
8. Rice bran oil (hydrogenated)
9. Sesame oil (crude)
10. Shortening (commercial)
11. Soybean oil (crude)
12. Soybean oil (bleached)

which necessitates a longer line, is not required, 12 spectra can be photographed on a single plate.

The line-width method of quantitative spectrochemical analysis has been described by Pierre Coheur (2). It is based on the principle that if the physical and instrumental factors which normally affect line width, such as natural width, Doppler effect, Stark effect, pressure broadening, self-absorption effects, size of source, path of the light beam through the spectrograph, diffraction by the slit, aberrations of the optical parts, diffusion of light in the photographic emulsion, etc., are all maintained rigorously constant, the physical width of any line will remain constant. Consequently, if the physical width of a major line of the spectrogram—for example, a magnesium line in the spectrogram of our magnesium oxide-carbonate buffered ash—is measured in microns at a density corresponding to the maximum density of a line of the element to be determined, the resulting width will be dependent only upon the concentration of this element. This principle is shown schematically in Fig. 2.

In the present work Coheur's method of obtaining line widths has been modified to take advantage of the automatic recording microphotometer. The spectrogram is densitometered as usual through the selected line of the major element, i.e., magnesium, and through all of the selected lines of the elements to be determined. From these profiles the widths in millimeters can be read directly, thus eliminating the need for a wave length comparator. Fig. 2 is a reproduction of a portion of such an actual microphotometer tracing, showing the magnesium 2942.11 Å. line and the manganese 2949.20 Å. line for two different concentrations of manganese.

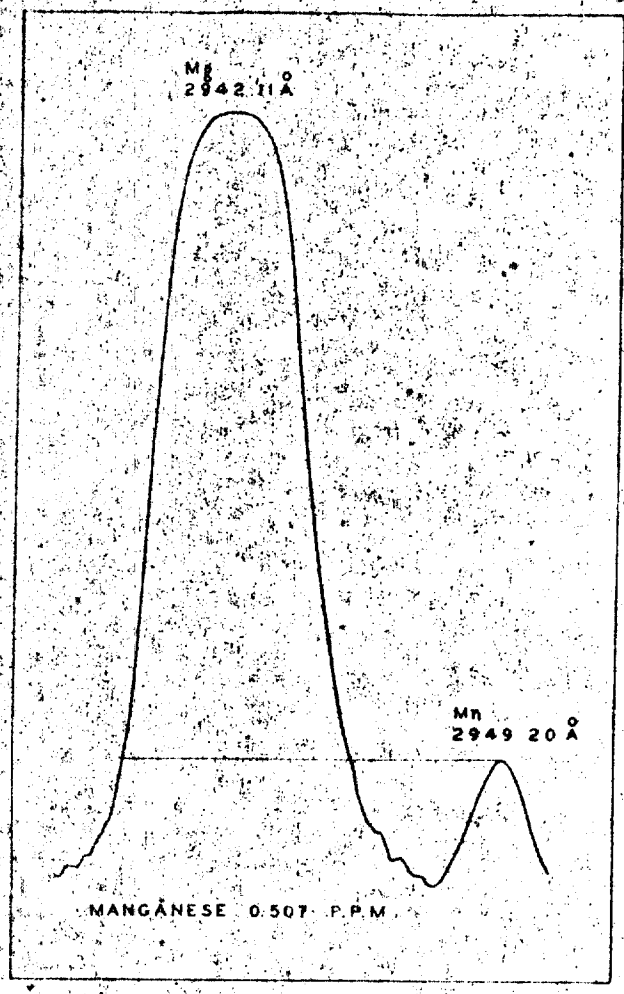
Working, or calibration, curves were prepared by ashing samples of a molecularly distilled oil, shown to be free of metallic impurities (3), containing a series of added amounts of the elements to be determined. The spectrograms and their microphotometric

TABLE I

Spectrochemical—Vegetable Oil Analysis Comparison of Ashing Techniques

Sample	Determination of Trace Elements—Parts Per Million											
	Copper			Iron			Manganese			Nickel		
	Av. Conc. P.P.M. Ashing Technique No. 1	Av. Conc. P.P.M. Ashing Technique No. 2	Av. Conc. P.P.M. Ashing Technique No. 3	Av. Conc. P.P.M. Ashing Technique No. 1	Av. Conc. P.P.M. Ashing Technique No. 2	Av. Conc. P.P.M. Ashing Technique No. 3	Av. Conc. P.P.M. Ashing Technique No. 1	Av. Conc. P.P.M. Ashing Technique No. 2	Av. Conc. P.P.M. Ashing Technique No. 3	Av. Conc. P.P.M. Ashing Technique No. 1	Av. Conc. P.P.M. Ashing Technique No. 2	Av. Conc. P.P.M. Ashing Technique No. 3
Crude peanut oil from Texas	0.20	<0.1	0.13	4.25	1.48	2.70	0.66	0.33	0.51			
Refined, bleached cottonseed oil	<0.1	0	0									
Refined, bleached cottonseed oil hydrogenated, refined										6.73	3.42	5.23
Crude cottonseed oil from Arkansas							0.36	0.26	0.27			
Refined, bleached, and hydrogenated cottonseed oil with added hard fat, refined and rebleached				0.36	0.20	0.16						

Ashing Technique No. 1—As recommended in this communication.
Ashing Technique No. 2—Method of A.O.C.S. Committee on Analysis of Commercial Fats and Oils, $Mg(NO_3)_2$ added after ashing, Journal of the American Oil Chemists' Society, *XXIV*, 76 (1947).
Ashing Technique No. 3—Method of A.O.C.S. Committee on Analysis of Commercial Fats and Oils, $Mg(NO_3)_2$ added before ashing, Journal of the American Oil Chemists' Society, *XXIV*, 76 (1947).
Values given for concentrations found are averages for at least four independent determinations by each ashing technique.



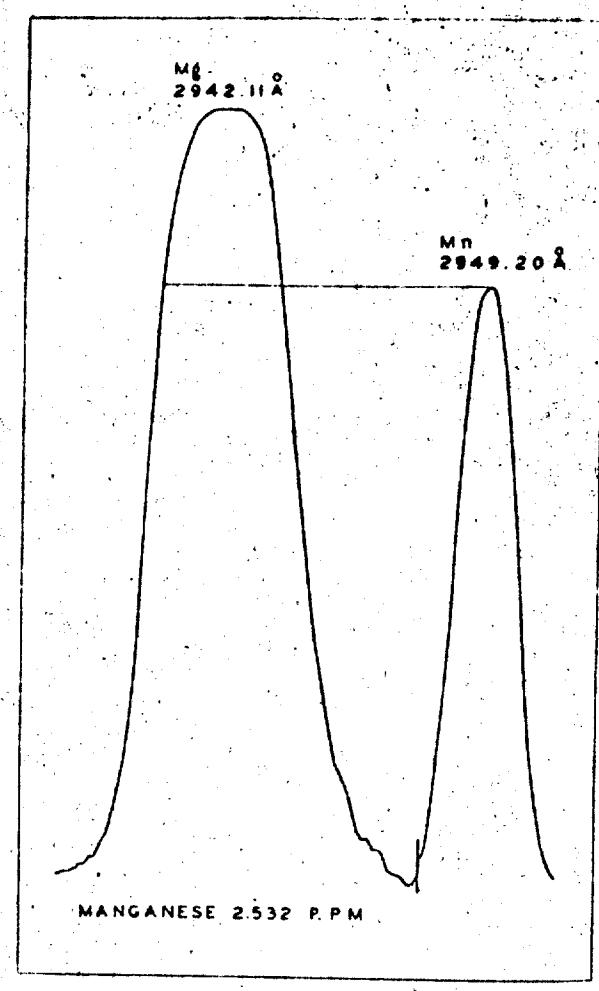


FIG. 2. Spectrochemical—Vegetable Oil Analysis. The Photometric Line-Width Method.

tracings were obtained and from the latter the widths of the profiles of the selected magnesium lines were measured in millimeters. These widths for a series of concentrations of the elements were plotted against the logarithms of the known concentrations and the smooth curves shown in Figs. 3 and 4 were obtained. Fig. 3 shows the working curves for the five elements studied at the lowest concentration range, that were usually encountered in actual vegetable oil analyses. Fig. 4 shows a series of curves for copper covering the concentration range from 0.1 to 4,000 parts per million. Similar curves have been prepared for each of the other four elements. The lines selected for the analyses with their accompanying selected magnesium line and the concentration range covered by each are given in Table II. The most probable interferences in the various determinations listed in the table will be discussed later.

Presumably the lower range might be extended by further increasing the amount of sample ashed with the 0.50 gram of magnesium nitrate buffer, if there is any practical value in precise measurements below a concentration of 1 part of the trace elements in 10 million parts of vegetable oil. The practical lower limit is reached on one hand when the amount of oil to be ashed is too great to permit sufficiently rapid ashing for routine analysis and on the other where the amount of magnesium nitrate ashed is too low to provide enough ash to obtain a satisfactory spectrogram. There is no upper limit. A ten-fold increase from 4,000 parts per million (that is, 0.4%) to 4 [illegible] and a second similar ten-fold increase to 40% can readily be attained either by decreasing the amount of vegetable oil and increasing the amount of magnesium nitrate buffer or by selecting weaker spectrum lines for the analysis, or by both. The practical upper limit is at the value where the usual chemical methods can provide the analysis with a greater accuracy than the spectrochemical methods, a concentration of about 4%.

TABLE II

Spectrochemical—Vegetable Oil Analysis. Spectrum Lines Selected for Various Analyses

Element	Line Identification Angstrom Units	Mg. Line Angstrom Units	Range With 16.67 g. Sample P.P.M.	Range With 2.5 g. Sample P.P.M.	Most Probable Interferences Angstrom Units
Cu	2961.16 Cu I	2942.11		400-4000	
Cu	3273.96 Cu I	3336.68	1 - 30	10- 600	
Cu	3273.96 Cu I	2942.11	0.1- 1		
Fe	3017.63 Fe I	3336.68		400-4000	3017.88 P; 3017.57 C[illegible]
Fe	3020.64 Fe I	3336.68	0.1- 5.0		3020.67 C[illegible]
Fe	3057.45 Fe I	3336.68	4.0-150	60-2400	3057.64 N[illegible]
Mn	2576.10 Mn II	3336.68	0.1- 1.5		
Mn	2949.20 Mn	3336.68	0.5- 12		
Mn	3054.36 Mn	3336.68	12 -260	200-4000	3054.32 N[illegible]
Sn	2571.59 Sn	3336.68	10 -240	160-4000	
Sn	3175.02 Sn I	3336.68	0.3- 15		
Ni	2943.91 Ni I	3336.68	10 -240	160-4000	
Ni	3002.49 Ni I	3336.68	0.3- 40		3002.27 F[illegible]

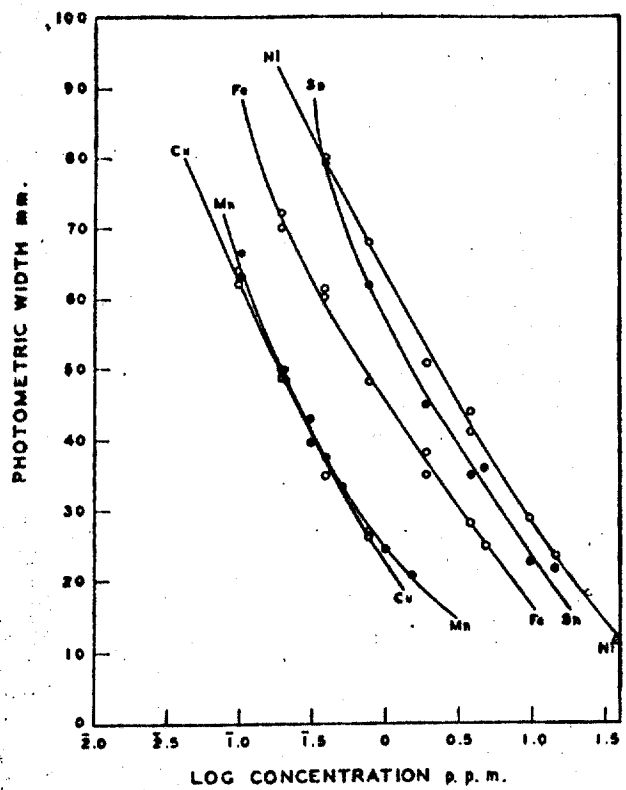


FIG. 3. Spectrochemical—Vegetable Oil Analysis. Working Curves, Lowest Concentrations.

Working directions for the preparation and evaluation of the spectrograms follow. Exactly 5 mg. of the sample ash are weighed into the electrode crater and the sample is completely consumed by arcing in a direct-current arc at 235 v., 20 a., for exactly 90 seconds. The excited sample is photographed on Eastman Spectrum Analysis No. 1 plates, using a large Littrow spectrograph set to photograph the ultraviolet region from 2500 to 3500 Å. Any spectrograph which will cover this range with equal or higher dispersion could, of course, be used. The processed plates are densitometered with a recording microdensitometer through the selected magnesium lines and all the selected lines of the elements to be determined. From measurements of the width of these magnesium lines at the maximum densities of each of the lines of the elements being determined, by use of the previously prepared working curves, the concentrations of the elements in parts per million are obtained.

Results and Discussion

The results obtained from at least 2 to 4 completely independent analyses of 30 samples of vegetable oils and fats are listed in Table III. The table gives the mean value obtained for each element indicated, the number of analyses made for each element, and the average per cent deviation of the individual values from the mean. When the amount of element found is of the order of 0.1 or 0.2 of a part per million in the oil, the deviation from the mean will be seen to be of the order of 10 to 15% of the amount found. If the concentration is of the order of 1 to 5 parts per million, a deviation of only from 3 to 9% is encountered. This deviation is based on the average value found for the particular element. For example, in the analysis of a crude peanut oil from Texas, 16 determinations were made for copper with a mean value of 0.20 parts per million and an average deviation of 16%. This means that the expected value of copper in this sample upon subsequent analyses should be within the range of 0.17 to 0.23 parts per million. Similarly, the average of the two values found for the iron content of the crude cottonseed oil from Mississippi of 3.02 with deviation of 6.6% (which is about the average deviation for this concentration range) implies that the values for this element in this sample lie within the rather close range of 2.9 to 3.2 parts per million.

In Table IV are given the results obtained by applying the method to the analysis of oils of known metal concentration. These samples were prepared by adding to metal-free molecularly distilled oil, traces of each of the five elements studied in varying amounts, covering and extending beyond the concentrations usually found in vegetable oil samples. Approximately 20 analyses have been made for each element. The results indicate that the accuracy with which these metals can be determined in vegetable oils, within the concentration range from 0.1 to about 250 parts per million, lies between the mean values of 3.93% for manganese to 8.02% for tin.

As further tests of the accuracy of the procedure, additional amounts of the metals found to be present were added to oils selected from those listed in Table III, and these samples were reanalyzed. Results of these recovery tests are given in Table V. From the results of the tests for precision described earlier, variations of twice the 10 to 15% for the lowest concentrations, or of the 3 to 9% for the concentrations

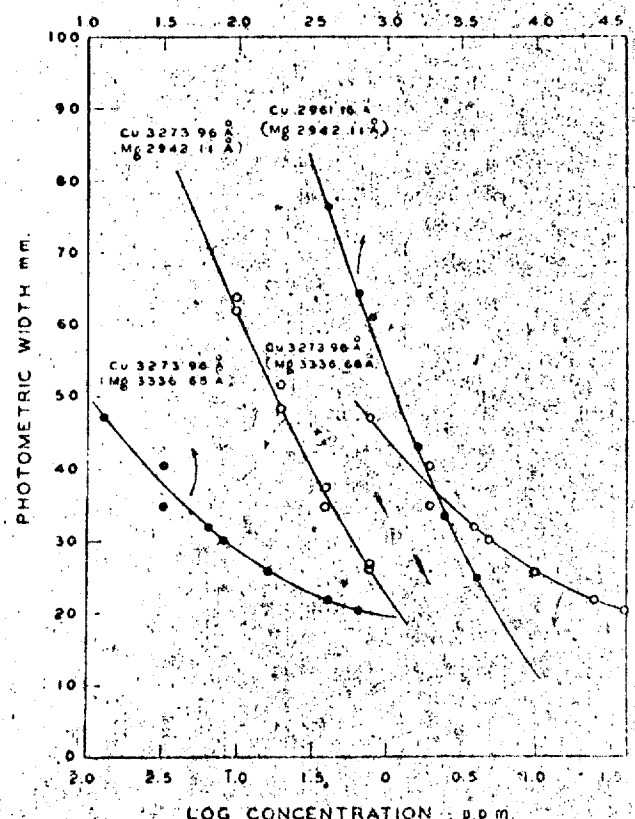


FIG. 4. Spectrochemical—Vegetable Oil Analysis. Working Curves for Copper from 0.1 to 4000 p.p.m.

TABLE III

Spectrochemical—Vegetable Oil Analysis. Analysis of Representative Vegetable Oils

Sample	Concentration Trace Metal—Parts Per Million														
	Copper			Iron			Manganese			Nickel			Tin		
	Av. Conc. P.P.M.	# Detn.	Av. % Deviation from Mean	Av. Conc. P.P.M.	# Detn.	Av. % Deviation from Mean	Av. Conc. P.P.M.	# Detn.	Av. % Deviation from Mean	Av. Conc. P.P.M.	# Detn.	Av. % Deviation from Mean	Av. Conc. P.P.M.	# Detn.	Av. % Deviation from Mean
Crude cottonseed oil. No treatment	<0.1	5	.....	2.10	5	6.5	0.46	5	6.1	0	5	.....	0	5	.....
Deodorized cottonseed oil	<0.1	2	.....	0	2	.....	0	2	.....	0	2	.....	0	2	.....
Crude cottonseed oil from Arkansas	0.11	4	.....	2.60	4	4.6	0.86	4	7.7	0	4	.....	0	4	.....
Crude cottonseed oil from Louisiana	<0.1	4	.....	6.30	4	7.2	<0.1	4	.....	0	4	.....	<0.1	4	.....
Crude cottonseed oil from Oklahoma	<0.1	4	.....	2.74	4	8.4	0.50	4	7.5	0	4	.....	0	4	.....
Expeller crude cottonseed oil from Mississippi	<0.1	2	.....	2.50	2	2.4	0.16	2	6.3	0	2	.....	<0.1	2	.....
Crude cottonseed oil from Tennessee	<0.1	4	.....	1.97	4	8.1	0.36	3	9.1	0	4	.....	0	4	.....
Crude cottonseed oil from Mississippi	<0.1	2	.....	3.02	2	6.6	0.26	2	13.5	0	2	.....	0	2	.....
Crude cottonseed oil from Alabama	<0.1	3	.....	4.92	3	3.4	0.14	3	14.3	0	3	.....	0	3	.....
Crude peanut oil from Texas	0.20	16	16.0	4.25	16	8.0	0.66	16	7.0	0	16	.....	0	16	.....
Refined and bleached cottonseed oil	<0.1	2	.....	0	2	.....	0	2	.....	0	2	.....	0	2	.....
Refined, bleached, and hydrogenated cottonseed oil with added cottonseed hard fat	<0.1	4	.....	<0.1	4	.....	0	4	.....	10.1	4	6.4	0	4	.....
Refined, bleached, and hydrogenated cottonseed oil with added cottonseed hard fat—rerefined	0	6	.....	0.92	6	3.5	0	6	.....	6.75	7	9.1	0	6	.....
Refined, bleached, and hydrogenated cottonseed oil with added cottonseed hard fat—rerefined and rebleached	0	2	.....	0.36	2	0	0	2	.....	0	2	.....	0	2	.....
Commercial shortening	<0.1	2	.....	<0.1	2	.....	0	2	.....	0	2	.....	0	2	.....
A.O.C.S. crude degummed soybean oil	<0.1	3	.....	0.59	3	12.2	0	3	.....	0	3	.....	<0.1	3	.....
A.O.C.S. refined degummed soybean oil	<0.1	4	.....	0	4	.....	0	4	.....	0	4	.....	<0.1	4	.....
A.O.C.S. bleached degummed soybean oil	<0.1	3	.....	0	3	.....	0	3	.....	0	3	.....	<0.1	3	.....
A.O.C.S. crude degummed soybean oil	0.28	2	14.3	0.62	2	2.4	0.20	2	12.5	0	2	.....	<0.1	2	.....
A.O.C.S. refined degummed soybean oil	<0.1	4	.....	<0.1	4	.....	0	4	.....	0	4	.....	<0.1	4	.....
A.O.C.S. crude cottonseed oil	0.11	4	10.9	5.06	4	10.4	0.14	4	8.9	0	4	.....	0.78	3	14.1
A.O.C.S. refined cottonseed oil	<0.1	2	.....	0	2	.....	0	2	.....	0	2	.....	<0.1	2	.....
Extracted rice bran oil No. 1	0.18	2	8.3	2.81	2	7.1	30.8	2	2.3	0	2	.....	<0.1	2	.....
Extracted rice bran oil No. 2	0.10	2	5.0	1.34	2	7.5	3.98	2	6.8	0	2	.....	<0.1	2	.....
Calabash seed oil, crude	<0.1	2	.....	1.18	2	1.7	0.16	2	3.2	0	2	.....	0	2	.....
Extracted okra seed oil	0.12	2	16.6	4.45	2	2.2	0.12	2	0	0	2	.....	0	2	.....
Extracted sesame oil	0.15	2	13.3	2.29	2	9.1	<0.1	2	.....	0	2	.....	0	2	.....
Rice bran oil, hydrogenated	0.12	7	11.7	0	6	.....	0	6	.....	5.28	5	9.8	1.49	5	10.8
Commercial lard	0	4	.....	0.46	4	13.0	0	4	.....	0	4	.....	0	4	.....
Dried bland lard	0	4	.....	<0.1	4	.....	0	4	.....	0	4	.....	0	4	.....

TABLE V

Spectrochemical—Vegetable Oil Analysis. Recovery Tests

Sample	Copper					Iron					Manganese				
	Amount Added P.P.M.	Amount Orig. Present from Table 3 P.P.M.	Total P.P.M.	Found P.P.M.	Diff. P.P.M.	Amount Added P.P.M.	Amount Orig. Present from Table 3 P.P.M.	Total P.P.M.	Found P.P.M.	Diff. P.P.M.	Amount Added P.P.M.	Amount Orig. Present from Table 3 P.P.M.	Total P.P.M.	Found P.P.M.	Diff. P.P.M.
Crude Cottonseed Oil from Arkansas	0.48	0.11	0.59	0.40	−0.19	1.92	2.60	4.52	3.59	−0.93					
Crude Cottonseed Oil from Tennessee						1.92	1.97	3.89	3.75	−0.14	0.75	0.36	1.11	0.96	−0.15
Crude Cottonseed Oil from Texas	0.48	0.20	0.68	0.57	−0.11	12.0	4.25	16.2	14.4	−1.8	1.26	0.66	1.92	1.52	−0.40
A.O.C.S. Crude Cottonseed Oil	0.48	0.11	0.59	0.50	−0.09						0.25	0.14	0.39	0.29	−0.10
Hydrogenated Rice Bran Oil	0.48	0.12	0.60	0.58	−0.02										
Extracted Okra Seed Oil	0.48	0.12	0.60	0.60	0.00	12.0	4.45	16.4	16.9	+0.5	0.25	0.12	0.37	0.25	−0.12
Extracted Sesame Oil	0.48	0.15	0.63	0.60	−0.03	1.92	2.29	4.21	3.97	−0.24					

Sample	Nickel					Tin				
	Amount Added P.P.M.	Amount Orig. Present from Table 3 P.P.M.	Total P.P.M.	Found P.P.M.	Diff. P.P.M.	Amount Added P.P.M.	Amount Orig. Present from Table 3 P.P.M.	Total P.P.M.	Found P.P.M.	Diff. P.P.M.
Crude Cottonseed Oil from Arkansas										
Crude Cottonseed Oil from Tennessee										
Crude Cottonseed Oil from Texas										
A.O.C.S. Crude Cottonseed Oil										
Hydrogenated Rice Bran Oil										
Extracted Okra Seed Oil	12.0	5.28	17.28	15.98	−1.3	0.48	0.78	1.26	1.08	−0.18
Extracted Sesame Oil						1.92	1.49	3.41	4.62	−1.21

Sample	Copper			Iron			[illegible]			[illegible]			[illegible]		
	Av. Conc. P.P.M.	# Detn.	Av. % Deviation from Mean	Av. Conc. P.P.M.	# Detn.	Av. % Deviation from Mean	Av. Conc. P.P.M.	# Detn.	Av. % Deviation from Mean	Av. Conc. P.P.M.	# Detn.	Av. % Deviation from Mean	Av. Conc. P.P.M.	# Detn.	Av. % Deviation from Mean
Crude cottonseed oil. No treatment	<0.1	5	.....	2.10	5	6.6	0.46	5	6.1	0	5	.....	0	5	.....
Deodorized cottonseed oil	<0.1	2	.....	0	2	.....	0	2	.....	6	2	.....	0	2	.....
Crude cottonseed oil from Arkansas	0.11	4	.....	2.60	4	4.6	0.36	4	7.7	0	4	.....	0	4	.....
Crude cottonseed oil from Louisiana	<0.1	4	.....	6.80	4	7.2	<0.1	4	.....	0	4	.....	<0.1	4	.....
Crude cottonseed oil from Oklahoma	<0.1	4	.....	2.74	4	8.4	0.50	4	7.5	0	4	.....	0	4	.....
Expeller crude cottonseed oil from Mississippi	<0.1	2	.....	2.50	2	2.4	0.16	2	6.3	0	2	.....	<0.1	2	.....
Crude cottonseed oil from Tennessee	<0.1	4	.....	1.97	4	8.1	0.36	3	9.1	0	4	.....	0	4	.....
Crude cottonseed oil from Mississippi	<0.1	2	.....	3.02	2	6.6	0.26	2	13.5	0	2	.....	0	2	.....
Crude peanut oil from Alabama	<0.1	3	.....	4.92	3	3.4	0.14	3	14.3	0	3	.....	0	3	.....
Crude peanut oil from Texas	0.20	16	16.0	4.25	16	8.0	0.66	16	7.0	0	16	.....	0	16	.....
Refined and bleached cottonseed oil	<0.1	2	.....	0	2	.....	0	2	.....	0	2	.....	0	2	.....
Refined, bleached, and hydrogenated cottonseed oil with added cottonseed hard fat	<0.1	4	.....	<0.1	4	.....	0	4	.....	10.1	4	6.4	0	4	.....
Refined, bleached, and hydrogenated cottonseed oil with added cottonseed hard fat—rerefined	0	6	.....	0.92	6	3.5	0	6	.....	6.73	7	9.1	0	6	.....
Refined, bleached, and hydrogenated cottonseed oil with added cottonseed hard fat—rerefined and rebleached	0	2	.....	0.36	2	0	0	2	.....	0	2	.....	0	2	.....
Commercial shortening	<0.1	2	.....	<0.1	2	.....	0	2	.....	0	2	.....	0	2	.....
A.O.C.S. crude degummed soybean oil	<0.1	3	.....	0.59	3	12.2	0	3	.....	0	3	.....	<0.1	3	.....
A.O.C.S. refined degummed soybean oil	<0.1	4	.....	0	4	.....	0	4	.....	0	4	.....	<0.1	4	.....
A.O.C.S. bleached degummed soybean oil	<0.1	3	.....	0	3	.....	0	3	.....	0	3	.....	<0.1	3	.....
A.O.C.S. crude degummed soybean oil	0.28	2	14.3	0.62	2	2.4	0.20	2	12.5	0	2	.....	<0.1	2	.....
A.O.C.S. refined degummed soybean oil	<0.1	4	.....	<0.1	4	.....	0	4	.....	0	4	.....	<0.1	4	.....
A.O.C.S. crude cottonseed oil	0.11	4	10.9	5.06	4	10.4	0.14	4	8.9	0	4	.....	0.78	3	14.1
A.O.C.S. refined cottonseed oil	<0.1	2	.....	0	2	.....	0	2	.....	0	2	.....	<0.1	2	.....
Extracted rice bran oil No. 1	0.18	2	8.3	2.81	2	7.1	30.8	2	2.3	0	2	.....	<0.1	2	.....
Extracted rice bran oil No. 2	0.10	2	5.0	1.34	2	7.5	3.98	2	6.8	0	2	.....	<0.1	2	.....
Calabash seed oil, crude	<0.1	2	.....	1.18	2	1.7	0.16	2	8.2	0	2	.....	0	2	.....
Extracted okra seed oil	0.12	2	16.6	4.45	2	2.2	0.12	2	0	0	2	.....	0	2	.....
Extracted sesame oil	0.15	2	13.3	2.29	2	9.1	<0.1	2	.....	0	2	.....	0	2	.....
Rice bran oil, hydrogenated	0.12	7	11.7	0	6	.....	0	6	.....	5.28	5	9.8	1.49	5	10.8
Commercial lard	0	4	.....	0.46	4	13.0	0	4	.....	0	4	.....	0	4	.....
Dried bland lard	0	4	.....	<0.1	4	.....	0	4	.....	0	4	.....	0	4	.....

TABLE V

Spectrochemical—Vegetable Oil Analysis. Recovery Tests

Sample	Copper					Iron					Manganese				
	Amount Added P.P.M.	Amount Orig. Present from Table 3 P.P.M.	Total P.P.M.	Found P.P.M.	Diff. P.P.M.	Amount Added P.P.M.	Amount Orig. Present from Table 3 P.P.M.	Total P.P.M.	Found P.P.M.	Diff. P.P.M.	Amount Added P.P.M.	Amount Orig. Present from Table 3 P.P.M.	Total P.P.M.	Found P.P.M.	Diff. P.P.M.
Crude Cottonseed Oil from Arkansas	0.48	0.11	0.59	0.40	—0.19	1.92	2.60	4.52	3.59	—0.93					
Crude Cottonseed Oil from Tennessee						1.92	1.97	3.89	3.75	—0.14	0.75	0.36	1.11	0.96	—0.15
Crude Cottonseed Oil from Texas	0.48	0.20	0.68	0.57	—0.11	12.0	4.25	16.2	14.4	—1.8	1.26	0.66	1.92	1.52	—0.40
A.O.C.S. Crude Cottonseed Oil	0.48	0.11	0.59	0.50	—0.09						0.25	0.14	0.39	0.29	—0.10
Hydrogenated Rice Bran Oil	0.48	0.12	0.60	0.58	—0.02										
Extracted Okra Seed Oil	0.48	0.12	0.60	0.60	0.00	12.0	4.45	16.4	16.9	+0.5	0.25	0.12	0.37	0.25	—0.12
Extracted Sesame Oil	0.48	0.15	0.63	0.60	—0.03	1.92	2.29	4.21	3.97	—0.24					

Sample	Nickel					Tin				
	Amount Added P.P.M.	Amount Orig. Present from Table 3 P.P.M.	Total P.P.M.	Found P.P.M.	Diff. P.P.M.	Amount Added P.P.M.	Amount Orig. Present from Table 3 P.P.M.	Total P.P.M.	Found P.P.M.	Diff. P.P.M.
Crude Cottonseed Oil from Arkansas										
Crude Cottonseed Oil from Tennessee										
Crude Cottonseed Oil from Texas										
A.O.C.S. Crude Cottonseed Oil						0.48	0.78	1.26	1.08	—0.18
Hydrogenated Rice Bran Oil	12.0	5.28	17.28	15.98	—1.3	1.92	1.49	3.41	4.62	—1.21
Extracted Okra Seed Oil										
Extracted Sesame Oil										

above 1 part per million, must be expected as statistical extremes. The values in Table V show that the recoveries obtained were well within these limits.

The unexpected result from the data in the table is that, in nearly every case, the recovery is low. In attempts to locate the sources of error, tests were made not only by repeatedly analyzing the oils but also by multiple examinations of the same ash and multiple evaluations of the same spectrograms. These tests indicate that over half of the total variation was incurred in the ashing step. Repeated analysis of the same ash resulted in average deviations of a magnitude less than half that encountered for the whole procedure. Repeated evaluations of the same spectrogram showed that this step contributed nothing significant to the total variation. This last test constitutes a verification of the satisfactory performance of the line-width method for the evaluation of spectrum lines. These results coupled with the usual low values obtained in recovery tests indicate that, even with the slow and careful ashing technique employed, very small losses may occur during ashing.

In Table II are listed the spectrum lines most likely to interfere with the determinations as described, using an instrument having a dispersion in the ultraviolet region from 2500 to 3500 Å. of from 2 to 8 Å. per millimeter. The most serious interference is that of the chromium line at 3020.67 Å. with the most persistent iron line at 3020.64 Å. In the presence of any considerable amount of chromium this means that the sensitivity for which iron could be determined would be somewhat less than 1 part in 10 million. Actually, chromium has not been found in the vegetable oils analyzed. However, if it should be present in quantities sufficient to interfere with the determination of small traces of iron as described, substitution of the iron line at 3021.07 Å. could be made with no great loss of sensitivity.

Chromium also interferes with the iron line at 3017.63 Å. and nickel interferes with both iron at 3057.45 Å. and with manganese at 3054.36 Å., but in these cases the interferences are with the weaker lines of the element being determined and if sufficient chromium or nickel is present to cause interferences, substitutions of other lines can readily be made.

Platinum interferes with the most sensitive line of nickel and, if small traces of this element are to be obtained, the use of platinum dishes and spatulas must be avoided. Platinum also interfers with the

TABLE IV

Spectrochemical—Vegetable Oil Analysis. Analyses of Known Samples

Line λ in Å.	Amount in Sample p.p.m.	Amount Found Spectrochemically p.p.m.	Difference Per cent
Copper			
2961.16	24.0	25.0	4.17
3273.96*	9.60	8.80	8.33
3273.96*	4.80	4.40	8.33
3273.96*	3.84	4.04	5.21
3273.96*	0.768	0.759	1.17
2961.16	144.0	148.0	2.78
2961.16	24.0	25.0	4.17
2961.16	38.4	36.7	4.43
3273.96	0.096	0.100	4.17
3273.96*	9.60	8.87	7.60
3273.96*	4.80	4.06	15.40
3273.96*	0.768	0.851	10.80
2961.16	240.0	238.9	0.46
Average			5.92
Iron			
3057.45	3.84	4.10	6.77
3057.45	4.80	4.82	0.42
3020.64	0.768	0.713	7.16
3020.64	3.84	4.04	5.20
3020.64	4.80	5.32	10.80
3057.45	4.80	4.74	1.25
3020.64	4.80	4.64	3.33
3057.45	38.4	39.5	2.86
3057.45	24.0	21.8	9.17
3057.45	96.0	98.5	2.60
3017.63	96.0	94.7	1.35
3057.45	24.0	22.0	8.33
3057.45	38.4	39.8	3.65
3017.63	24.0	22.3	7.08
3017.63	38.4	40.4	5.21
3017.63	24.0	24.0	0.00
Average			4.79
Tin			
2571.59	48.0	44.5	7.29
3175.02	0.384	0.337	12.20
3175.02	1.92	1.92	0.00
3175.02	9.60	10.19	6.15
3175.02	14.40	12.19	15.30
2571.59	14.40	12.25	14.90
2571.59	192.0	181.2	5.63
2571.59	240.0	265.0	10.40
2571.59	192.0	173.6	9.48
3175.02	0.384	0.387	0.78
3175.02	0.768	0.721	6.12
Average			8.02

Line λ in Å.	Amount in Sample p.p.m.	Amount Found Spectrochemically p.p.m.	Difference Per cent
Manganese			
2576.10	0.507	0.508	0.20
2576.10	1.02	1.10	7.84
2949.20	1.02	1.10	7.84
2949.20	7.62	7.47	1.97
2949.20	12.66	12.59	0.55
3054.36	12.66	12.98	2.53
3054.36	26.28	26.19	0.34
3054.36	26.34	27.43	4.14
3054.36	52.68	48.21	8.49
2949.20	0.507	0.507	0.00
3054.36	210.6	216.8	2.94
3054.36	263.4	251.3	4.59
2949.20	1.014	1.014	0.00
2949.20	12.66	11.17	11.77
2576.10	0.101	0.101	0.00
2576.10	1.518	1.406	7.38
2949.20	1.518	1.507	0.72
2576.10	0.507	0.455	10.26
2576.10	1.014	0.73	4.04
2576.10	1.518	1.439	5.20
3054.36	26.84	26.80	1.15
Average			[illegible]
Nickel			
2943.91	144.0	142.3	1.18
2943.91	192.0	205.7	7.14
2943.91	14.40	15.04	4.44
2943.91	24.00	24.50	2.08
2943.91	38.40	41.5	8.07
2943.91	48.00	46.0	4.17
2943.91	192.0	188.9	1.61
3002.49	9.60	10.00	4.17
3002.49	14.40	14.90	3.47
3002.49	24.00	22.19	7.54
3002.49	38.40	42.9	11.70
2943.91	14.4	15.7	9.03
2943.91	24.0	23.9	4.17
2943.91	192.0	208.0	8.34
3002.49	0.384	0.370	3.65
3002.49	3.84	4.20	9.38
3002.49	9.60	10.92	13.84
3002.49	14.40	16.34	13.50
2943.91	192.0	215.3	[illegible]
3002.49	0.384	0.461	[illegible]
3002.49	0.60	8.37	[illegible]
Average			[illegible]

* For these determinations of copper the reference line was Mg. 3336.68 Å. For all other determinations the Mg 2942.06 Å. line was used.
All values within the ruled black lines were obtained from the same spectrographic plate.

weak 3017.63 Å. iron line, and the same precaution is necessary.

Though no attempt was made to correlate metal content with processing procedure, presence of hydrogenation catalyst, etc., it is obvious from an inspection of Table III that the results obtained by the method would readily permit such studies. Most vegetable oils examined contained a very small trace of copper, usually less than 1 part in 10 million, probably about 0.03 to 0.04 part per million. In some samples as much as 0.1 to 0.3 part per million were found. There is some indication that these larger quantities were acquired during extraction. Similarly, tin is seldom found except in very small traces; and again, when present, it would appear to have been picked up from metal parts of the equipment during extraction or from metal containers during storage. Iron is frequently present in concentrations of a few parts per million, and manganese is often found in concentrations of a few tenths of a part per million. Nickel is found only in the hydrogenated oils where this metal has been used as a catalyst. It is, apparently, removed quantitatively if the hydrogenated products are rebleached. In general, bleaching, as would be expected by the use of an adsorbent, decreases the trace metallic content.

Correlations of the metal content of the oils and their stability are beyond the scope of this paper. Use of a method capable of determining the metal content of an oil to the sensitivity and with the accuracy indicated should, however, make such studies readily possible. The method described has been applied to a few fats, such as commercial shortening and lards. It is readily applicable to such materials and can, in fact, be used for any organic material having a low ash content.

Summary

The application of the line-width method to the spectrochemical analysis of oils and fats in conjunction with the use of an improved ashing technique has been shown to permit the quantitative determination of copper, iron, manganese, nickel, and tin in quantities as low as 1 part in 10 million.

The procedure has been critically examined by precision tests on commercial and experimental samples, by accuracy tests on synthetic samples, and by recovery tests.

Results of actual analysis of 30 vegetable oils and fats indicate the use of the procedure as a research tool.

The procedure may be used for the trace element analysis of organic material low in ash content.

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Preparation of Ash and Spectrochemical Determination of Traces of Metallic Elements in Oils, Fats, and Related Substances*

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EMISSION spectrographic techniques offer means for the determination of traces of metallic elements in fats and oils. Successful application of them should be of particular interest in researches on the effect of traces of such elements on stability and on the measurement of metallic contamination obtained from processing equipment or resulting from incomplete removal of hydrogenation catalysts. Markley and Goss (1) have commented that "In view of the fact that a few parts per million of copper salts (oleate) adversely affect the stability or keeping quality of most refined oils, it would be of interest to know how much of the total copper, contained in soybeans, finds its way into the refined oil." Lea (2) showed the extremely small concentrations of many metals which may affect the keeping quality of oils.

As the amount of ash and, consequently, of any of the metallic elements present in fats and oils is very small, a preliminary preparation of the sample by special ashing methods is essential in order that the full amount may be recovered and at the same time the organic matter may be eliminated by combustion. The purpose of the present communication is to describe an ashing procedure that has proved adequate and to discuss briefly some features of the spectroscopic techniques involved in making the essential measurements.

Preparation of the Sample and Standards

A modification of the magnesium-nitrate method, widely used for the ashing of samples of organic materials for the determination of phosphorus, was found satisfactory and was adopted.

A sample of exactly 2.5 gm. of the fat or oil is accurately weighed into a 100-ml. Vycor dish (90 mm. across the top) and 5 ml. of an alcoholic solution of magnesium nitrate [250 gm. $Mg(NO_3)_2 \cdot 6H_2O$ per liter of 95% C_2H_5OH] is added. The dish is covered with an inverted short-stemmed Pyrex funnel, whose maximum diameter is less than the maximum diameter of the dish. The sample is heated on a hot plate and the temperature gradually and cautiously raised until fuming occurs. When the fuming has ceased, the temperature is again raised gradually until the maximum temperature of the hot plate, approximately 300° C., is attained and heating is prolonged for at least an additional 30 minutes. The charred sample is cooled and a solution of the selected internal standard is added. The sample is then dried overnight in a vacuum desiccator over P_2O_5 or an equally efficient desiccant. The dried sample is then ashed in a muffle furnace, with an initial temperature of 225° C. and increasing in increments of 25° C. at 30-minute intervals until a temperature of 450° C. is reached. The samples are held at this temperature in the muffle furnace overnight and then cooled and quantitatively transferred to a small mortar with the aid of a camel-hair brush. The ash is then finely ground, very thoroughly mixed, and transferred to a weighing bottle for storage.

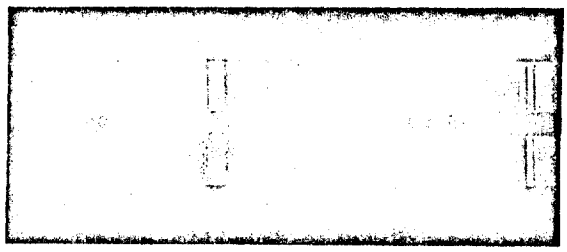

FIG. 1. Spectra of molecularly distilled oil ashed in Vycor dish (upper spectrogram) and in porcelain dish (lower spectrogram).

Certain precautions must be observed. The magnesium nitrate used must be entirely free of the metallic elements to be determined. Vycor dishes, being essentially silica, were not found to impart any measurable quantity of a metallic element to the sample. Porcelain dishes could not be used because they were found to add copper, aluminum, and probably iron (Fig. 1). Truncated filter paper cones could not be used instead of Pyrex funnels to prevent loss by spattering, because the papers examined were found to contain measurable traces of copper and iron (Fig. 2).

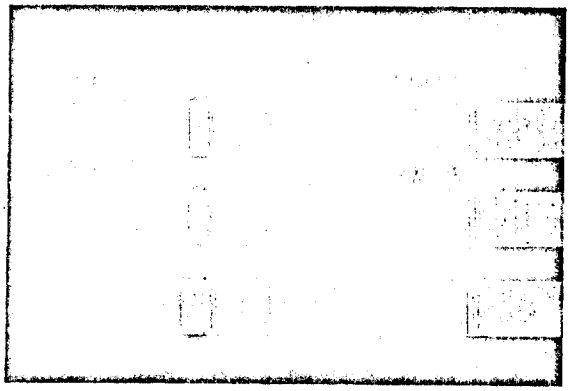

FIG. 2. Spectra of molecularly distilled oil ashed in Vycor dish with filter paper cone (upper spectrogram) and Pyrex glass funnel (lower spectrogram). Middle spectrogram is that of filter paper ash.

The initial heating of the sample and alcoholic magnesium nitrate must be slow in order to avoid flash and perhaps violent ignition of the mixture.

*Presented at the 20th fall meeting of the American Oil Chemists' Society Oct. 30-Nov. 1, 1946 in Chicago, Ill.

[1] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

The addition of the acid solution of the internal standard must be deferred until the sample has been thoroughly charred and then evaporated without heating for the same reasons.

The magnesium nitrate performs a triple function: (1) It acts as an ashing aid. (2) The resulting oxide-carbonate mixture acts as a carrier for the extremely small amount of ash obtained from the fat or oil sample. (3) It performs the service of a spectrochemical buffer. The last function is required because the intensity of a particular atomic transition, and consequently the density of a given spectral line of a photographic plate, is dependent, somewhat, upon the matrix in which the particular element is found. Thus, for example, 1 part per million of copper in an otherwise pure sample of silica would produce a considerably more dense line than the same concentration of copper in an otherwise pure sodium chloride when arced and photographed under identical conditions.

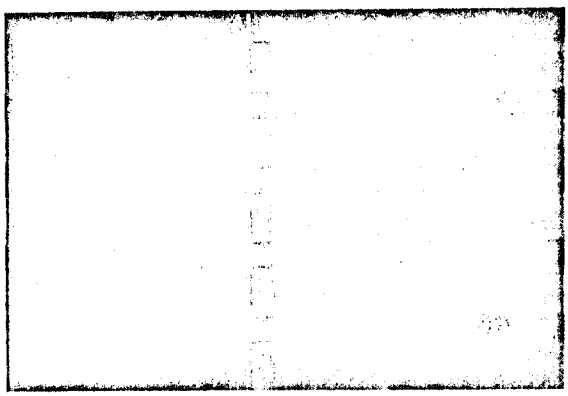

FIG. 3. Spectrograms of standards for metal analysis. Middle spectrogram is that of the molecularly distilled oil ashed with magnesium nitrate.

This method of preparing the sample reduces the spectrochemical analysis, actually, to the determination of the impurities in the magnesium oxide, contributed by the ash constituents of the original oil or fat sample. It is, also, applicable to any material that has a relatively low ash content.

The ash obtained on completion of the final ignition, at not higher than 450° C., is usually gray in color and may contain some free carbon. The low temperature lessens the possible loss by evaporation of some of the more volatile metals or their compounds. Free carbon, however, not only does not interfere with the analysis, but actually aids it. In fact, many procedures recommend the addition of small quantities of pure carbon powder in preparing the sample for spectrochemical analysis.

For the preparation of the standards required to obtain the working (or calibration) curves samples of a molecularly distilled oil are ashed as specified. Every precaution must be taken to avoid any contamination with small traces of the element to be determined. While mathematical methods have been proposed to deal with small residual traces of metals, such corrections have not been entirely satisfactory and their use further complicates the calculations. Figure 3 shows that the molecularly distilled oil and the alcoholic magnesium-nitrate solution were free of traces of metallic contaminants. Solutions of the internal standards were shown similarly to be free of the elements to be determined. At the time the internal standard solution is added to the charred sample, graded amounts of a solution of the element to be determined, varying from 0.0001% to 0.4%, as the element based on the 2.5-gm. sample, are added to a series of 2.5-gm. samples of the molecularly distilled oil. Preparation of standards by ashing individual series of molecularly distilled oil for each element is desirable in order to avoid contamination of one element by traces of impurities in another.

Preparation and Evaluation of the Spectrograms

Though the general details of the emission spectrochemical analysis are adequately given in the literature, some features of the method as applied to fats and oils warrant consideration. The spectrograph employed must have sufficient dispersion to separate the analytically important spectrum lines in the most commonly used ultraviolet region from about 2500 Å. to about 3500 Å. It has been demonstrated that the alternating current arc is not suitable (3) and that the direct current arc, operating at a relatively high current, is the most sensitive source for ash of organic materials (4). For the present work a direct current arc operating at 235 volts and 20 amperes was used. The heat generated from such a source makes water cooling of the electrode holders necessary, if high plate background, due to noncharacteristic temperature radiation, is to be avoided. For this reason hollow steel cylinder electrode holders, through which cool water may be continuously circulated, are used (Fig. 4).

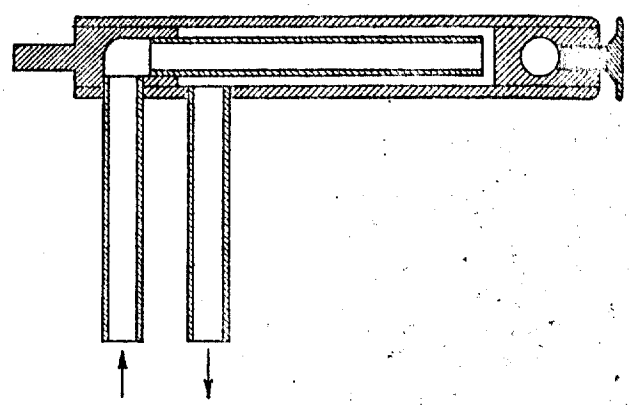

FIG. 4. Water-cooled electrode holder.

The carbon rods used must be made of the purest carbon available. Extreme care must be taken to avoid contamination of the rods during the tooling and storage of the electrodes. The positive craters, which are to become the lower electrodes and hold the sample, are shaped with a special electrode cutting tool which makes a flat-bottom cup cut to a depth of 4 mm. Figure 5 shows the spectrum of special graphite spectroscopic electrodes before and after replacement of a brass guide of the special cutting tool with a steel one. The upper electrodes may be pointed with an ordinary pencil sharpener reserved for this purpose.

In order to avoid any possibility of differences in plate densities arising from self-absorption in the arc at the high current used (5), exactly 5 mg. of the sample ash are weighed into the electrode craters for

arcing. Moving plate spectrograms have shown that this size of sample of magnesium oxide will be entirely consumed in from 60 to 80 seconds. A standard exposure time of 90 seconds was adopted to assure complete burning.

By use of a rotating sector having four steps, each of the atomic lines appears on the photographic plate at four different densities, each bearing a known intensity ratio to one another. Spectrograms made in this manner are shown for typical vegetable oils in Figure 6. Most of the analytical lines for such samples do not, however, appear intense enough to be readily detected in a reproduction.

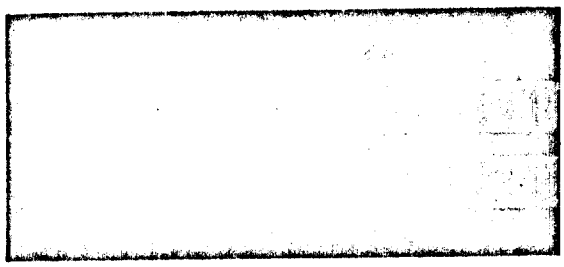

FIG. 5. Spectra of carbon rods tooled with brass guide (upper spectrogram) and with steel guide (lower spectrogram).

Eastman Spectrum Analysis No. 1 plates are satisfactory for photographing the spectral region between 2500 Å. and 3500 Å. and Eastman spectroscopic plates No. 103 A-O u.v. for photographing the region between 2100 Å. and 2500 Å. The latter are coated with a special ultraviolet sensitizing agent and have been shown to be suitable for routine analytical work (6).

Photographing of the entire four steps of the rotating sector enables all lines to be measured at suitable densities to permit either reading of all intensities from straight line portions of the plate calibration curve, or measurement of both the lines of the unknown element and of the internal standard at equal densities, as required by the D–Log E method of measurement.

In general, the spectral lines of the element being determined and of the selected internal standard should lie close to one another either to avoid differences in the slope (gamma) of the plate calibration curve if the plate calibration methods are used or to provide more or less equal background at equal densities if the D–Log E method of plate measurement is used. In addition, the lines selected for both metals should represent the same energy conditions in the arc to avoid saturation, photographic reversal, or self-absorption effects, and both metals should volatilize at about the same rate in order to assure complete burning of the entire sample. Also, all lines of both elements must be free from all interfering lines in the spectrum of the sample. Lists of suitable lines for the several elements for determination with appropriate internal standards have been published (7, 8, 9, 10, 11).

Germanium meets the requirements for a suitable internal standard for the determination of aluminum, copper, iron, nickel and manganese, and lead for the determination of tin and zinc, using the spectrum lines given in Table 1.

TABLE 1

Analytical Lines for the Spectrochemical Determination of Metals in Oils

Element	Wave length of atomic line	Internal Standard	Wave length of atomic line	Sensitivity
	Å		Å	*Parts per million*
Al	3082.16	Ge	3039.06	10
Cu	3273.96	Ge	3039.06	>0.1
Fe	3020.64	Ge	3039.06	>1
Mn	2593.73	Ge	2592.54	1
Ni	3050.82	Ge	3039.06	3
Sn	3175.02	Ge	3039.06	1
Sn	2429.50	Pb	2476.38	10
Zn	2138.56	Pb	2169.99	1

A germanium solution containing 0.1000 gm. of the dioxide (GeO_2) and 15 ml. of concentrated hydrochloric acid per liter and an aqueous solution of lead nitrate [$Pb(NO_3)_2$] containing 3.312 gm. per liter were used. One milliliter of the internal standard solution was added to each sample as specified.

The four steps of the selected lines of the elements to be determined and of the internal standards are conveniently measured with a recording densitometer.

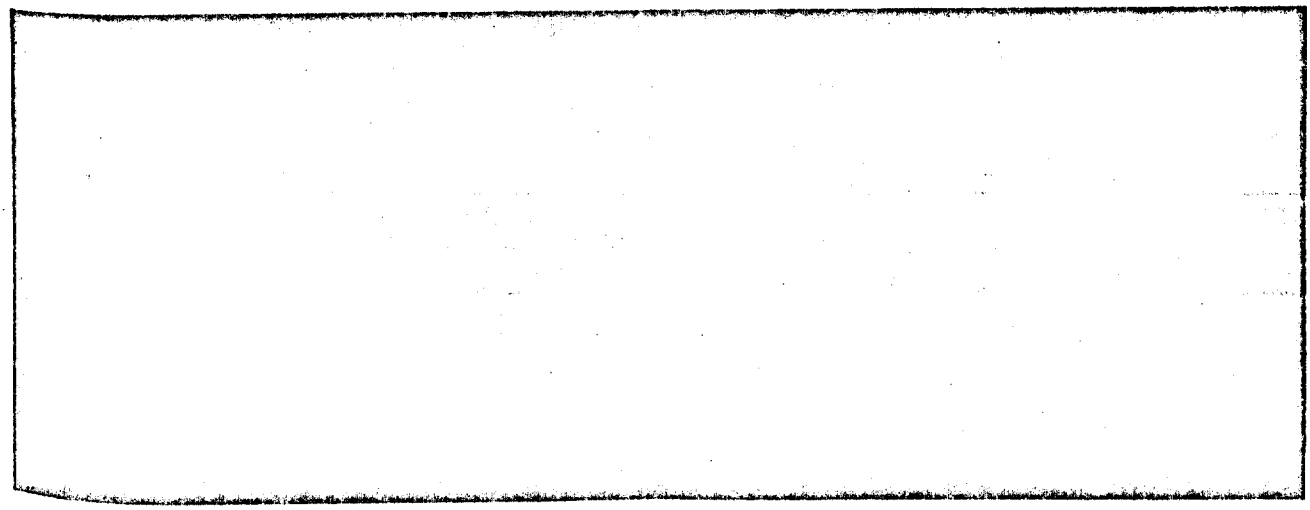

FIG. 6. Spectra of vegetable oils.

Upper spectrogram—Crude peanut oil. Lower spectrogram—Crude cottonseed oil.

This instrument gives directly the densities of the various lines. The two methods in general use for obtaining the concentration are the plate calibration (7, 12, 13) and the so-called D–Log E separation method (14, 15, 16, 17, 18, 19, 20).

The plate calibration method is illustrated by Fig-7 which shows a plate calibration curve together with the working curve used in the determination of nickel by use of the line at 3050.8 Å. and of the line of germanium (internal standard) at 3039.1 Å. To obtain the plate calibration curve (Fig. 7A) the photographic densities of the successive steps of any group of suitable lines are measured and plotted against the logarithms of the relative intensities from the known step sector factor. From this curve the measured densities of the nickel and germanium lines are converted into intensities, and to obtain the working curve for nickel (Fig. 7B) the logarithms of the ratios of these intensities are plotted against the logarithms of the known concentrations of the standards. In the analysis of oil samples the plate calibration step is repeated for each plate, and then, after obtaining the logarithm of the intensity ratio, the corresponding logarithm of the concentration is read from the prepared working curve.

The D–Log E separation method is illustrated by the measurements for the same determination in Figure 8. The densities of each of the four steps of the lines of nickel and germanium are measured, the eight values are plotted against the corresponding logarithms of the relative intensities obtained from the known step sector factor, and the separation of the two curves at a constant density of 0.3 is measured (Fig. 8A). This separation is measured for each of the standards for nickel, and plotted against the logarithms of the known concentrations to obtain the working curve (Fig. 8B). For determination of the concentrations of nickel in the samples analyzed the intensity separations are obtained in a like manner and the concentrations obtained from the working curve.

Discussion

The preparation of the oil sample for the actual spectrochemical analysis is the most time-consuming step in the method. The initial heating and drying *in vacuo* take one day and night and the final ashing in the furnace takes an additional 24 hours. The number of samples prepared at a time, however, is limited only by capacity of the equipment available.

Some preliminary tests have been made of the precision that can be expected by use of the method. For the most difficult cases, where the concentration of the metal in the oil is so low as to require densitometric measurements to be made of the minimum value that can be detected, an overall precision within ±7% can be obtained. Thus, if a particular sample is reported as containing 0.000125% nickel, repeated analyses will be expected to yield results varying from 0.000116 to 0.000134%. Tests have shown that the imprecision observed is due to the sum of very small experimental errors incurred throughout the determination from the first step in ashing through the final densitometry and calculations.

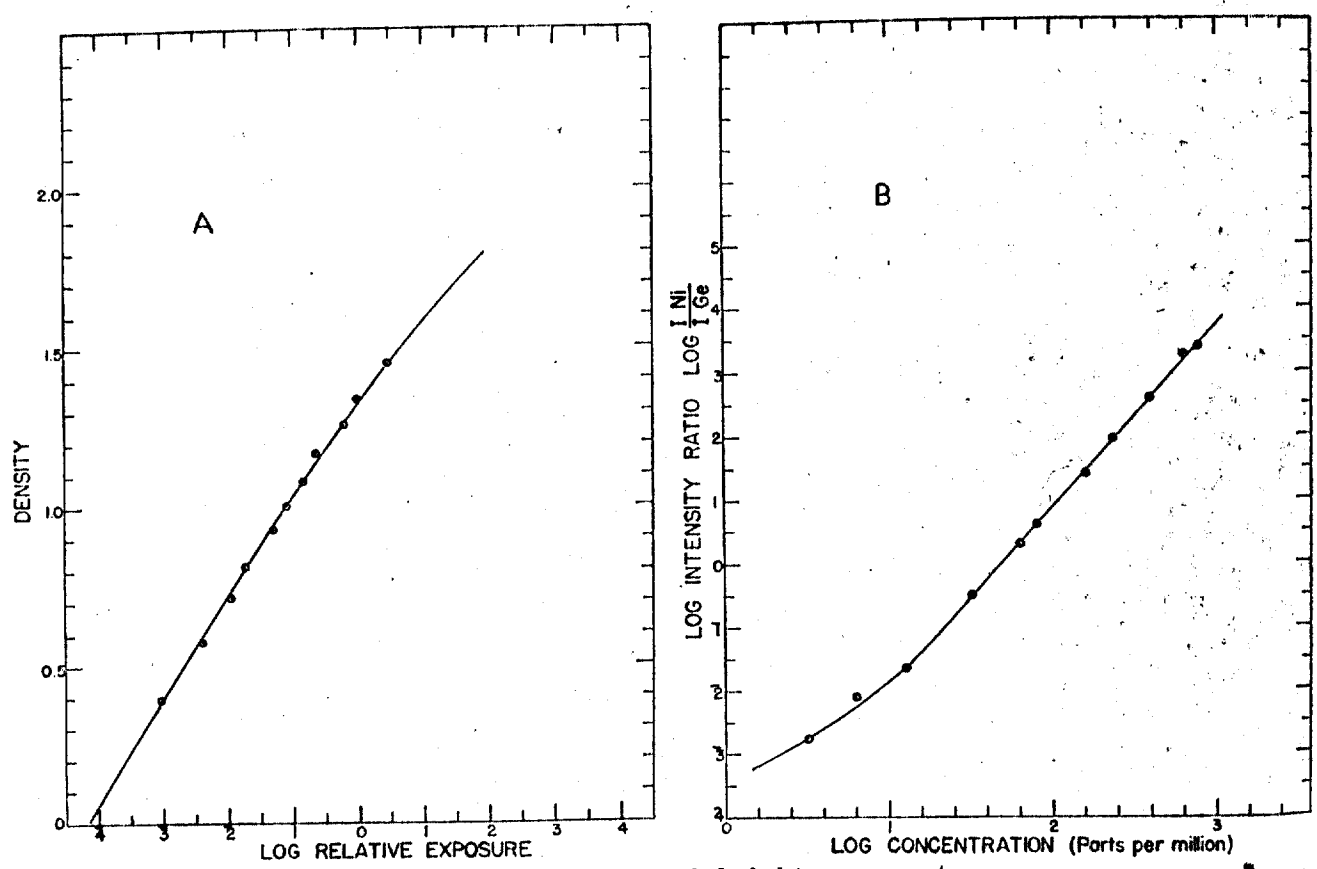

FIG. 7. Plate calibration method of plate measurement.

A. A typical plate calibration curve. B. Working curve based on intensity ratios obtained by plate calibration.

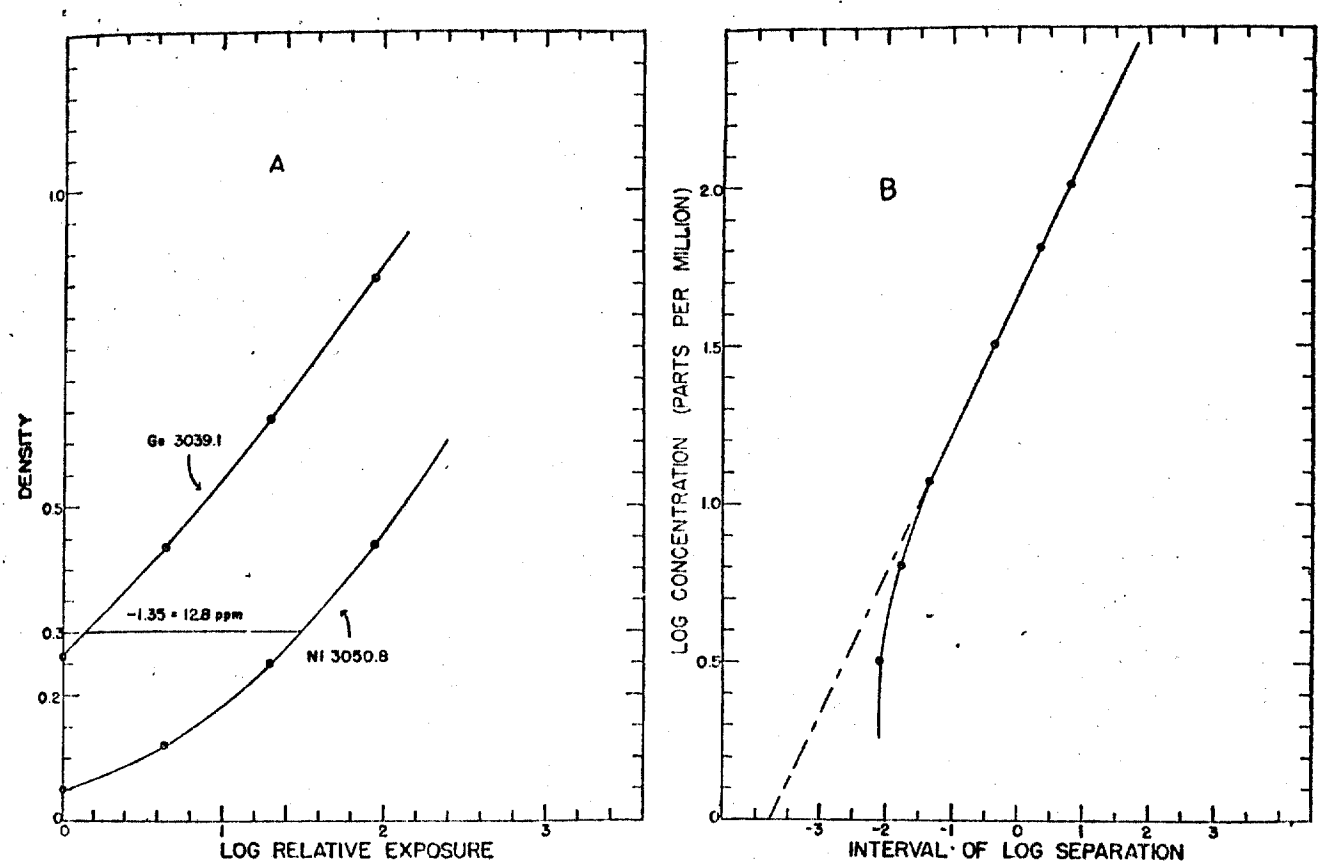

FIG. 8. The D-Log E method of plate measurement.

A. A typical interval of log separation measurement. B. Working curve based on intervals of log separation.

No tests of the accuracy of the method have been possible due to the lack of other methods of proven accuracy. However, recovery tests have indicated that the accuracy is within the variations encountered in successive determinations of a metal in the same sample of oil. The manner in which the various concentrations of the standards fall into a straight line on the working curves is in itself an indication of the accuracy of the method.

The sensitivity of the method for the determination of traces of metals in oils is of considerable importance for research purposes. Approximate limits of the sensitivity of the method for 2.5-gm. samples of oil for the determination of seven metals are given in Table 1. These data show that in general the method will permit the quantitative determination of traces of these elements when present in only a few parts per million. Increase in the size of the sample of oil ashed would enhance the sensitivity and perhaps the precision of the results.

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EFFECT OF DIETARY NICKEL LEVEL ON EXCRETION AND NICKEL CONTENT OF TISSUES IN MALE CALVES[1]

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TOXIC effects of nickel have been shown in mice (Weber and Reid, 1969), chicks (Weber and Reid, 1968) and calves (O'Dell *et al.*, 1970). Tissue distribution of this metal was widespread when ^{63}Ni was administered intraperitoneally (Wase, Goss and Boyd, 1954), intravenously (Smith and Hackley, 1968) and when stable Ni was included in large quantities in the diet of rats (Phatak and Patwardham, 1950). The route of nickel excretion appears to be influenced by the method of administration. Urine has been shown to be the primary route of excretion after intravenous injections (Kent and McCance, 1941; Smith and Hackley, 1968). Wase *et al.* (1954) observed greater amounts of ^{63}Ni in feces than in urine after mice had been injected intraperitoneally. This work was conducted to determine the effect of ingestion of large amounts of nickel on tissue distribution and route of excretion in the bovine.

Experimental Procedure

Twelve male Holstein calves (three per group) were fed, from 13 to 21 weeks of age, a basal diet supplemented with 0, 62.5, 250, or 1,000 ppm elemental nickel (Ni) as nickelous carbonate. The basal diet, on a percentage basis, consisted of the following: ground yellow corn, 42.1; oats, 18.9; soybean meal, 13.2; alfalfa leaf meal, 2.6; cottonseed hulls, 21.0; defluorinated rock phosphate, 1.1; trace mineralized salt, 1.1; and vitamin A and D supplement. On a dry matter basis, the nickel content of these feed ingredients were: corn, .4 ppm; oats, 1.0 ppm; soybean meal, 3.6 ppm; alfalfa leaf meal, 1.4 ppm and cottonseed hulls, .6 ppm. At the end of the 8-week treatment period, total urine and feces were collected from all animals for a period of 5 days. The animals were then sacrificed for tissue analysis.

Duplicate tissue samples were analyzed for nickel by atomic absorption spectrophotometry.[5] These samples were from the following organs: (a) liver, omasal impression area; (b) lung diaphragmatic lobe; (c) heart, left ventricle; (d) spleen, midway between the ventral and dorsal extremities; (e) kidney, lobe of the cortex; (f) testis, midway between the ventral and dorsal extremities after stripping the tunica albuginea; (g) eye, vitreous humor; (h) brain, anterior part of cerebrum; (i) tongue, anterior to prominence of dorsum area; (j) gall bladder, bile; (k) pancreas, midway between the ventral and dorsal extremities; (l) 13th rib, midway between the sternal end and the neck; (m) reticulo-rumen, ventral sac of rumen; (n) omasum, laminae; (o) abomasum, fundus gland region, and from the middle of (p) duodenum; (q) first half of small intestine (exclusive of duodenum); (r) second half of small intestine. Samples were wet-ashed with a sulfuric-perchloric-nitric acid mixture, and quantitatively transferred to Babcock milk test bottles to which 1 ml of 2% ammonium pyrrolidine dithiocarbamate and 5 ml of methylisobutyl ketone were added. Deionized water was added to bring the organic phase which contained chelated Ni into the neck of the bottle from which direct aspiration was made. The spectrophotometer was equipped with a hollow cathode nickel lamp with a 25 milliamp current. The wave length was 2320A. A stable flame was obtained with air and acetylene flow of .42 and .32 kg/cm^2, respectively. The lower limit of reliable detectability of this method was .1 ppm. Results were subjected to analysis of variance (Snedecor, 1956) and to Duncan's multiple range test (Duncan, 1955).

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Results and Discussion

Average daily intake of supplemental Ni was 0, 363, 1281 and 1557 mg for 0, 62.5, 250 and 1,000 ppm Ni treatment groups respectively (table 1). Those animals receiving the 250 ppm Ni diet continued to grow and to consume increased quantities of feed during the 8-week treatment period while those on 1,000 ppm Ni stopped growing and consumed approximately the same total quantity of feed weekly throughout the trial. Because of this, the 250 ppm Ni group was consuming significantly more Ni ($P<.05$) during the last 2 weeks of the trial than the first 2 weeks and at a daily level only 32 mg less than the 1,000 ppm Ni group (1,565 and 1,597 mg, respectively). Based on the final weight of the animals, the Ni intake per kilogram of body weight was 1.8, 7.0 and 14.6 mg for the 62.5, 250 and 1,000 ppm treatment groups, respectively.

The small difference in total Ni intake between 250 and 1,000 ppm Ni diet groups might suggest small differences in tissue Ni content. However, much higher levels of Ni were found in most tissues from animals on the 1,000 ppm Ni diet (table 2). This suggests that if a homeostatic mechanism exists for nickel as it does for zinc (Miller *et al.*, 1968) and other essential metals (Underwood, 1962), it has become, at least partially, inoperative at the 1,000 ppm Ni level. Differences in eating patterns were observed. Those fed 250 ppm Ni followed a normal eating pattern, distributed over a 24-hr. day but those fed 1,000 ppm Ni ate small quantities immediately following feeding and then ate infrequently or not at all at other times. Therefore, the Ni ingested by those animals receiving the 1,000 ppm diet entered the digestive tract in a shorter time segment of the 24-hr. day. The faster rate of Ni intake by animals fed 1,000 ppm Ni diet could have contributed to increased absorption and to differences observed in tissue nickel content.

TABLE 2. EFFECT OF NICKELOUS CARBONATE IN THE DIET ON NICKEL LEVEL OF BOVINE TISSUE

Tissue	Level of nickel supplementation, ppm: 0	62.5	250	1,000	SE[a]
	μg Ni/ml				
Serum	0.00[b]	0.00[b]	0.25[b]	2.88[c]	0.29
	μg Ni/g dry matter				
Serum	0.00[b]	0.00[b]	3.10[b]	37.68[c]	4.25
Kidney	2.08[b]	1.85[b]	2.26[b]	22.83[c]	1.91
Vitreous humor	0.00[b]	0.04[b]	0.35[b]	6.42[c]	0.69
Lung	2.94[b]	2.51[b]	13.64[b]	5.88[b]	4.15
Testis	0.46[b]	0.55[b]	0.95[b]	4.78[c]	0.13
Bile	0.00[b]	0.00[b]	0.27[b]	3.84[c]	0.22
Tongue	0.35[b]	0.37[b]	0.82[b]	3.14[c]	0.18
Pancreas	0.00[b]	0.22[b]	0.41[b]	2.73[c]	0.31
Rib	0.25[b]	0.39[b]	0.90[b]	2.32[c]	0.21
Spleen	0.00[b]	0.00[b]	0.00[b]	1.85[c]	0.05
Brain	0.06[b]	0.00[b]	0.02[b]	1.33[c]	0.09
Liver	0.76[b]	0.88[b]	0.37[b]	0.55[b]	0.27
Heart	1.11[b]	0.00[b]	0.32[b]	0.50[b]	0.58

[a] Standard error of a mean, three animals per treatment.
[b-c] Mean values within a row that are not followed by the same superscript are significantly different ($P<.01$).

No Ni was detected in blood serum of animals fed 0 or 62.5 ppm Ni diets. Ni content in serum from animals given 1,000 ppm Ni was 12 times greater than those fed 250 ppm. The level of Ni in the kidney did not change in animals fed 62.5 or 250 ppm but was elevated in animals at 1,000 ppm even though the daily Ni intake of the groups fed the 250 and 1,000 ppm did not differ greatly. Some of the observed Ni in kidneys could arise from residual urine. High levels of nickel were found in the vitreous humor of animals fed 1,000 ppm Ni.

Although not statistically significant, both 250 and 1,000 ppm Ni treatments increased lung tissue Ni levels with the greatest effect observed at 250 ppm. The animals on the 250 ppm Ni diet were observed to spend more time eating and, therefore, had their nostrils in contact with feed for a longer period of time. Thus, it is possible that a part of the lung Ni concentration found in animals fed 250 ppm

TABLE 1. AVERAGE INITIAL WEIGHT, DAILY FEED CONSUMPTION, LIVE WEIGHT GAINS AND SUPPLEMENTAL NICKEL INTAKE OF MALE HOLSTEIN CALVES

Item	Level of nickel supplementation, ppm: 0	62.5	250	1,000	SE[a]
Initial weight, kg	116	114	111	112	....
Daily rate of gain, kg	1.3[b]	1.5[c]	1.3[b]	—.1[d]	0.06
Daily feed consumption, kg	5.6[bc]	5.8[b]	5.1[c]	1.6[d]	0.18
Daily nickel intake, mg	...	363[a]	1281[b]	1557[c]	79.15

[a] SE—Standard error of a mean, three animals per treatment.
[b-d] Mean values within a row that are not followed by the same superscript are significantly different ($P<.05$).

TABLE 3. EFFECT OF DIETARY NICKEL LEVELS ON NI CONTENT OF DIGESTIVE TRACT TISSUES

Tissue	Level of nickel supplementation, ppm				
	0	62.5	250	1,000	SE[a]
	μg Ni/g dry tissue				
Rumen-reticulum	0.46[b]	10.59[c]	8.62[d]	7.64[c]	0.28
Omasum	0.37[b]	4.45[c]	10.63[d]	8.88[d]	0.63
Abomasum	0.07[b]	0.44[b]	1.46[b]	6.13[c]	1.00
Duodenum	0.54[b]	0.90[b]	8.96[b]	29.12[c]	4.60
Small intestine, 1st half	0.37[b]	1.12[b]	7.16[c]	14.02[d]	1.83
Small intestine, 2nd half	0.13[b]	0.82[c]	1.54[d]	5.83[e]	0.14

[a] SE—Standard error of a mean, three animals per treatment.
[b–e] Mean values within a row that are not followed by the same superscript are significantly different ($P<.05$).

Ni could result from inhalation of dietary $NiCO_3$. References to respiratory tract neoplasia among workers in nickel refineries have been quoted by Wase *et al.* (1954) and Schroeder, Balassa and Tipton (1962). Neoplasia, with its carcinogenic implications, could result from continued exposure to this feeding program. Testicular tissue from animals fed 1,000 ppm Ni contained 5 to 10 times as much Ni as the same tissue from animals at other levels of supplementation.

The elevated Ni content in bile at 1,000 ppm might be accepted as prima facie evidence of increased absorption of Ni by animals receiving this diet. No Ni was detected in bile at 0 or 62.5 ppm. Tongue tissue of all animals had detectable Ni levels with no difference observed at the three lower levels of supplementation. The 1,000 ppm diet increased Ni in this tissue to a level of 4 times greater than the 250 ppm treatment. This occurred in spite of the fact that the animals on 1,000 ppm ate for a much shorter period of time and therefore had physical contact between the tongue and the diet for a shorter period of time. Nickel content increased in the pancreas with increased dietary levels of Ni. The accumulation of Ni in rib, spleen, and brain was significantly greater in animals fed the 1,000 ppm dietary level of Ni but was less than in other tissues of animals fed the same level of Ni. There were no differences found in Ni concentration in liver and heart at any dietary level of Ni supplementation.

These data on tissue Ni levels vary widely from those reported by Phatak and Patwardham (1950) who fed rats Ni at levels similar to those used in this work and for the same period of time. In rats fed a diet containing 1,000 ppm Ni, these investigators found concentrations of tissue Ni in the following order: bones > heart > kidney > spleen > testis > blood > liver. At the same level of Ni supplementation, the order of tissue Ni concentrations observed in this work was: serum > kidney > testis > bones > spleen > liver > heart. While a comparison of data for monogastric and ruminant animals may not be valid, it is of interest to note that the data indicate the skeleton to be the main storage depot for Ni even though the Ni concentration in bone (leg *vs.* rib) differs greatly between the experiments. The data agree on the limited capacity of the liver to store Ni which is in contrast to most of the trace elements (Underwood, 1962). One major disagreement between the data for rats and calves is in the Ni level of heart muscle. Very little Ni was found in heart muscle in this work while large levels were found in rat heart muscle. High intakes of trace elements usually do not result in increased levels in heart muscle (Underwood, 1962).

Data of Ni content in digestive tract tissues are in table 3. There was no trend observed in the Ni content of ruminal or omasal tissues that could be attributed to supplementary dietary Ni levels. Abomasal, duodenal and intestinal Ni content increased as the level of Ni increased in the diet, but the differences were not always statistically significant. In digestive tract tissue of those animals fed 1,000 ppm Ni, the greatest amount of Ni was observed in the duodenum and first half of the small intestine which probably indicates these sites to be the major absorptive area for ingested Ni. In both the 250 and 1,000 ppm Ni fed groups, the level of Ni declined approximately 80% between tissue of the duodenum and the 2nd half of the small intestine.

The primary route of excretion of supplemental dietary Ni was feces (table 4). Fecal material contained from 22 to 51 times as much Ni as urine, with animals receiving 250 ppm in the diet having the largest total daily fecal excretion of Ni. During the excreta collection period, animals on 1,000 ppm Ni consumed only 6.7% more Ni than those on the 250 ppm diet but retained more than 20 times as much Ni, which accounts for the greatly elevated Ni content of many of the analyzed tissues. The analysis for excreted Ni cannot differenitate between unabsorbed and reexcreted Ni, thus the fecal Ni necessarily included both.

Absorption of Ni apparently increased with increased dietary levels of supplemental Ni as

TABLE 4. EFFECT OF DIETARY NICKEL LEVEL ON AVERAGE NICKEL CONTENT OF EXCRETA COLLECTED OVER A 5-DAY PERIOD

Item	Level of nickel supplementation, ppm				
	0	62.5	250	1,000	SE[a]
Urinary Ni					
μg/ml	0.08	1.86	6.18	33.90	.....
Total daily excretion, mg	0.47[b]	7.16[b,c]	24.75[c]	45.31[d]	5.61
Fecal Ni					
μg/g dry matter	5.40	158.25	713.76	2299.78	.....
Total daily excretion, mg	9.83[b]	260.56[b]	1285.30[c]	1020.90[d]	80.71
Total excreta, mg Ni	10.30[b]	267.72[b]	1310.05[c]	1066.21[c]	85.58
Supplemental Ni intake, mg daily	.....	335.37	1325.62	1414.20	.....

[a] Standard error of a mean with three animals per treatment.
[b–d] Mean values within a row that are not followed by the same superscript are significantly different ($P<.05$).

shown by urinary Ni values in table 4 and bile Ni values in table 2. Also, pancreas Ni values increased at higher levels of supplementation indicating the possibility of reexcretion via pancreatic juice. The kidney has limited capacity for excretion of elements like Zn, Fe and Cu (Underwood, 1962) which appears to be in contrast to its ability to excrete Ni. The excretion of Ni in urine, expressed as a percent of the total Ni excreted, was 2.7, 1.9 and 4.3 for 62.5, 250 and 1,000 ppm diets, respectively.

These data show conclusively that absorption and tissue retention of dietary Ni can be increased and that this increase is related to the rate of, as well as the total, Ni intake. The long term effect of high tissue Ni levels has not been determined but growth of animals receiving 1,000 ppm Ni in the diet was stopped during the 8-week treatment period. This effect on growth may indicate an inhibitory relationship between Ni and vital biological system(s) within the animal's body. Ni has been shown to inhibit both growth and vitamin B_{12} synthesis of certain bacteria (Rainbow and Rose, 1963). In other work (O'Dell *et al.*, 1970) the growth inhibition disappeared quickly when Ni fed animals were returned to a basal diet without supplemental Ni. It should be pointed out that the daily caloric intake by the group receiving the highest level of Ni was insufficient to support weight gains. Even without weight gain, growth of long bones should have occurred. This growth was not observed (O'Dell *et al.*, 1970).

Summary

Tissue distribution and excretion of nickel were determined for 12 male Holstein calves fed supplemental dietary nickel as nickelous carbonate ($NiCO_3$) at 0.0, 62.5, 250.0 and 1,000.0 ppm of the total diet. Feed intake and rate of gain were greatly reduced at a dietary Ni level of 1,000 ppm. The average daily supplemental Ni intake was 0.4, 1.3 and 1.6 g for the 62.5, 250 and 1,000 ppm Ni treatments, respectively. Tissue Ni content did not differ statistically among the first 3 treatments but at 1,000 ppm Ni, a highly significant ($P<.01$) increase in Ni content was found in many tissues. Tissues from animals on 1,000 ppm Ni supplementation accumulated Ni in the following order: serum>kidney>vitreous humor>lung>testis>bile>tongue>pancreas>rib>spleen>brain while liver and heart Ni levels did not differ statistically at any treatment level. Of the total Ni excreted, 97.3, 98.1 and 95.8% appeared, respectively, in the feces of the calves receiving the 62.5, 250 and 1,000 ppm treatments. One thousand ppm Ni in the diet appeared to overcome any existing homeostatic control mechanism even though the total daily Ni intake at this level was not greatly different from that on the 250 ppm level.

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SPECTROCHEMICAL DETERMINATION OF MAGNESIUM, CHROMIUM, NICKEL, COPPER AND ZINC IN HUMAN PLASMA AND RED CELLS

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Recently this laboratory[1] reported a spectrochemical procedure for the determination of magnesium, chromium, nickel, copper and zinc in human plasma. It was part of a long-range study of the trace element content of human blood being conducted in co-operation with BYRD, LEAVELL *et. al.* in the Hematology Laboratory of the University of Virginia School of Medicine. The work reported in this communication is a continuation of these studies and includes values for magnesium, chromium, nickel, copper and zinc in human red cells, as well as additional values for these metals in human plasma. All samples of blood were taken from persons believed to be normal in health; a number of the samples were taken from the same individuals at intervals of several weeks to several months in order to determine the variation for the metal concentrations in the sample donors over a period of time. Similar studies are being made on samples taken from diseased persons but these will be reported later in a joint publication with the Hematology Laboratory.

The procedures given in this paper require only about half the overall time required by those previously reported[1] for plasma (35 hours), being 14 h for plasma and 17 h for red cells.

APPARATUS

Spectrograph. Applied Research Laboratories 2-meter grating spectrograph, modified with exterior optics and ignitor[2]. The camera was set so that all five elements could be determined on one film, covering a range from 4200 to 7000 Å. Eighteen spectra were photographed on one film.

Excitation source. Applied Research Laboratories rectifier unit, 0–15 A direct current.

Densitometer. Applied Research Laboratories film projection comparator-densitometer.

Electrodes. National Carbon special spectrographic graphite, 3/16-inch-diameter rod, cut to 1-inch lengths. The sample end of the electrode was drilled to a depth of 1/16 inch with a 3/16-inch drill. The cup thus formed was treated with a drop of paraffin solution in carbon tetrachloride to prevent absorption of the sample by the electrode. The sample electrode is lower and negative. The upper and counter electrode is 1/8-inch in diameter.

Muffle furnaces. Electric furnaces operated by automatic controllers (Wheelco Model 241 P) were used in ashing the samples. The temperature was checked with a thermocouple and was held within 465–475°.

Hypodermic syringes. 50-ml syringes, fitted with special platinum (89%)–ruthenium (11%) alloy needles (J. Bishop and Co., Platinum Works, Malvern, Pa.). The needles are size No. 19, and their collars are heavily gold plated.

References p. 514

REAGENTS

Ammonium hydroxide. Baker and Adamson, reagent grade. Free of molybdenum and the metals being determined as shown spectrographically.

Hydrochloric and nitric acids. Baker's analysed reagents, distilled in an all-Pyrex distilling apparatus until spectrographic analyses showed the absence of molybdenum and the metals to be determined.

Hydrogen peroxide. Fisher certified reagent, high purity, 30%, with only 0.0001% heavy metals, 0.00002% Fe and free of Mg, Cr, Ni, Cu, Zn and Mo.

Sodium heparin, solid salt. USP, Organon, Inc., Orange, N.J. Qualitative spectrographic analysis of a sample showed it to be free of molybdenum and metals to be determined.

Standard solutions. Solutions of sodium, potassium, calcium, magnesium, chromium, iron, nickel, copper and zinc were made from spectroscopically pure salts or metals (Mathey's "specpure") and were used in the preparation of "synthetic" blood fractions.

Molybdenum trioxide (internal standard). Add 1.500 g of molybdenum trioxide ("specpure") to a 1-l volumetric flask containing 100 ml of concentrated ammonium hydroxide and gently warm on a hot plate till solution is complete. Then add 300 ml triply distilled hydrochloric acid, dilute to the mark with triply distilled water and mix. This solution contains 1 mg Mo per ml.

Triply distilled water. Laboratory distilled water was redistilled twice. Two Pyrex stills in series and automatically controlled were used, the second condenser being fitted with a fused silica tube leading into a large polyethylene bottle protected from dust.

EXPERIMENTAL

A. Cleaning procedure

1. All glassware and polyethylene bottles, etc. used in the analyses should be cleaned in the following manner: (a) Wash thoroughly with detergent and water; (b) Immerse in a 1:1 mixture of concentrated sulfuric and nitric acids for at least one hour; (c) Rinse six times with distilled water, followed by six rinsings with triply distilled water; (d) Dry in an oven at 80°.

2. The needles are cleaned in the same manner as described above except that they are dipped in the acid bath for 10 to 15 sec only. The clean needles and syringes are placed in glass jackets, which have also been cleaned, and are sterilized in an oven at 180° overnight.

B. Synthetic samples and calibration curves

Calibration curves were obtained from synthetic samples whose composition closely approximated that of the blood fraction being analysed (see Table I). These samples were stored in sealed polyethylene bottles and were used periodically for checking and/or recalibration of the standard curves.

C. Preparation of samples

The blood samples must be drawn from the donor in a careful manner in order to avoid contamination. They were drawn as described by THIERS, WILLIAMS AND

References p. 514

YOE[3] in their work on cobalt, using a 50-ml hypodermic syringe, fitted with a special platinum-ruthenium alloy needle. Sodium heparin (1.6 mg per 50 ml of sample) is placed in the syringe before the sample is drawn so as to prevent coagulation of the blood. Immediately after being drawn, the blood is transferred to a 60-ml polyethylene bottle and the red cells and plasma fractions separated by centrifugation. Samples

TABLE I

DATA ON SYNTHETIC SAMPLES

Element	Concentrations in p.p.m. Cells	Plasma
Sodium	1200	2000
Potassium	400	200
Calcium	20	100
Magnesium	30–96	12–36
Iron	510	1
Chromium	0.024–0.08	0.02–0.08
Nickel	0.020–0.08	0.04–0.125
Copper	0.4–1.4	0.6–2.0
Zinc	4–20	1–7.5

ranging from four to six grams for the cells and from nine to eleven grams for the plasma, are drawn from the bottle with a syringe and are individually transferred to previously weighed 50-ml Erlenmeyer flasks that have been tightly covered with thin polyethylene caps. The flasks are uncapped, reweighed and the sample weights obtained by difference. The samples are dried under a set of 250-W lamps; this requires about 2 h. They are then transferred to a hot plate under another infrared lamp, charred for about one hour and placed in a muffle furnace at 465–475°. The plasma requires about 7 h for ashing; the red cells about 10 h. Only one sample out of sixty required a longer ashing time. "Synthetic" samples containing known amounts of zinc were given the same analysis steps as the plasma and red cells and there was no indication of loss of zinc due to volatilization (see Table III). However, a word of warning is in order because of the danger of local overheating in the case of large samples, due to the heat resulting from oxidation[1, 4, 5].

The samples are removed from the furnace, cooled and to each is added 2 ml of concentrated nitric acid and 0.25 ml of 30% hydrogen peroxide. They are evaporated to dryness under infrared lamps and given successive treatments of 1 ml of nitric acid to insure complete removal of any organic matter left from the ashing. The salts are converted to chlorides by the addition of hydrochloric acid and evaporation to dryness. They are then ready for dilution and spectrographic analysis.

Analytical procedure

The chloride salts are dissolved in triply distilled water and made to volume in 5-ml volumetric flasks to which has been added 0.13 ml of the molybdenum trioxide solution as the internal standard. To facilitate dissolving the plasma samples, two drops of hydrogen peroxide were added to the chloride salts. An appropriate volume of sample solution (see Table II) is transferred dropwise with a syringe to a graphite electrode and evaporated to dryness. A soapstone block drilled to hold electrodes serves as a convenient holder; it is placed on a hot plate at 130°.

References p. 514

The arcing, exposure, and photographic processing conditions are summarized in Table II. After developing the film, the percentage transmission of the analytical lines (all in the second order) of the elements being determined the internal standard lines are measured with a densitometer and the concentration of the metals are read off from the calibration curve, prepared by plotting p.p.m. against the ratio of the intensity of the internal standard line to that of the analytical line.

TABLE II

DATA ON SPECTROGRAPHIC CONDITIONS OF THE METHOD

Spectral region	4200–7000 Å	Emulsion	SA No. 2 film
Slit width	0.04 mm	Development	Eastman Kodak D 19 5 min at 70 °F
Preburn time	none	Fixing	Eastman Kodak, rapid liquid fixer, 5 min duration
Exposure period	35 sec		
Amperage	12 A	Washing	10 min
Electrode gap	6 mm		

	Mg	*Cr*	*Ni*	*Cu*	*Zn*
Analytical lines (2nd order)	2795.53	4254.34	3414.76	3247.54	3345.02
Internal stand lines (2nd order)	2816.15	2816.15	2816.15	3208.83	2816.15
Amount/electrode (cell)	0.22 ml	0.22 ml	0.22 ml	0.05 ml	0.22 ml
Amount/electrode (plasma)	0.35 ml	0.35 ml	0.35 ml	0.03 ml	0.35 ml

RESULTS

A. Accuracy of the method

A comparison with those reported in the literature indicates that our results are consistent. However, a more rigorous evaluation of the method was desired. This was

TABLE III

DATA ON ACCURACY OF METHOD

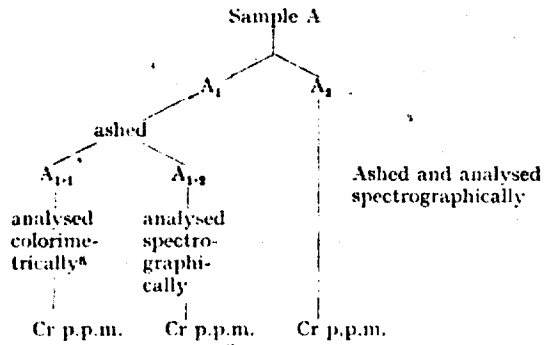
		Cells		*Plasma*	
Run	*Element*	*Amount added in p.p.m.*	*Amount recovered in p.p.m.*	*Amount added in p.p.m.*	*Amount recovered in p.p.m.*
1	Magnesium	40.0	38.7	10.0	9.79
2		70.0	70.6	25.0	26.1
3		100.0	98.8	40.0	40.75
1	Chromium	0.01	0.011	0.01	0.01
2		0.025	0.024	0.025	0.027
3		0.04	0.04	0.04	0.044
1	Nickel	0.05	0.052	0.05	0.054
2		0.10	0.11	0.10	0.10
3		0.20	0.21	0.20	0.19
1	Copper	0.40	0.414	0.50	0.54
2		1.00	1.03	1.00	0.97
3		2.00	1.89	2.00	1.98
1	Zinc	5.00	4.78	1.00	0.98
2		12.00	12.36	3.00	3.06
3		20.00	19.67	5.00	5.13

References p. 514

done by preparing "synthetic" samples of the five metals under consideration and analyzing them. The results are shown in Table III. In the case of chromium the method was further checked against a spectrophotometric method recently developed in

TABLE IV

DATA ON ACCURACY OF METHOD FOR CHROMIUM



Cr p.p.m.	Cr p.p.m.	Cr p.p.m.	
0.027	0.028	0.030	
0.032	0.030	0.027	Sample B
0.026	0.026	0.024	Sample C
0.034	0.032	0.035	Sample D
0.029	0.032	0.031	Sample E
0.017	0.017	–	Sample F[b]
0.026	0.025	–	Sample G[b]

[a] Analyses by DWIGHT O. MILLER.
[b] Samples F and G were separated after they had been ashed.

this laboratory[6]. The steps followed in the two methods and a comparison of the results are presented schematically in Table IV. As is noted, the two methods gave satisfactory values for chromium and are in excellent agreement with each other.

B. Precision of the method

A series of samples was analyzed at intervals of three months in order to determine any fluctuation in the standard values obtained from the calibration curves. The results for the blood fractions of one of these samples are shown in Table V. The coefficients of variation are given in Table VI.

TABLE V

DATA ON PRECISION OF THE METHOD

Element	*Blood fraction*	*Run 1 p.p.m.*	*Run 2 p.p.m.*	*Run 3 p.p.m.*	*Average p.p.m.*
Magnesium	Plasma	14.0	16.2	15.0	15.1
	Cells	52.7	58.6	50.2	53.8
Chromium	Plasma	0.024	0.025	0.025	0.025
	Cells	0.017	0.019	0.016	0.017
Nickel	Plasma	0.00	0.00	0.00	0.00
	Cells	0.00	0.00	0.00	0.00
Copper	Plasma	0.58	0.60	0.67	0.62
	Cells	0.59	0.92	1.07	0.99
Zinc	Plasma	2.30	3.00	2.60	2.63
	Cells	8.80	9.20	9.30	9.10

References p. 514

TABLE VI

COEFFICIENT OF VARIATION[a]

Element	*Blood fraction*	*Number of determinations*	*Average concentration p.p.m.*	*Coefficient of variation, v*
Magnesium	Plasma	39	19.4	0.30
	Red cell	40	64.8	0.37
Chromium	Plasma	39	0.024	0.19
	Red cell	40	0.022	0.31
Nickel	Plasma	39	0.023	1.95
	Red cell	40	0.051	0.72
Copper	Plasma	39	0.99	0.13
	Red cell	40	0.94	0.63
Zinc	Plasma	39	2.70	0.44
	Red cell	40	11.40	0.27

[a] Calculated as follows:

$$v = \frac{1}{C}\sqrt{\frac{\Sigma d^2}{n}}$$

where: d = difference of the determination from the mean.
n = number of determinations.
C = average concentration.

TABLE VII

MAGNESIUM, CHROMIUM, NICKEL, COPPER AND ZINC IN HUMAN PLASMA

Element	*Average concentration p.p.m.*	*Spread*	*Number of determinations*	*Method*	*Investigator*
Magnesium	19.4	13.1–37.2	39	Spectrochemical	PAIXAO AND YOE
	20.4	17.0–28.8		Colorimetric	ALBRITTON[7]
	24.0	10.0–42.0	25	Spectrochemical	MONACELLI *et al.*[1]
	22.7			Colorimetric	ORANGE[8]
		17.0–25.0		Spectrochemical	SHIMP *et al.*[9]
Chromium	0.024	0.016–0.038	39	Spectrochemical	PAIXAO AND Y[illegible]
	0.180	0.080–0.300	25	Spectrochemical	MONACELLI *et al.*[illegible]
	0.020	0.007–0.050	17	Colorimetric	KOCH *et al.*[10]
Nickel	0.023	0.00–0.18	39	Spectrochemical	PAIXAO AND Y[illegible]
	0.040	0.01–0.06	12	Spectrochemical	MONACELLI *et al.*
	0.030	0.01–0.09		Spectrochemical	KOCH *et al.*[10]
Copper	0.99	0.50–2.47	39	Spectrochemical	PAIXAO AND [illegible]
	1.10	0.76–1.42		Colorimetric	ALBRITTON[7]
	1.20	1.00–2.10	22	Spectrochemical	MONACELLI *et al.*[illegible]
	0.92	0.67–1.30	12	Spectrochemical	KOCH *et al.*[10]
	0.98	0.65–1.35	58	Colorimetric	KOCH *et al.*[10]
Zinc	2.70	0.48–4.80	39	Spectrochemical	PAIXAO AND [illegible]
	3.00	0.00–6.13		Colorimetric	ALBRITTON[7]
	1.30	0.60–2.30	14	Spectrochemical	MONACELLI *et al.*[illegible]
	1.20	0.32–1.70	58	Colorimetric	KOCH *et al.*[10]
	1.95	1.37–2.84	100	Colorimetric	WOLFF[11]
	3.50	1.20–11.40	30	Colorimetric	VALLEE[12]
	1.25	0.84–1.63	62	Colorimetric	VICKBLADH[13]
	1.13	0.72–1.60	24	Colorimetric	BERFENSTRAM[14]
	0.71	0.35–0.80		Spectrochemical	SHIMP *et al.*[9]

References p. 514

C. Discussion

The results obtained in the analyses are grouped separately for plasma and red cells and are listed in Tables VII and VIII. From these results, values for whole blood were calculated based on 55 and 45 volume percents for plasma and cells, respectively.

TABLE VIII

MAGNESIUM, CHROMIUM, NICKEL, COPPER AND ZINC IN HUMAN RED CELLS

Element	*Average concentration p.p.m.*	*Spread*	*Number of determinations*	*Method*	*Investigator*
Magnesium	64.8	26.6 –112.0	40	Spectrochemical	PAIXAO AND YOE
	61.2			Colorimetric	ALBRITTON [7]
Chromium	0.022	0.011–0.041	40	Spectrochemical	PAIXAO AND YOE
Nickel	0.051	0.00 – 0.16	40	Spectrochemical	PAIXAO AND YOE
Copper	0.94	0.28 – 3.5	40	Spectrochemical	PAIXAO AND YOE
	0.75	0.49 – 1.01		Colorimetric	ALBRITTON [7]
	0.85	0.21 – 1.97	60	Colorimetric	KOCH *et al.* [10]
Zinc	11.4	3.80 –16.6	40	Spectrochemical	PAIXAO AND YOE
	14.4	9.10 –19.7		Colorimetric	ALBRITTON [7]
	11.8	7.65 –15.1	57	Colorimetric	KOCH *et al.* [10]
	13.0		20	Colorimetric	WOLFF [11]
	14.3		30	Colorimetric	VALLEE [12]

These results are given Table IX. The accuracy and precision of the method are good. The time required to prepare and analyze a sample has been reduced to less than half the time required for the plasma analysis procedure recently reported by this laboratory [1]. About 17 h are required for the red cells.

TABLE IX

MAGNESIUM, CHROMIUM, NICKEL, COPPER AND ZINC IN HUMAN WHOLE BLOOD

Element	*Average concentration p.p.m.*	*Spread*	*Number of determinations*	*Method*	*Investigator*
Magnesium	40.0[a]				PAIXAO AND YOE
	41.4	36.0–44.4		Colorimetric	ALBRITTON [7]
Chromium	0.023[a]				PAIXAO AND YOE
Nickel	0.036[a]				PAIXAO AND YOE
Copper	0.97[a]				PAIXAO AND YOE
	0.94	0.73 - 1.15		Colorimetric	ALBRITTON [7]
	1.06	0.50 - 1.88	54	Colorimetric	KOCH *et al.* [10]
Zinc	6.64[a]				PAIXAO AND YOE
	8.80	4.90–12.7		Colorimetric	ALBRITTON [7]
	6.30	4.50– 9.35	54	Colorimetric	KOCH *et al.* [10]
	7.00	5.60– 9.01	30	Colorimetric	WOLFF [11]
	8.80	5.20–14.8	30	Colorimetric	VALLEE [12]
	8.10	6.90– 9.80	18	Colorimetric	VIKBLADH [13]
	6.60	5.60 - 7.50	24	Colorimetric	BERFENSTAM [14]

[a] Calculated from the values for plasma and red cells (Tables VII and VIII).

References p. 514

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The blood samples for this investigation were collected by Mrs. JOYCE MAHON, technician in the Hematology Laboratory of the University of Virginia, School of Medicine. This assistance is gratefully acknowledged.

SUMMARY

Procedures are given for the collection and digestion of human plasma and red cells for the spectrochemical determination of Mg, Cr, Ni, Cu and Zn. Values found for the five metals are compared with those reported in the literature. The accuracy and precision of the method are good. The time required to prepare and analyze a sample has been reduced to less than half (14 h) required for the plasma analysis procedure recently reported by this laboratory. The red cells require about 17 h.

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197 **Carcinogenicity of Nickel Compounds in Experimental Animals.** W.W.Payne, National Cancer Institute, Bethesda, Maryland.

The production of cancers in animals by powdered nickel, nickel sulfide and nickel oxide has been reported by others. We sought to determine whether the carcinogenicity was dependent upon solubility or the valence state of nickel compounds.

Nine nickel compounds (7 mg.) of varying solubility were formed into pellets with sheep fat and implanted surgically in the muscle tissue of NIH Black rats. Pellets of sheep fat were implanted in control animals. Treatment was repeated three times in groups of 35 rats each. After 18 months, 12 rats implanted with nickel sulfide had developed tumors at the site of implantation, 8 had died without tumor, 15 still survive; for nickel carbonate there were 6 with tumor, 12 without tumor and 17 surviving; for nickelous oxide there were 4 with tumor, 14 without tumor and 17 surviving. Tumors were not observed in rats implanted with Ni chloride, Ni anhydrous acetate, Ni ammonium sulfate, Ni^{+++} oxide and sheep fat control. Single tumors were observed with Ni sulphate and Ni acetate. Histologically the tumors were rapidly growing anaplastic sarcomas with metastases in some cases.

The soluble compounds were more toxic and were excreted more rapidly in the urine and feces. Carcinogenicity appears to be a reflection of the solubility of the compound and the valence state of the nickel. Rat mesenchymal tissue appears to be capable of distinguishing carcinogenic members of a related group of compounds.

Dermatology

Minn Med
51:289-90 (1963)

Nickel Sensitivity

A 38-year-old woman was seen with an eruption involving both ear lobes (Figure). In addition, she had bilateral circumscribed eczematous lesions on the anterolateral aspects of both thighs. A tentative diagnosis of contact sensitivity to metal was made which was later confirmed by a patch test using a 5% aqueous solution of nickel sulfate. The patient was instructed to avoid earrings and to use protective pads under her garters. With these measures and a topical ointment the eruption promptly cleared.

This relatively simple case serves to illustrate a not too simple problem, that of contact sensitivity to metal. Of the metals producing such sensitivity, nickel is by far the most common. Nickel is usually present alloyed with other metals; some of the more common include alnico, duralium, nichrome, stainless steel and coin alloys. In years past this sensitivity was confined mainly to industrial workers, though today contact allergy to nickel is increasing in the general population and especially among women. Today nickel is found in an ever-increasing number of silver and gold articles with which people may come in

Figure

contact, such as jewelry, buckles, zippers, etc.

Although nickel dermatitis may occur anywhere on the body, certain areas are more common, such as ear lobes, neck, wrists and thighs. Clinically, nickel dermatitis often appears as a chronic eczema with lichenification resembling atopic or neurodermatitis. A characteristic of this dermatitis is its spread to distant areas without apparent direct nickel contact. At present this phenomenon has not been adequately explained.

Of the factors influencing nickel dermatitis, sweating is felt to be of prime importance. Sweat chlorides apparently are necessary in converting the metallic nickel to its soluble salts which in turn produce the sensitivity. In conjunction with sweat, pressure and friction are also important. This may explain the relative frequency of ear-lobe dermatitis secondary to nickel sensitivity in women who wear earrings. Articles containing nickel which are not worn under pressure or are worn on areas where sweating is minimal may cause little or no reaction. A frequently raised question is whether atopic individuals have a greater tendency to develop this type of reaction. To date the evidence does not support this contention. However, there does appear to be increased susceptibility in those individuals with dermatitic and soap-alkali-damaged skin. The mechanism of nickel sensitivity is not clearly defined, although studies tend to support the concept of delayed hypersensitivity in the absence of a hapten* allergic mechanism.

Once this sensitivity develops it tends to remain for many years. Therefore, management of the patient with nickel sensitivity consists of avoidance of direct contact with nickel-containing metals and the use of substitutes such as plastic garter clips. To date, desensitization attempts have been a failure. In addition to avoidance, the use of topical corticosteroid preparations is most helpful in clearing the eruption. Also, the use of a topical corticosteroid prior to the use of a nickel-containing article may be of help in mitigating the reaction.

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*See Test Your Word Score Page 217.

MINNESOTA MEDICINE Cover Photographs

In order to encourage more physicians to submit color slides for use on the cover of MINNESOTA MEDICINE the competition is being discontinued. The picture editor now invites all physician-photographers to send 35 mm. color slides of suitable subjects for consideration as cover pictures. The format must be horizontal because of printing limitations. Pictures high-lighting Minnesota seasons, places, and activities will have an automatic advantage. No limit as to the number of slides sent will be set, but all slides should be individually identified. All slides will be returned. Physicians whose pictures are used as covers will be recognized in MINNESOTA MEDICINE. We can use many more pictures than we have received in the past.

Paradoxical

Two things fill the mind with ever new and increasing wonder—the starry heavens above me, and the moral law within me.—Immanuel Kant

Australian J. Exp. H. Biol. Med. Sci. 22 (pt. I) 21-7 (1944)

THE ACTION OF METALS ON BACTERIAL INTOXICATION

by MARY H. PETHERICK[1] AND E. SINGER.

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(Accepted for publication, 23rd December, 1943.)

Recently Petherick and Singer (1943) described an investigation of the detoxication of diphtheria toxin by metals. In guinea pigs iron, copper or zinc, injected simultaneously with a skin dose of diphtheria toxin, abolished the skin reaction. Manganese, nickel and cobalt were inactive. This detoxication was reproduced *in vitro* by incubating diphtheria toxin in high dilution with iron, copper or zinc compounds together with redox substances such as ascorbic acid, adrenaline or glutathione. It was suggested that detoxication of toxin in the *in vivo* experiments was due to a similar mechanism, the tissue apparently furnishing the redox substances necessary for detoxication.

This paper deals with the effect of the same metals on staphylococcal toxin and the toxins of *Cl. welchii* A and D. Preliminary experiments showed that staphylococcal toxin, injected intradermally into the guinea-pig skin produces reactions bearing a superficial similarity to those caused by diphtheria toxin. Welchii toxins A and D do not produce well-defined skin reactions. Using the method applied with diphtheria toxin, the metal compounds had no effect on the skin reaction produced by staphylococcal toxin.

Accordingly a different technique was adopted and the curative effect of the metals on mice injected with a fatal dose of toxin was investigated.

CURATIVE ACTION OF METALS.

Material and methods. White mice, bred at the Institute, were used throughout. They averaged 20 gm. weight and were kept on a diet of bread, bran, bone meal, sunflower seeds and greens.

The metal compounds used were A.R. reagents with the exception of ferricitrate B.P.

Injections of metals and toxins were made subcutaneously into different sites.

Diphtheria toxin, staphylococcal toxin and welchii A toxin were kindly supplied by the Commonwealth Serum Laboratories and purified welchii D toxin by Dr. Turner of the C.S.I.R.

Method of treatment. As a preliminary, the doses of the different metal compounds which were fatal to mice when given in a single injection were determined.

TABLE 1.

Fatal doses of metal compounds expressed as mg. metal per 20 gm. mouse.

Compound.	Fatal dose.
$FeSO_4.7H_2O$	1·0 mg.
Ferricitrate	0·5 mg.
$CuSO_4$	0·6 mg.
$NiSO_4$	0·5 mg.
$CoSO_4$	1·0 mg.
$MnSO_4$	2·0 mg.
$ZnSO_4$	2·0 mg.

In the experiments with staphylococcal and welchii A and D toxins, the following treatment was adopted:

[1] Working under a full-time grant from the National Health and Medical Research Council.

The mice were given, over a period of 2 days, 4 injections of one-tenth the fatal dose of the metal. On the morning of the third day a fatal dose of toxin was injected followed one hour later by a fifth injection of metal compound. The total amount of metal administered was, therefore, one-half the dose fatal if given in a single injection.

As it is difficult to produce fatal diphtheria intoxication in mice, the treatment was modified to suit the particular conditions of these tests.

An amount of toxin, which would kill all the controls when injected daily over a period of from 10 to 12 days, was used for the experiments with diphtheria toxin and a control experiment with welchii D toxin. Two injections of the metal compounds totalling one-fifth of the fatal dose were given on the day preceding the first injection of toxin. Thereafter one injection of one-twentieth of a fatal dose of the metal compound, followed after 3 hours by an injection of toxin, were given daily until the death of the last control.

Survival for 3 days after the death of the last control was accepted as the end point of the experiment since very few delayed deaths occurred in such experiments. These are mostly due to intercurrent infections precipitated by the toxaemia.

TABLE 2.

Curative experiments on mice injected with fatal dose of toxin.

Figures in italics give number of survivors, figures in Roman type give number of deaths.

Toxin.	Fatal doses of of toxin.	Metal compounds used for injection and total amount of metal administered in mg. per 20 gm. mouse. $FeSO_4$ 0·5.		Ferricitrate 0·4.		$CuSO_4$ 0·5.		$ZnSO_4$ 2·0.		$CoSO_4$ 0·5.		$NiSO_4$ 0·25.		$MnSO_4$ 1·0.		Controls.	
Staphylococcal	0·045 ml.	*7*	9	*4*	8	*4*	8	*4*	4	*2*	6	*2*	6	*7*	1	*1*	19
Welchii A	0·18 ml.	*10*	2	*4*	4	*5*	3	*6*	2	*6*	2	*6*	2	*5*	3	*2*	14
Welchii D	0·075 mg.	*1*	7	—		*1*	6	*4*	4	*0*	7	*2*	6	*4*	4	*1*	11
Diphtheria	12 × 0·1 ml.	*6*	1	—		*3*	5	*2*	6	*0*	8	*2*	6	*2*	6	*0*	8
Welchii D	10 × 0·0125 mg.	*0*	4	—		*0*	4	*2*	2	*0*	4	*2*	2	*2*	2	*0*	4

Only simple metal compounds were used in these tests. As these caused severe infiltration and, in some cases, necrosis, it was necessary to determine whether the curative action was due to a specific effect of the metal or to a non-specific increase of resistance. In further experiments the metals were administered in the drinking water. The greatest amount that could be administered in this way was limited by the concentration that the mice would accept without undue restriction of the fluid intake. As the average daily consumption of fluid per mouse was approximately 2 c.c., the amount of metal introduced in this way was rather less than that administered by injection. Variations in uptake and irregular absorption from the intestines must be considered.

Administration of metal compounds in the drinking water began 2 days prior to the injection of toxin and was continued throughout the experimental period, which lasted 10 days in the case of staphylococcal, welchii A and D toxins, and 15 days in the experiment with diphtheria toxin. To prevent cumulative toxic effects, the concentration of the metal solution was reduced to one-quarter on the fifth experimental day.

TABLE 3.

Curative experiments on mice injected with fatal dose of toxin.

Figures in italics give number of survivors, figures in Roman type give number of deaths.

Toxin.	Fatal doses of Toxin.	Metal compounds administered in drinking water and metal content of water at commencement of experiment in mg. per ml. Ferricitrate 0·32.		$CuSO_4$ 0·25.		$ZnSO_4$ 0·5.		$CoSO_4$ 0·2.		$NiSO_4$ 0·1.		$MnSO_4$ 0·5.		Controls.	
Staphylococcal	0·04 ml.	*6*	2	*2*	6	*1*	7	*4*	4	*5*	3	*6*	2	*2*	6
Welchii A	0·15 ml.	*4*	3	*3*	5	*4*	4	*4*	3	*4*	4	*5*	3	*2*	6
Diphtheria	10 × 0·1 ml.	*3*	3	*1*	5	*2*	4	*2*	4	*0*	6	*2*	4	*0*	6
Welchii D	0·075 mg.	*0*	6	*0*	6	*0*	6	*1*	5	*1*	5	*3*	3	*0*	6

In judging the results of these experiments, it must be realized that they were designed for only one purpose. It had to be decided whether it was possible to prevent the death of mice injected with a fatal dose of different toxins by the administration of metal compounds. The experiments in which the metals were administered in the drinking water were performed to

obviate the possibility that the increase in resistance observed when metal compounds were injected subcutaneously was caused by a non-specific inflammatory effect. No attempts were made to determine optimal dosage or timing of administration. Refinements in experimental technique would have required a much larger number of mice. This was not justified, as the simple compounds tested are unsuited for exact determination of curative action.

The results with the two methods of administration are similar. Much lower doses of the metals may have been effective when given by injection.

Diphtheria intoxication is strongly influenced by iron in the form of ferrous sulphate, and moderately by ferricitrate and copper. The effect of zinc, nickel, cobalt and manganese is doubtful.

The results of the protection experiments on mice are practically identical with those obtained in the guinea-pig skin and with *in vitro* methods. The metals which were able to prevent the skin reaction or to detoxify the toxin *in vitro* could prevent the death of mice injected with a fatal dose of the toxin. Zinc, which had a slight effect only when tested on the guinea-pig skin or *in vitro*, was almost inactive in experiments on mice as were the metals which were inactive in the guinea-pig or with *in vitro* tests.

Experiments with the other toxins showed that iron and manganese were very active with staphylococcal toxin, zinc and copper were slightly active, and cobalt and nickel were inactive. Ferrous sulphate was very active with welchii A toxin and all the other metals had a marked, but lesser, curative effect. Striking differences were observed in the experiments with welchii D toxin. Only manganese was active in both types of experiment, zinc was active to a moderate extent when injected but was inactive when administered in the drinking water, and all the other metals were inactive.

The fact that the activity of the different metals varied with the toxin attracts attention, especially the effect of manganese on intoxication with staphylococcal, welchii A and D toxins. This makes the ambiguous results with diphtheria toxin rather more significant.

The strong activity of nickel and cobalt against welchii A toxin and their slight activity in some other intoxications suggest that the clear cut results which were obtained in the experiments on the guinea-pig skin and *in vitro* indicate only one of the possible mechanisms of detoxication in which biologically active metal compounds are implicated. The activity of the metals varies apparently not only with the toxin but with the organ affected. In experiments with diphtheria toxin, iron, copper and zinc are active in the guinea-pig skin, whereas manganese is devoid of activity but has some slight activity in generalized intoxication of mice. This fact could be explained by assuming that manganese cannot be activated in the skin but by some other organ.

To elucidate this point the effect of differences in the properties of the metals and the toxins was investigated.

Two fundamentals had to be disregarded, namely, the differences in the chemical constitutions of the toxins and in the distribution and chemical combinations of the different metals in the body of the experimental animals. Investigation of these questions would lead into fields of such complexity and difficulty that chances of success would be practically non-existent. If one accepts these restrictions only two problems remain, the solution of which could contribute to an understanding of the observed results:

(1) What are the differences between the reaction of the different toxins and the animal body which cause the varying activity of the metals?

(2) By what substances are manganese, nickel and cobalt activated as iron, copper and zinc are activated by ascorbic acid, adrenaline or glutathione?

For technical reasons the experiments were restricted to welchii D and staphylococcal toxins and manganese.

AFFINITY OF TOXINS FOR GUINEA-PIG SKIN.

In experiments with skin doses of staphylococcal toxin, the first signs of the reaction were observed after from 3 to 4 hours. In analogous experiments with diphtheria toxin, the reaction appeared after 24 to 48 hours. The rapid action of staphylococcal toxin might explain our failure to influence the skin reaction by the simultaneous injection of metal compounds.

The fate of intradermally injected toxins was investigated.

Method:

Diphtheria toxin and staphylococcal toxin. Several intradermal injections, each containing 20 skin doses of toxin in a volume of 0·1 ml., were given at varying intervals. The animal was then killed and the cutis excised, finely minced, mixed with saline, frozen, thawed three times and centrifuged. The total volume of tissue and extraction fluid was adjusted to 1 ml. If all the toxin were recovered, 0·1 ml. of the extract would contain 2 skin doses. This amount was injected intradermally into a guinea-pig.

Welchii D toxin. This toxin did not produce skin reaction. Ten fatal mouse doses were injected in a volume of 0·2 ml., the same technique was used for preparing the extracts, and 0·2 ml. was injected subcutaneously per 20 gm. mouse, 2 mice being used for each test. If all the toxin were recovered this volume would contain 2 fatal doses.

The results of these experiments are set out in Table 4.

TABLE 4.

Time after injection. (min.).	Toxin. Diphtheria.	Staphylococcal.	Welchii D.
10	+	—	++
30	+	—	+0
60	+	—	+0
2 hours	+	—	0 0
4 "	+	—	0 0

Diphtheria toxin and staphylococcal toxin: + Reaction equivalent to that produced by one skin dose. — No reaction.

Welchii toxin: ++ Both mice dead. +0 One mouse dead. 0 0 No mice dead.

These experiments show that the affinity of diphtheria toxin for the skin of the guinea-pig is very low as toxin was recovered up to 4 hours after injection. In comparable experiments with staphylococcal toxin, all toxin had disappeared within 10 minutes. The reaction of staphylococcal toxin with the guinea-pig skin is, therefore, instantaneous, because the wheal raised by the intradermal injection is clearly visible for 5 minutes, and during this time contact between toxin and cells must be poor.

Welchii toxin was demonstrated up to 1 hour after the injection of 10 fatal doses. However, the experiments with welchii D toxin are not strictly comparable with those with diphtheria and staphylococcal toxins.

Skin doses were used in the experiments with diphtheria and staphylococcal toxins and fatal doses for mice in the experiments with welchii D. The amount of toxin-protein injected is not known but it is probable that the fatal dose for mice contains a larger amount of toxin-protein than that necessary to produce a local skin reaction in the guinea-pig. It is probable that, in the experiments with welchii D toxin, the amount of toxin injected was excessive and could not be absorbed therefore as quickly as the small amounts of staphylococcal toxin.

One is justified in concluding that both staphylococcal and welchii toxins have a much greater affinity for guinea-pig skin than has diphtheria toxin.

DETOXICATION OF DIPHTHERIA TOXIN BY MANGANESE.

The technique applied in the investigation of the activity of iron, copper and zinc was used in these experiments. High dilutions of diphtheria toxin in phosphate buffer or inactivated rabbit serum were exposed at room temperature and 37° C., to the action of various combinations of manganese and other substances. The effect on the toxin was then tested by intradermal injection of appropriate dilutions into guinea-pigs.

A considerable number of possibilities had to be investigated and a short list of combinations which were found to be inactive is given below. The reasons which prompted the various investigations are given at the beginning of each series.

1. Increasing the detoxifying effect of iron, copper and zinc plus redox substances:
   (i) Mixtures of toxin, manganese and iron, cytochrome c, copper or zinc, plus ascorbic acid, adrenaline or glutathione in phosphate buffer or inactivated rabbit serum.
   (ii) Mixtures of toxin, manganese and iron or cytochrome plus cysteine in inactivated serum.
2. Stimulating the production of ascorbic acid by manganese:
   Mixtures of toxin and manganese with glucose, galactose or mannose in phosphate buffer, inactivated rabbit serum and extracts from mouse and guinea-pig organs (Rudra, 1939a and b).

3. **Activation of manganese by complement:**
   Mixtures of toxin and manganese with and without addition of cysteine in active rabbit serum.
4. Specific activation of manganese by vitamin B_1.
   Mixtures of toxin and manganese with vitamin B_1 in phosphate buffer (Foster *et al.*, 1942; Perla, 1939).
5. Activation of proteolytic enzymes by manganese:
   Mixtures of toxin and manganese with organ extracts of mouse and guinea-pig with and without addition of cysteine.

The only experiments which gave positive results are discussed below. Hydrogen peroxide activated the detoxication of diphtheria toxin by manganese. Dilutions of diphtheria toxin in Sörensen's phosphate buffer of pH 6·6 were set up in test tubes and the appropriate additions of manganese, as manganese sulphate, and hydrogen peroxide were made.
The total volume was 3 ml., the toxin content being 30 skin doses per 0·1 ml. After incubation at 37° C. for 5 hours, 0·1 ml. of a 1/30 dilution of each mixture was injected into guinea-pigs. The results are given in Table 5.

Slight varintions in results occurred, hence every experiment is recorded, the figures indicating the number of tests made, e.g. with a concentration of hydrogen peroxide of 1×10^{-4} and no addition of manganese, in six different experiments the intradermal reaction following injection of the toxin dilution was ++. This is given in the Table as 6++.

TABLE 5.

Compound tested.	Metal mg. p.c.	Concentration of H_2O_2.	Skin reaction.
—	—	1×10^{-4}	6++
		5×10^{-5}	1++
		1×10^{-5}	1++
		5×10^{-4}	1++
		$2{\cdot}5 \times 10^{-4}$	1++, 1±
$MnSO_4$	1·5	1×10^{-4}	3++, 2+, 1±
		5×10^{-5}	1++
		1×10^{-5}	1 0
		5×10^{-4}	1±
		$2{\cdot}5 \times 10^{-4}$	2 0

++ Signifies the reaction obtained with one skin dose of diphtheria toxin consisting of redness and infiltration one cm. in diameter. +, ± Reactions weaker than one skin dose. 0 No reaction.

The effect of mixtures of other metals with hydrogen peroxide was then investigated. Detoxication occurred when toxin was incubated with iron, copper or zinc and hydrogen peroxide. Cobalt and nickel were not activated. The results with iron, copper and zinc are shown in Table 6.

TABLE 6.

Compound tested.	Metal mg. p.c.	Concentration of H_2O_2.	Skin reaction.
$CuSO_4$	1·5	1×10^{-4}	2++, 2±, 1 0
		5×10^{-5}	1 0
		1×10^{-4}	1 0
		5×10^{-4}	1 0
		$2{\cdot}5 \times 10^{-4}$	1 0
$ZnSO_4$	1·5	1×10^{-4}	1++, 3±
		$2{\cdot}5 \times 10^{-4}$	1 0
$FeSO_4$	1·5	1×10^{-4}	1++, 3±
		$2{\cdot}5 \times 10^{-4}$	1 0

The controls given in Table 5 apply to both series of experiments.

Iron, copper and zinc are effective with both redox substances and hydrogen peroxide as activators. The question arises whether hydrogen peroxide is the direct activator of the detoxication of toxin by these metals. The aerobic oxidation of the redox substances and the production of hydrogen peroxide would thus be the first step in a two stage reaction. The second step would then be the oxidation of the toxin molecule by the combined action of hydrogen peroxide and metal compounds. This is a possibility which deserves serious consideration.

The active concentrations of redox substances and hydrogen peroxide are in fair agreement. The different activities of iron, copper and zinc on the one hand and manganese on the other could be explained by different distribution of these metals in the animal body. Recent biochemical investigations indicate that hydrogen peroxide has greater importance in metabolic processes than has been ascribed to it previously. (Abrams *et al.*, 1942; Altschul *et al.*, 1940; Huszak, 1942).

According to these investigators hydrogen peroxide is an important metabolite and should not be considered as a noxious by-product of oxidative metabolism, which is at once destroyed by catalase.

Facile acceptance of this hypothesis is unwarranted, because knowledge of oxidative processes outside of carbohydrate metabolism is extremely scanty, and not applicable to the problem of detoxication of toxins.

DISCUSSION.

In these studies on diphtheria, staphylococcal and welchii A and D toxins a detoxication mechanism has been demonstrated which operates by the combined action of metal compounds and either redox substances or hydrogen peroxide.

A number of iron compounds has been tested with diphtheria toxin, and those which contained iron in the ionized form showed the highest activity. Metal-protein-combinations may be more active than inorganic salts, as inorganic salts are more active when dissolved in serum than when dissolved in broth or saline.

The detoxication might be an oxidative process, the toxin molecule being directly oxidized by activated oxygen, the components of the detoxication system varying with the different toxins. Iron, copper and zinc and ascorbic acid, glutathione and adrenaline are most active with diphtheria toxin, experiments with the guinea-pig skin, general intoxication of mice, and *in vitro* tests giving identical results.

The toxin of welchii D, however, is resistant to this mechanism, but is subject to detoxication by a system consisting of manganese plus hydrogen peroxide; staphylococcal and welchii A toxin can be detoxified by both. Iron is more active in an intoxication in which the toxin reacts slowly with the tissues; manganese is more active where the toxin reacts more quickly. These differences recall the results of Walbum (1925-26) who tested systematically the influence of nearly all the elements of the periodic system in a variety of bacteriological and serological problems. He found manganese active in anti-toxin production, beryllium in agglutinin production, caesium and iridium in mouse typhoid infections, and aluminium, manganese and gold in guinea-pig tuberculosis. In intoxication of rabbits with staphylococcal, diphtheria or dysentery toxin, best results were obtained with manganese. Walbum could not give an explanation of these results but considered activation of enzymes and increase in antibody production. According to our results this explanation seems to be erroneous, but the general conclusions that different metals are active in different infections and toxic states are confirmed by our observations.

As toxin is detoxified in the organs and not in the blood, and as iron in the form of cytochrome was more active than in the form of haemoglobin or ferritine, it appears that the metal proteins which determine the resistance against infectious diseases and intoxications are constituents of organ cells.

Investigations will have to be undertaken to determine the metallo-protein or proteins which are most severely affected in the course of infectious diseases. Iron alone will probably not be effective in the maintenance of resistance to infectious diseases as it has been shown that iron, by itself, is insufficient to restore the normal enzymatic activity of depleted organs. (Cohen and Elvehjem, 1934; Frost, Spitzer, Elvehjem and Hart, 1941; Schultze, 1939; Whipple, 1942).

The activity of the metals is not restricted to bacterial intoxication but extends to other toxaemic states. It has long been known that anaphylactic shock can be prevented by manganese, ascorbic acid, sodium thiosulphate or cysteine (Hochwald, 1936; Klopstock, 1925).

In preliminary experiments it was found that iron can prevent local anaphylactic reactions in the guinea-pig skin, that iron and copper reduce the intradermal tuberculin reaction in tuberculous cows, and that iron and manganese reduce the reaction following a Mantoux test in humans.

These experiments indicate that the detoxication mechanism which has been discussed is active in a very wide range of toxaemic conditions and furnish a rational basis for the well-known fact that resistance against infectious diseases is lowered by malnutrition.

SUMMARY.

The detoxication of diphtheria, staphylococcal and welchii A and D toxins has been investigated.

Two detoxication mechanisms have been described. In the first, iron, copper or zinc and ascorbic acid, adrenaline or glutathione are implicated. The second operates by the combined action of manganese and hydrogen peroxide.

The theoretical importance of these observations has been discussed.

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Toxicity of Nickel—Accumulation of Nickel in Rats Fed on Nickel-containing Diets & Its Elimination

S. S. PHATAK & V. N. PATWARDHAN

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The general condition and growth of rats fed on nickel-containing diet for 16 months were comparable with those of rats fed on nickel-free diets. On withdrawing nickel from the diet, the nickel was eliminated by excretion through urine and faeces. The urine was free from nickel within 40 days and faeces within 20 days.

INVESTIGATIONS on the effects of feeding nickel-containing diets to rats and monkeys had shown that no toxic effects could be demonstrated in these animals even after feeding nickel continuously for 4 to 6 months at levels of 25 to 100 mg. nickel per 100 g. of diet[1]. It was also observed that small quantities of nickel were retained in the body of the animals. Appreciable quantities of nickel were found stored in long bones, liver, kidney, spleen, heart, intestines, testes and skin. Blood also contained nickel. The amounts of nickel found in the tissues at the end of 9 weeks' feeding depended upon the level of nickel intake and the form in which nickel was incorporated in the diets. Two aspects of the question remained to be investigated. Firstly, it was necessary to find out whether, on continued feeding, the amount of accumulated nickel in various tissues would continue to increase. Secondly, how soon after discontinuing the feeding of nickel-containing diet would the body be freed of nickel? The present communication records the results of experiments designed to throw light on these points.

Accumulation of Nickel

Forty-two young rats, 4 to 5 weeks old, were fed on nickel containing diet (25 mg. nickel/100 g. of diet) of the following composition: wheat, 58 parts; Bengal gram (without husk), 20 parts; skim milk powder, 10 parts; vegetable fat, 10 parts; $Ca_3(PO_4)_2$, 1·5 parts; and NaCl, 0·5 parts; vitamin A, 70 I.U./rat/day; and vitamin D, 13 I.U./rat/day were administered in weekly doses.

100 g. of the diet were mixed with a nickel catalyst to give the desired concentration of nickel.

Six animals of the same age group kept on nickel-free diet of the above composition served as controls. Weights were recorded once a week. Fig. 1 records the rate of growth of rats on these diets. It will be seen from Fig. 1 that continuous feeding of nickel over a period of 16 months has had no deleterious effect on experimental animals and the general condition of the rats was excellent throughout. The average life span of a rat is a little over 2 years and continuous feeding at the level of 250 p.p.m.

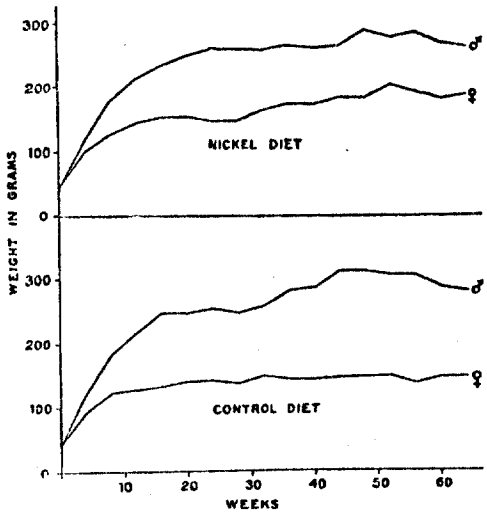

FIG. 1 — GROWTH OF RATS ON NICKEL DIET: 25 MG. NICKEL/100 GM. DIET.

TABLE 1 — DISTRIBUTION OF NICKEL IN TISSUES

(All the figures are based on 100 g. of fresh tissues)

Duration of feeding, months	No. of animals	Bone, mg.	Liver, mg.	Kidney, mg.	Spleen, mg.	Heart, mg.	Intestine, mg.	Testes, mg.	Blood, mg.	Skin, mg.
4	4	7·50	0·28	0·55	2·70	2·10	0·61	0·43	0·76	0·07
8	4	8·40	0·36	2·82	5·36	2·57	0·71	1·10	0·91	0·09
12	4	8·63	0·17	2·00	2·77	3·00	0·52	0·64	0·50	—
16	4	7·30	0·13	2·30	1·90	2·00	0·46	0·52	0·45	—

parts of diet for a period extending over half the life span of the animal had no demonstrable effect.

At intervals of 4, 8, 12 and 16 months respectively, 4 rats were sacrificed and the nickel content of tissues was determined. The results are given in Table 1.

A comparison of the nickel contents of rat tissues at the end of 2 months (PHATAK & PATWARDHAN, loc. cit.) and at the end of 4 months shows an increase in the nickel content of bone, spleen, heart, intestine and blood, and a decrease in liver, kidney and skin of rats fed for 4 months on a nickel containing diet. However, the accumulation of nickel in the tissues continues up to 8 months when the maximum amounts of nickel are found in all the tissues. Thereafter there is a fall even though the nickel diet was continued. There may be 2 probable causes for the observed decrease in tissue nickel. (1) The fall may be due to decreased food intake by the rat resulting in a reduction of the total amount of nickel ingested and the continued excretion of nickel in urine at a rate greater than 0·04 mg. per day resulting in its gradual elimination from the tissues, particularly the soft tissues. The balance studies carried out in these rats at the end of 12 and 16 months, however, did not indicate any reduction in food intake. (2) The fall may be due to diminished absorption of dietary nickel which would bring about the same result. The results of balance experiments indicate that diminished absorption of nickel in the diet was probably the reason for the reduction in tissue nickel.

One fact clearly emerges from the data summarized in Table 1: with prolonged feeding (16 months), the accumulation of nickel in the tissues is not progressive and a state is reached after which there is no further accumulation of nickel in the tissues.

In the course of these experiments it was considered necessary to find out if a low protein diet, adequate in other nutrients, would in any way influence the accumulation of nickel in the tissues. Although no theoretical considerations suggested this line of investigation, it was felt that observations made on a low protein diet would approximate to conditions obtaining in the diet of poor people in India. The following experiment was, therefore, undertaken:

TABLE 2 — METABOLIC EXPERIMENTS ON NICKEL DIET (25 mg. NICKEL/100 g. DIET)

(All the figures relate to 4 days' collection and are averages of 4 rats)

Period of feeding, months	Nickel intake, mg. Ni/rat/4 days	Nickel in urine, mg.	Nickel in faeces, mg.	Nickel retained, mg.
4	13·1	0·14	12·3	0·66
8	13·9	0·20	12·9	0·80
12	15·3	0·11	14·9	0·29
16	15·8	0·14	15·3	0·36

TABLE 3 — NUTRIENT CONTENT OF EXPERIMENTAL DIETS

(Figures relate to 100 g. of diet.)

	Basal	Low protein
Fat, g.	11·4	11·1
Protein, g.	16·4	6·8
Calcium, mg.	754·0	750·0
Phosphorus, mg.	669·0	680·0
Iron, mg.	20·0	19·0

Twenty-four young rats, 4 to 5 weeks of age, were divided into 2 groups of 12 each. To one group was given a low protein diet containing nickel (25 mg. nickel in the form of nickel catalyst/100 g. of diet). To the other group was given a diet with protein and nickel content as in the previous experiments. The composition of the low protein diet was as follows: Wheat, 25 parts; skim milk, 10 parts; vegetable fat, 10 parts; starch (tapioca), 49·9 parts; Marmite, 2·0 parts; $Ca_3(PO_4)_2$, 1·5 parts; NaCl, 0·5 part; Na_3PO_4, 1·0 part; Fe(ic) citrate, 0·1 part; vitamin A, 70 I.U./rat/day; and vitamin D, 13 I.U./rat/day were administered in weekly doses. 100 g. of the diet were mixed with nickel catalyst to give a concentration of 25 mg. of nickel/100 g. of diet.

Table 3 gives the nutrient contents of the 2 diets.

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8	4	8·40	0·36	2·82	5·36	2·57	0·71	1·10	0·91	0·09
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Table 3 gives the nutrient contents of the 2 diets.

TABLE 4 — DISTRIBUTION OF NICKEL IN TISSUES ON LOW & NORMAL PROTEIN DIETS

(No. of animals killed, 4; all figures based on 100 g. of fresh tissues)

Diet	Duration of feeding, months	Mg. nickel in								
		Bones	Liver	Kidney	Spleen	Heart	Intestine	Testes	Blood	Skin
Low protein	4	4·15	0·28	0·43	1·66	0·62	0·92	0·28	0·57	—
Normal	4	6·80	0·31	0·69	2·65	2·13	0·57	0·66	0·59	0·01
Low	8	6·40	0·24	1·68	2·90	1·90	0·58	0·66	0·58	0·02
Normal	8	7·80	0·41	2·20	4·23	2·35	0·61	0·86	0·61	0·04

TABLE 5 — EXCRETION OF NICKEL AFTER DISCONTINUING THE NICKEL DIET

(Figures are for 4 days' collection and are average of 4 rats)

Period	Mg. of nickel excreted in urine & faeces after						
	Immediately after stopping nickel diet	10 days	15 days	20 days	25 days	30 days	40 days
After 4 months							
Urine	0·146	0·130	nil	—	—	—	—
Faeces	0·050	nil	nil	—	—	—	—
After 8 months							
Urine	0·155	—	0·150	—	0·062	0·036	nil
Faeces	0·052	—	0·009	—	nil	nil	nil
After 12 months							
Urine	0·110	0·100	—	0·050	—	nil	nil
Faeces	0·064	0·017	—	nil	—	nil	nil
After 16 months							
Urine	0·135	0·100	—	0·050	—	nil	nil
Faeces	0·053	0·016	—	nil	—	nil	nil

At intervals of 2, 4 and 8 months, rats were sacrificed and the nickel content of the tissues determined. At the end of the 2 months' period 2 rats from each group were sacrificed; in other cases, 4 animals from each group were killed. The results for 4 and 8 month periods are given in Table 4.

The results given in Table 4 show that inadequacy of protein did not contribute to an increased accumulation of nickel in the tissues. On the other hand, the general tendency seemed to be that on low protein diet there was less accumulation of nickel in the tissues.

Elimination of Nickel from the Body

After 4 months' feeding on nickel-containing diet (diet 1), 4 rats were removed to metabolic cages and balance experiments were carried out to determine the intake and excretion of nickel. The rats were then put on the nickel-free diet. The excretion of nickel in urine and faeces was determined at intervals, by placing the rats in metabolic cages continuously for 4 days and collecting the urine and faeces during the period. Similar experiments were carried out with rats after 8, 12 and 16 months of feeding on nickel-containing diet. The results are given in Table 5.

TABLE 6 — RETENTION OF NICKEL IN THE KIDNEY OF THE RAT

Period, months	No. of rats used	No. of rats showing nickel in kidney	Amount of nickel in kidney, mg./100 g.
4	4	—	—
8	4	2	0·54-0·71
12	4	3	0·09-0·16
16	4	2	0·14-0·16

The rats were killed after the urine and the faeces were found to be free from nickel and the tissues analysed for residual nickel. With the exception of the kidney, all the other tissues examined were free from nickel. Small amounts of nickel, however, were present in the kidneys (Table 6).

It appears, therefore, that nickel is eliminated from the body within a short period after nickel is withdrawn from the diets. In rats fed on nickel diet for 4 months, urine and faeces became free from nickel within 15 days of placing the rats on nickel-free diet; 30 to 40 days were required for rats fed on nickel diet for 12 to 16 months. The residual nickel remaining in the kidneys was extremely small. It is interesting to note that nickel occurred in faeces 10 to 15 days after it was withdrawn from the diet. It is unlikely that nickel ingested with food would remain in the intestines for such long

periods. It is possible that the nickel is derived from other tissues and is excreted via the intestines.

Summary

1. The general condition and growth of rats fed on nickel-containing diets for a period of 16 months was comparable with those of rats on nickel-free diets.

2. The maximum accumulation of nickel in bones and soft tissues was observed after 8 months' continuous feeding on nickel diets.

3. On a low protein (6·8 per cent) diet, the accumulation of nickel was less in all the tissues (with the exception of the intestines) than when the protein content of the diet was normal (16·4 per cent).

4. On withdrawing nickel from the diet, the nickel was eliminated by excretion through urine and faeces. The urine was free from nickel within 40 days, while the faeces was free from nickel within 20 days after discontinuing the nickel diet.

Acknowledgement

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760 *Analyst*, August, 1970, Vol. 95, *pp*. 760–762

Rapid Determination of Nickel in Edible Fats by Atomic-absorption Spectrophotometry

By W. J. PRICE, J. T. H. ROOS and A. F. CLAY
(*Pye Unicam Ltd., Cambridge*)

The determination, by atomic-absorption spectrophotometry, of small amounts of nickel in edible fats is described. Nickel is extracted from the samples with 10 per cent. nitric acid in the presence of carbon tetrachloride, to break the emulsion, and the aqueous layer is aspirated directly to determine the nickel. The method requires only 15 minutes for completion, and the detection limit for nickel is better than 0·1 p.p.m.

Small amounts of nickel are frequently used as catalysts during the production of edible fats. However, it is necessary to control the amount of nickel in the final product, and thus an accurate method is required for the determination of trace amounts of nickel in such samples. Furthermore, the method should be capable of providing results in the shortest possible time so as to cause the minimum amount of delay during the production process. Atomic-absorption spectrophotometry appeared to be an ideal technique for this purpose.

Experimental

Apparatus—

The work was performed with a Unicam SP90A atomic-absorption spectrophotometer, and a nickel hollow-cathode lamp from Pye Unicam Ltd. A Griffin-Christ "Universal Junior III" centrifuge, supplied by Griffin and George Ltd., was used to speed the separation of aqueous and organic phases.

Reagents—

Nickel stock solution, 1000 *mg* l^{-1}—Dissolve 1·000 g of Specpure grade nickel foil in the minimum volume of hydrochloric acid (sp.gr. 1·18) and make up the solution to 1 litre in a calibrated flask.

Nitric acid (*sp.gr.* 1·42)—Analytical-reagent grade.

Perchloric acid, 61 *per cent. w/v*—Analytical-reagent grade.

Carbon tetrachloride—Analytical-reagent grade.

Nitric acid, 10 *per cent. v/v*—Dilute 100 ml of nitric acid (sp.gr. 1·42) to 1 litre with de-ionised water.

Development of the method—

Slavin[1] has outlined methods for the determination of copper, iron, lead, manganese and nickel in vegetable oils. These methods involve the direct aspiration of a solution of the oil in isobutyl methyl ketone. Willis[2] found that samples of butter and butter oil could not be analysed in this way because of clogging of the burner slot by the samples. He suggested instead an extraction procedure with nitric acid, and determined copper.

Because it was required to determine nickel not only in true oils but also in samples approximating in consistency to butter, the possibility of using a nitric acid extraction technique was investigated. However, preliminary experiments revealed that a thick emulsion was produced, which proved extremely difficult to break. At the same time, when separation was achieved by centrifugation, the nitric acid extract was found to be the lower layer in the centrifuge tube. This rendered direct aspiration of the nitric acid (and extracted nickel) impossible.

Both of these difficulties were overcome by dissolving the oil sample in an equal volume of carbon tetrachloride prior to extraction with nitric acid. Under these conditions the aqueous extract forms the upper layer, and any emulsion produced is broken without difficulty. Centrifugation for 2 minutes at 2500 r.p.m. is sufficient to obtain complete separation of the two phases.

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To investigate the time required for complete extraction of the nickel, 10-ml portions of oil and 10 ml of carbon tetrachloride were shaken with 10 ml of 10 per cent. nitric acid for 2, 5, 10, 15 and 20 minutes. After shaking it, each sample was centrifuged and the nickel determined in the aqueous layer. From the results (see Fig. 1) it is seen that 10 minutes are sufficient for the extraction of virtually all of the nickel, further shaking producing less than 5 per cent. increase in the amount of nickel extracted.

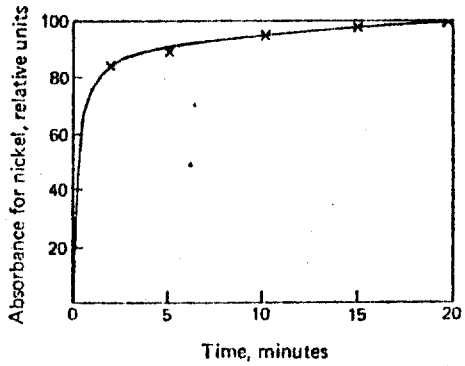

Fig. 1. Rate of extraction of nickel from edible fats

PROCEDURE—

A series of calibration standards (0 to 50 p.p.m. of nickel in 10 per cent. nitric acid) was prepared by suitable dilution of the nickel stock solution.

Either 10 ml or 10 g of the sample were dissolved in 10 ml of carbon tetrachloride in a 45-ml centrifuge tube. Solid samples were warmed if necessary to assist dissolution. Then 10 ml of 10 per cent. nitric acid were added. The tube was then stoppered and the contents shaken continuously for 10 minutes. The resulting mixture was centrifuged for 2 minutes at approximately 2500 r.p.m., and the upper layer aspirated directly for the determination of nickel. The experimental conditions used are shown in Table I.

TABLE I

INSTRUMENTAL CONDITIONS FOR THE DETERMINATION OF NICKEL

Wavelength	232·0 nm
Observation height	1·0 cm
Slit width	0·1 mm
Burner	10 cm air - acetylene
Air flow-rate	5 l minute^{-1}
Acetylene flow-rate	1300 ml minute^{-1}
Lamp current	15 mA
Scale expansion	× 2

RESULTS AND DISCUSSION

The results obtained by the proposed method for nickel in a number of edible fats are shown in Table II, together with the values found by a wet-chemical method. From these results it can be seen that the agreement between the two methods is satisfactory, with the atomic-absorption results being, as expected, slightly the lower of the two sets. This is probably caused by the incomplete (approximately 95 per cent.) extraction of the nickel by the proposed procedure.

The determination can be performed very rapidly, the time required for the analysis of a sample of the oil being about 15 minutes. Naturally the determination can be made more rapid by incorporating a shorter period of shaking, but with a corresponding loss of accuracy.

TABLE II

RESULTS FOR THE DETERMINATION OF NICKEL

Nickel found chemically, p.p.m.	Nickel found by A.A.S., p.p.m.	Nickel found chemically, p.p.m.	Nickel found by A.A.S., p.p.m.
17·5	16·2	7·0	6·7
35·0	33·6	14·0	14·0
0·9	1·0	24·5	24·4
1·5	1·5	0·9	0·9
3·6	3·2	1·8	1·8
3·5	3·2	2·7	2·7

The detection limit for nickel by the proposed procedure is better than 0·1 p.p.m. in the original oil sample. By using a smaller volume of nitric acid, however, the detection limit could be improved even further should this be necessary.

It is to be anticipated that the proposed procedure could, without modification, be used for the determination of trace amounts of copper, iron, manganese and zinc in samples of this type. Detection limits of better than 0·1 p.p.m. would be expected for all of these metals. However, such extensions of the method have not been investigated to date.

We thank Dr. P. Jowett, Kraft Foods Limited, Manchester, for preparing artificial samples with high nickel contents.

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DETERMINATION OF ADDED DISTINCTIVE CATIONS IN WHISKY. V. SPECTROPHOTOMETRIC DETERMINATION OF MOLYBDENUM AND NICKEL*

By MAYNARD J. PRO and RAYMOND A. NELSON (Alcohol and Tobacco Tax Division Laboratory, Internal Revenue Service, Washington 25, D.C.)

This is the fifth in a series of papers which describe certain phases of the investigation, in the Alcohol and Tobacco Tax Division Laboratory, of the use of markers for identifying different brands of whisky. In this paper analytical procedures are described for the microdetermination of molybdenum and nickel added to whisky.

* Received at the Association of Official Agricultural Chemists on July 2, 1956.

In earlier work, other investigators have carried out the determination of molybdenum and nickel by such procedures as colorimetry (1–5), polarography (6–8), and spectrography (9, 10). Since the colorimetric procedures are rapid and in many cases are free from interferences, adaptations were made to meet the present problem, and the details are presented in this paper.

The organic material in both methods is destroyed by a wet ash procedure. Samples containing molybdenum are treated with thiocyanate and stannous chloride reagents, and the quinquevalent molybdenum thiocyanate complex is extracted and concentrated with isoamyl alcohol. Solutions containing nickel are oxidized to the quadrivalent state with bromine and then are treated with dimethylglyoxime. The colored complexes of molybdenum and nickel are measured spectrophotometrically.

EXPERIMENTAL

Absorbance spectra.—The absorbance spectra, illustrated by Figs. 1 and 2, show maximum absorbances at the following wavelengths: molybdenum, 465 mμ; nickel, 450 mμ.

Calibration curves.—The calibration curves, illustrated by Fig. 3, were prepared from microgram quantities of molybdenum and nickel added to whisky and determined by the procedures described below. These curves are linear and pass through

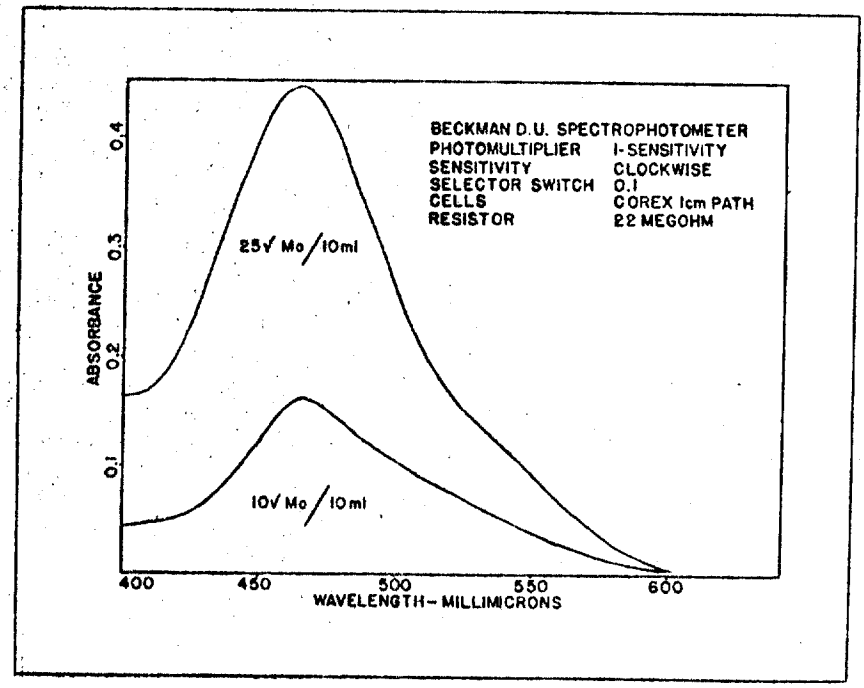

FIG. 1.—Absorbance spectra of molybdenum solutions at two wavelengths.

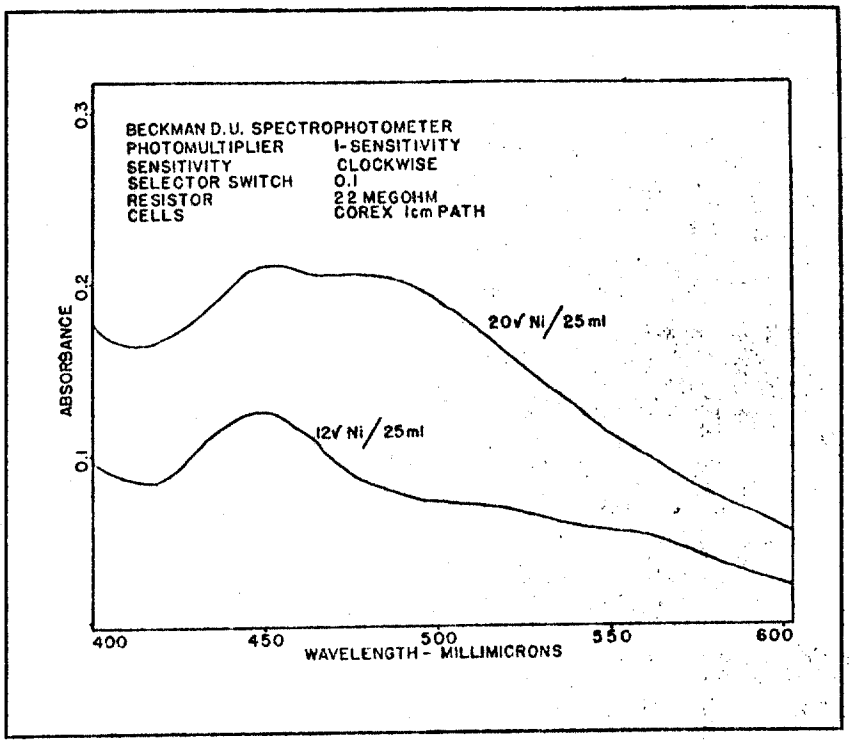

FIG. 2.—Absorbance spectra of nickel solutions at two wavelengths.

the origin after a correction has been applied for the absorbance of the reagent blank.

Effect of reagent concentration.—Hurd (3) reported that in the molybdenum procedure, hydrochloric acid concentrations both larger and smaller than 5% prevented full development of color and caused fading; that considerable fading of color occurred when the potassium thiocyanate concentration was less than 0.6%; and that variations in the stannous chloride concentration had no effect on the color. Further, in the work reported by Perrin (11), it was shown that approximately 1*N* hydrochloric acid was necessary for the development of maximum color when isoamyl alcohol was used as an extractant. Also, a 1% potassium thiocyanate concentration was recommended for full development of the molybdenum thiocyanate color.

Stability of color.—Although available literature references show little data concerned with the stability of the isoamyl extractant in the molybdenum procedure, in this experimental work it was found that the color remained stable for several hours.

Effect of pH.—Sandell (5) reported that in the nickel procedure, both the hue and color intensity were affected by changes in pH. Also, in his work it was shown that adding bromine water and then ammonia and dimethylglyoxime to an acidic nickel solution produced a brown color, and adding bromine water to a neutral or slightly ammoniacal nickel solution yielded an orange-red color. As shown in the following experimental work the ashed sample is heated to 500° to assure the loss of all acid so that a neutral solution is obtained.

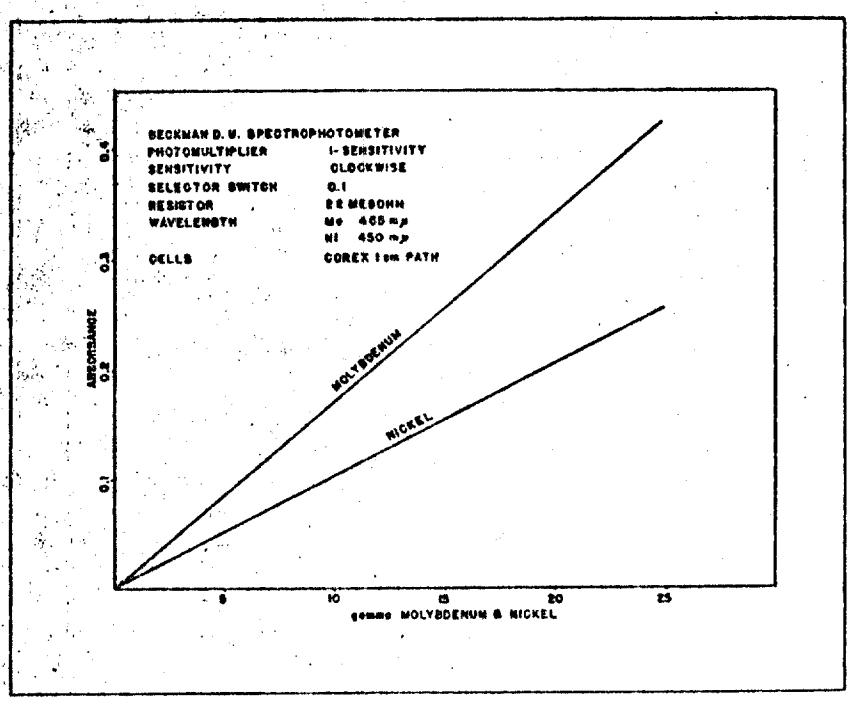

FIG. 3.—Standard calibration curves for whisky containing molybdenum and nickel, respectively.

Stability of color in the nickel procedure.—The experimental work performed in this laboratory showed that the nickel dimethylglyoxime color remained stable for at least 1 hour.

MOLYBDENUM PROCEDURE

REAGENTS AND STANDARDS[1]

(a) *Standard molybdenum solution.*—Add 1.5003 g reagent grade (99.8%) molybdenum anhydride to a 250 ml beaker containing 100 ml H_2O and 6 ml 6*N* NaOH. Swirl until a clear solution results and then make acid to litmus with concentrated HCl. Transfer to a 1 l volumetric flask and dilute to mark with H_2O. This solution contains 1,000 ppm Mo.

(b) *Sodium hydroxide solution.*—Approximately 6*N*. Dissolve 240 g reagent grade NaOH in 600 ml H_2O. Cool, and dilute to 1 l with H_2O.

(c) *Hydrochloric acid solution.*—Approximately 6*N*. Add 500 ml concentrated HCl to a 1 l volumetric flask and dilute to mark with H_2O.

(d) *Potassium thiocyanate solution.*—Dissolve 10 g reagent grade potassium thiocyanate in 100 ml H_2O. Prepare fresh daily.

(e) *Stannous chloride solution.*—Into a 250 ml beaker add 10 g stannous chloride dihydrate and 10 ml concentrated HCl. Heat until a clear solution results and dilute to 100 ml with H_2O. Prepare fresh daily.

(f) *Isoamyl alcohol.*—Boiling range 128–131°; reagent grade.

(g) *Phenolphthalein solution.*—0.5%. Dissolve 0.5 g reagent grade phenolphthalein crystals in 100 ml 0.1*N* NaOH.

[1] All reagents were reagent grade chemicals, and were stored in polyethylene bottles.

DETERMINATION

By pipet add 20 ml whisky to a Vycor dish, 50–100 ml capacity, containing 1 ml concentrated H_2SO_4, 1 ml 60–70% $HClO_4$, and 5 ml concentrated HNO_3. Cover the dish with a watch glass and heat over a low Meker flame or electric hot plate. If necessary, add more HNO_3 to prevent charring or to completely destroy the organic material. Remove the watch glass and evaporate most of the $HClO_4$ until the volume of liquids in the dish is approximately 1 ml.

Add 10 ml H_2O and make alkaline to phenolphthalein with concentrated NH_4OH. Evaporate on a steam bath to remove the excess ammonia, but do not allow the salts to go to dryness. Cool the residue and add 4 ml 6*N* HCl.

Transfer the solution in the Vycor dish to a 50 ml glass-stoppered separatory funnel with the aid of three 4 ml portions of H_2O. Add 2 ml concentrated HCl and mix; add 3 ml potassium thiocyanate solution, mix again, and add 5 ml isoamyl alcohol. Shake well for 30 seconds. Allow the 2 phases to separate completely; then transfer the aqueous layer to another separatory funnel. With the aid of a funnel containing a small plug of glass wool, transfer the isoamyl alcohol phase into a 10 ml volumetric flask. Add 4 ml isoamyl alcohol to the previously extracted aqueous phase, shake for 30 seconds, allow to separate, and discard the aqueous portion. Transfer the isoamyl alcohol extract to the 10 ml volumetric flask through the same funnel. Dilute to the mark with isoamyl alcohol, shake well, and allow to stand approximately 15 minutes. Read the absorbance at 465 mμ.[2] (A Beckman Model DU spectrophotometer was used in this work.)

NICKEL PROCEDURE

REAGENTS AND STANDARDS[1]

(a) *Standard nickel solution.*—Dissolve 4.0501 g reagent grade $NiCl_2 \cdot 6H_2O$ in H_2O and dilute to 1 l. This solution contains 1,000 ppm Ni.

(b) *Bromine water.*—Saturate 100 ml H_2O with bromine by adding 3 ml bromine, shaking, and allowing the mixture to stand overnight.

(c) *Dimethylglyoxime.*—1%. Dissolve 1 g reagent grade dimethylglyoxime crystals in 100 ml 95% ethanol.

DETERMINATION

By pipet add 20 ml whisky to a 50 ml beaker and evaporate to dryness on a steam bath. Add 0.2 ml concentrated H_2SO_4 and 2 ml concentrated HNO_3, cover the beaker with a watch glass, and heat over an electric hot plate or an asbestos centered wire gauze with a low Meker flame. Allow the HNO_3 to reflux for approximately 10 minutes, remove the glass cover, and heat to fumes of H_2SO_4. Add HNO_3 a few drops at a time to destroy any remaining organic material. Continue heating until fumes of H_2SO_4 cease. Transfer the beaker to a muffle furnace and heat at 500° for 5–10 minutes to remove the last traces of H_2SO_4.

To the beaker add 10 ml H_2O to dissolve the ash, and heat to boiling; then cool to room temperature and add 1 ml bromine water, mix, and add 1 ml concentrated NH_4OH. Stir the solution, add 1 ml dimethylglyoxime solution, again stir, and transfer to a 25 ml volumetric flask with the aid of three 3 ml portions of H_2O. Dilute to volume with H_2O and measure the absorbance of the solution at 450 mμ.

Along with the sample, determine the absorbance contributed by 20 ml of a standard solution (1 ppm Ni) and a reagent blank carried through the same procedure.

CALCULATIONS

$(A_{sample} - A_{blank})/(A_{standard} - A_{blank})$ = ppm marker in whisky sample, where A_{sample} is the absorbance of the sample, $A_{standard}$ is the absorbance of the standard, and A_{blank} is the absorbance of the blank.

[2] Calculations are shown in a separate section.

ANALYTICAL DATA

Approximately 50 brands of whisky were analyzed for molybdenum and nickel and none showed the presence of these elements. Molybdenum and nickel (0.5 and 1.0 ppm) were added to several brands, and the whisky was analyzed for these elements by the procedures described. The agreement of the experimental and theoretical results, shown in Table 1, is well within the limits of instrumental error.

TABLE 1.—*Analytical data for molybdenum and nickel added to whisky as markers*

WHISKY BRAND	MARKER ADDED		MARKER FOUND	
	MOLYBDENUM	NICKEL	MOLYBDENUM	NICKEL
	ppm	ppm	ppm	ppm
1	0.5	0.5	0.51	0.50
2	0.5	0.5	0.51	0.50
3	0.5	0.5	0.52	0.47
4	0.5	0.5	0.49	0.49
5	0.5	0.5	0.51	0.51
6	0.5	0.5	0.52	0.50
7	0.5	0.5	0.50	0.50
8	0.5	0.5	0.49	0.50
9	0.5	0.5	0.48	0.50
10	0.5	0.5	0.50	0.51
11	1.0	1.0	1.03	0.98
12	1.0	1.0	0.96	1.04
13	1.0	1.0	0.97	0.96
14	1.0	1.0	0.94	0.95
15	1.0	1.0	0.96	0.92
16	1.0	1.0	0.97	0.91
17	1.0	1.0	0.98	0.92
18	1.0	1.0	1.04	0.98
19	1.0	1.0	1.03	0.99
20	1.0	1.0	1.04	0.99

INTERFERENCES

In the work reported by Perrin (11) it was shown that the presence of 0.5 to 1.0 mg iron increased the development of color when more than 4 mmg of molybdenum was present. To circumvent errors from this source, earlier workers added iron in excess of 1 mg to give maximum enhancement. However, in the present work no iron was added because the concentration of iron in whisky (mmg/ml) had no effect on the molybdenum thiocyanate color.

To study the effect of numerous cations on these determinations, whisky solutions containing 1 ppm molybdenum and nickel were prepared with 1, 5, and 10 ppm of the following ions: Aluminum, barium, boron, cadmium, calcium, cerium, cesium, chromium, cobalt, copper, gold,

indium, iron, lanthanum, lithium, magnesium, manganese, molybdenum, neodymium, nickel, platinum, praesodymium, rubidium, samarium, selenium, strontium, thallium, thorium, tungsten, vanadium, zinc, and zirconium.

These ions in the concentrations given had no effect on the color produced from 1 ppm molybdenum in whisky. Also, combinations of these ions, in quantities not greater than 1 ppm each, showed no effect on the molybdenum thiocyanate complex.

Although 1 ppm or less of these ions did not interfere with the determination of 1 ppm nickel in whisky, more than 5 ppm of copper enhanced the nickel dimethylglyoxime color, and chromium in concentrations greater than 5 ppm caused fading. Manganese concentrations greater than 5 ppm precipitated in ammoniacal solution and the manganese had to be removed after the color was developed. Aluminum in concentrations larger than 5 ppm produced low absorbance which was presumed to be due to a coprecipitation of nickel with the aluminum. Further studies showed that, in combination, these ions in concentrations not greater than 1 ppm each had no effect on the nickel dimethylglyoxime color.

SUMMARY

Colorimetric methods are presented for the determination of micro quantities of molybdenum and nickel in whisky. Also, the effects of 31 cations on these color systems are described.

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668

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Recent dietary trace element research, exemplified by tin, fluorine, and silicon[1,2]

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ABSTRACT

New trace element requirements have become accessible to nutrition research over past years. To the seven elements known to be essential before 1957, another seven have been added: selenium, chromium, tin, vanadium, fluorine, silicon, and nickel. In most of these studies, ultraclean isolator systems and purified amino acid diets were applied. Selenium prevents a wide variety of fatal deficiency diseases, is related to vitamin E and sulfur amino acids in its effects, and has been identified as the active site of glutathione peroxidase. It also is a constituent of a special cytochrome in muscle and heart, and of two bacterial enzyme complexes, formic dehydrogenase and glycine reductase, which contain selenium enzymes. Chromium functions as a glucose tolerance factor in the organism. It is related to insulin in its biological role. Tin is necessary for growth in rats at a level of 1-2 ppm in the diet. It occurs normally in foods, feeds, and tissues. Tin salts, but also organotin compounds, are effective. Its biological chemistry is unexplored. Compared with tin, vanadium is required in much smaller amounts. It probably functions as an oxidation-reduction catalyst. Fluorine, studied for effects on dental caries, is essential for growth and general development. As fluoride, 2.5 ppm produce optimum growth responses and beneficial effects on incisor pigmentation. Fluorine concentration in blood plasma is homeostatically controlled, bone and teeth serve as storage organs containing normally between 100 to 7,000 ppm, the fetus contains fluorine, the placenta plays an active role in fluoride transfer, rather constant concentrations are maintained in soft tissues such as liver, heart, and brain, the element occurs in practically all foods and feeds, and food is the main source for animals and man, aside from drinking water. Not only fluoride, but also organically bound fluorine may be important. Basic questions, such as the chemical nature of fluorine in food, discrepancies in the utilization of fluorine compounds, its biochemistry in soft tissues, and its mode of action remain to be clarified. Silicon is necessary for growth in rats, meta-silicate producing optimal effects at 50 mg/100 g in the diet. Bone deformations develop in silicon deficiency. Silicic acid in bound form is a building block of connective tissue, serving as a cross-linking agent. High levels of bound silicon were detected in acid mucopolysaccharides and in connective tissue proteins, notably collagen. The silicon atom, having the same stereochemistry as carbon is structurally rigid and thus contributes to the architecture of connective tissues and membranes. These findings open up new possibilities in biochemistry and medicine. An overview reveals that a continuous set of 8 elements essential for warm-blooded animals in the first series of transition elements constitutes an area of special emphasis where most trace element requirements are consolidated. There are 20 additional elements which may qualify for trace element function. Thus, additional essential trace elements may be discovered in the future.—SCHWARZ, K. Recent dietary trace element research, exemplified by tin, fluorine, and silicon. *Federation Proc.* 33: 1748-1757, 1974.

The search for unidentified dietary factors has taken a new and unexpected direction over the past two decades, following almost a century of preoccupation with the clarification of amino acids and vitamin requirements. The present phase deals with inorganic trace factors and their biochemistry. These have become accessible since amino acids, vitamins, and other organic constituents were made available in pure form. Progress in this field is closely tied to modern developments in trace element analysis and the introduction of ultraclean isolator techniques (31, 45). Seven elements were known to be essential before 1957 (Table 1): iron, iodine, copper, manganese, zinc, cobalt, and molybdenum. To these, we have added another seven essential trace elements since then: selenium (36), chromium (38), tin (42), vanadium (13, 39), fluorine (40), silicon (3, 41), and nickel (22).

These developments, which have been summarized elsewhere (31, 33, 35), have contributed to the emergence of bioinorganic chemistry as a separate category of research. Few realize that bioinorganic chemistry is just as complex as organic biochemistry and, in some respects, more difficult. Problems in nutritional trace element research arise mainly from the following circumstances: *a*) Some elements are required and effective at levels which are so small that we do not have satisfactory methods of analysis for their determination in biological materials, diets, and the like. Because of inaccurate methods, many of the data in the older literature appear to be incorrect. *b*) It is very difficult to exclude certain elements from the environment. They are ubiquitous, and special precautions have to be taken to isolate the animals from them. *c*) Any animal used in trace element experimentation contains, to begin with, a given store of the element which one may wish to study. It has an initial endowment which it must eliminate or outgrow before deficiencies can be expected to develop. *d*) The chemicals needed in diets to produce trace element deficiencies (sugars, amino acids, vitamins, fats and, especially, inorganic salts) must be of a degree of purity which greatly exceeds that normally encountered in chemistry. Trace elements are effective in the parts

[1] From the American Institute of Nutrition Symposium on *Newer Candidates for Essential Trace Elements* presented at the 57th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N.J., April 20, 1973.

[2] This work was supported by National Institutes of Health grant AM 08669.

per million (ppm) or parts per billion (ppb) range of concentration yet most hemicals are at best 99.9% pure. They 100–1,000 ppm of impurities w re mostly inorganic by nature.

The physiological essentiality of selenium and chromium was discovered in our laboratory in rats maintained under conventional conditions of animal experimentation. Following these, a new approach to trace element deficiencies was developed. This technique, in essence, consists of the application of ultrapurean, more or less trace element sterile (but not bacteriologically sterile) isolators, highly purified amino acid diets, and a rigid weaning routine which prevents accumulation of trace elements prior to the initiation of the experiment (31, 33, 45). All compounds tested are added to the diet. Figure 1 shows the three isolators in which four elements, tin, vanadium, fluorine, and silicon, were discovered to be essential for growth.

Rats maintained on highly purified amino acid diets under normal, conventional conditions grow well and look normal while those in the trace element-controlled isolator system are severely deficient (Fig. 2). They are delayed in growth and show alopecia, seborrhea and other symptoms, such as a lack of pigmentation of the incisors.

Some of the seven older trace elements are associated with well-known clinical disease entities, e.g., iron-deficiency anemia or goiter due to lack of iodine. Others, however, are not connected with specific deficiency syndromes. Their deficiencies may not be of practical importance or may be so subtle that they have not yet been detected. They could also simply have been overlooked. The situation is quite analogous to that existing in the vitamin field where some vitamins are associated with severe deficiency diseases in the human while those of certain other vitamins, even though they are indubitably required, have not yet been detected. Just as each vitamin is an entity by itself without any immediate relation to any other vitamin, each essential trace element is an entity of its own. In the organism it is not replaceable by any other one, as far as we know. Each newly discovered essential trace element opens new prospects in biochemical and medical research. Below, brief reviews are presented on tin, fluorine, and recent research on silicon from our laboratory; nickel (22), vanadium (12) and the work by Carlisle on silicon (2) are the subject of separate reports.

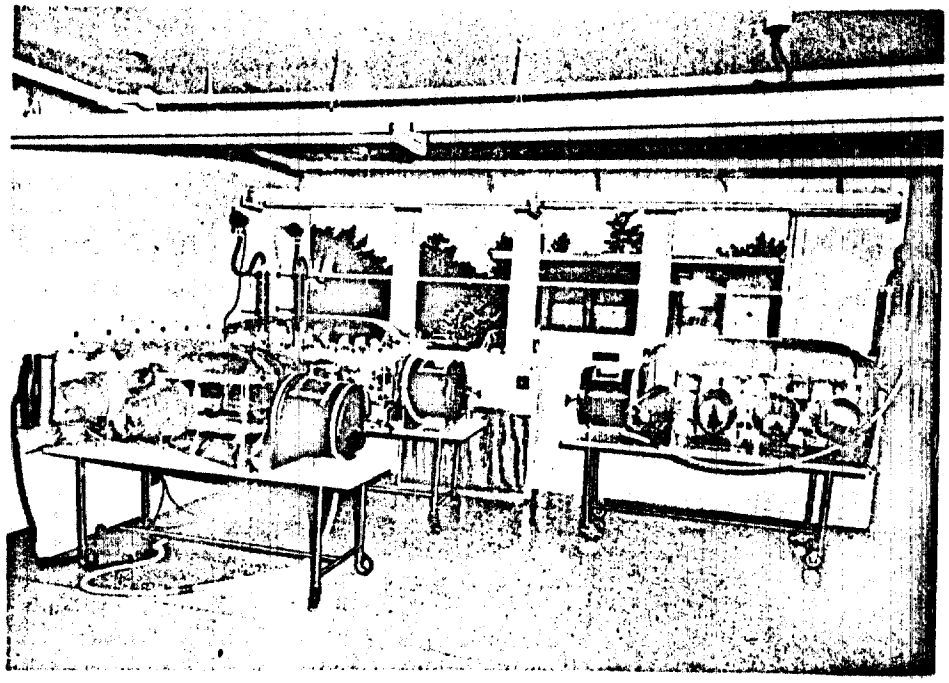
TABLE 1. Discovery of trace element requirements

Iron	17th Century	
Iodine	1850	Chatin, A.
Copper	1928	Hart, E. B., H. Steenbock, J. Waddell, and C. A. Elvehjem
Manganese	1931	Kemmerer, A. R., and W. R. Todd
Zinc	1934	Todd, W. R., C. A. Elvehjem, and E. B. Hart
Cobalt	1935	Underwood, E. J., and J. F. Filmer; Marston, H. R.; Lines, E. W.
Molybdenum	1953	deRenzo, E. C., E. Kaleita, P. Heytler, J. J. Oleson, B. L. Hutchings, and J. H. Williams; Richert, D. A., and W. W. Westerfeld
Selenium	1957	Schwarz, K., and C. M. Foltz
Chromium	1959	Schwarz, K., and W. Mertz
Tin	1970	Schwarz, K., D. B. Milne, and E. Vinyard
Vanadium	1971	Schwarz, K., and D. B. Milne; Hopkins, L. L., and H. E. Mohr
Fluorine	1972	Schwarz, K., and D. B. Milne
Silicon	1972	Schwarz, K., and D. B. Milne; Carlisle, E. M.
Nickel	1973	Nielsen, F. H.

SELENIUM

Reviews of the earlier work on selenium as an essential trace element, selenium-deficiency diseases, its relationships to other essential dietary factors, organic selenium chemistry, and the structure–activity relationship of organoselenium compounds are available (15, 29, 30, 37, 43). Selenium serves well to illustrate several points of general importance in trace element research. Its deficiency can document itself in a wide diversity of deficiency symptoms. Different symptoms occur in different species. The pathological changes preventable by selenium can be very profound; they often are fatal (Fig. 3). Selenium is biologically effective in very small amounts, 22 ppb of selenite-selenium affording 50% protection against death from dietary liver necrosis in rats. Both inorganic and organic selenium compounds are utilized, the potency of organic derivatives being strongly de-

Figure 1. Trace element controlled isolators.



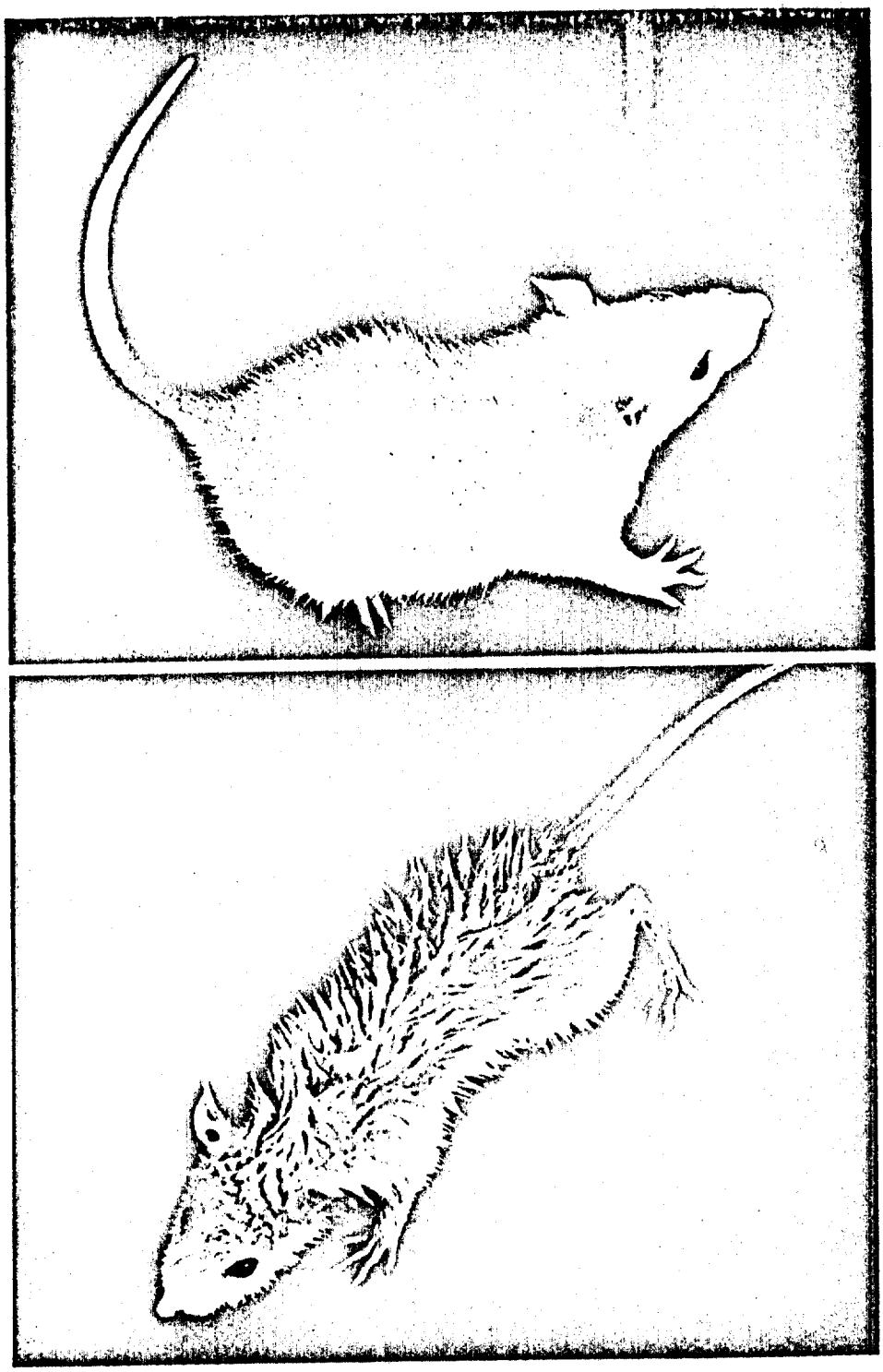


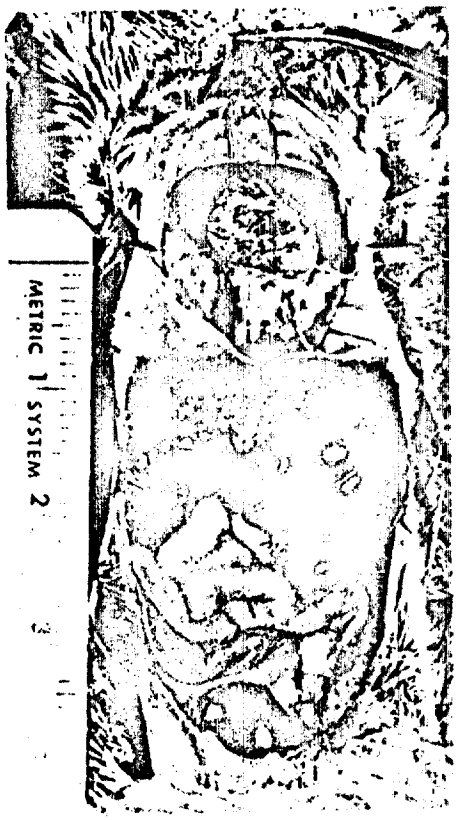
Figure 2. Trace element deficiency. *Upper animal:* outside control, kept under conventional conditions on purified diet. *Lower animal:* maintained for 20 days on the same diet in the trace element sterile environment system.

pendent on their structure. Selenium shows, furthermore, that trace elements are interrelated with other nutrients in their effects. Selenium deficiency is intimately connected to vitamin E-deficiency diseases on the one hand, and to the lack of sulfur amino acids on the other hand, even though these two dietary constituents are chemically very different from selenium and from each other. The interaction can be explained in terms of metabolic interrelationships.

It is a characteristic feature of trace element activity clearly illustrated by organic selenium derivatives that different compounds of one and the same element may vary profoundly in biological activity, as well as in their toxicities. Clear-cut regularities were observed which determine whether or not a compound is biologically active and also whether or not it is toxic (43). Through the synthesis of "tailor-made" selenium compounds, which combine features accounting for biological activity with those that make for low toxicity, active substances have been prepared that show only 1/300th of the toxicity of selenite-selenium. This demonstrates that it is nonsensical to talk about the toxicity of an element. One can talk only about the toxicity of certain defined compounds of an element. Carbon, for instance, is one of the most toxic "elements," in form of cyanide or gramicidin.

Selenium has recently been identified as the active site of the enzyme glutathione peroxidase, an enzyme of great metabolic significance in the reoxidation of reduced glutathione (7, 26). It utilizes the oxygen of physiologically occurring peroxides as the electron acceptor. This finding should put to rest the simplistic belief that selenium acts merely as an antioxidant in the animal (50). Identification of selenium as an integral, active site of glutathione peroxidase came from the original observation that selenium-deficient erythrocytes in the presence of reduced glutathione are highly labile and hemolyze more readily than those of

Figure 3. Multiple necrotic degeneration in the mouse. Note heart muscle degeneration, liver necrosis, and also muscular dystrophy (4).



selenium-supplemented animals (25). Glutathione peroxidase has been isolated from sheep red blood cells. It has a molecular weight of approximately 80,000 and contains one atom of selenium per protein subunit of approximately 20,000 molecular weight. The enzyme contains 0.34% of selenium.

Recently a selenium-containing protein of approximately 10,000 molecular weight resembling cytochrome b_5 in protein composition but containing a heme group identical to that of cytochrome *c* has been isolated from muscle extracts of lambs injected with selenium (46). This protein is absent in selenium-deficient lambs. The selenium-containing cytochrome appears to be present in the soluble fraction of muscle and heart homogenates, in contrast to cytochromes normally isolated from mitochondria or microsomes. It was not found in liver.

Two other enzymes containing selenium have been described: formic dehydrogenase from *Escherichia coli* and *Clostridium thermoaceticum* catalyses the oxidation of formic acid to carbon dioxide and water (1, 24), and an enzyme, protein A, which is a component of the glycine dehydrogenase complex (51). In the presence of a sulfhydryl donor, the latter actively deaminates glycine to acetic acid and generates ATP in doing so. Protein A is a small, very heat stable protein which travels close to cytochrome *c* in centrifugation gradients. Protein A contains no cysteine moieties and is effective in combination with two other, much larger molecules.

CHROMIUM

Chromium, in contrast to selenium, shows us that trace element deficiencies can be very subtle, but nonetheless of fundamental importance. The only symptom easily detectable is a disturbance of glucose utilization (Fig. 4). In its biological effects, chromium may be intimately related to insulin of which it may be a cofactor. Chromium also demonstrates that different oxidation states have different biological activities. Hexavalent chromium compounds are inactive; only those which are derived from trivalent chromium or chromium of lesser oxidation states show glucose tolerance factor activity. Different chromium (III) complexes have different potencies depending on their solubility. The organism appears to convert chromium (III) into a special form, most likely in the liver. This form of glucose tolerance factor is released into the bloodstream whenever a glucose load reaches the bloodstream or insulin is secreted or injected (17).

THE ESSENTIALITY OF TIN

Tin affords a typical example of misleading conclusions arising from inadequate analytical procedures. In an extensive review on tin in man and foods, Schroeder et al. (28) treated tin as an "abnormal" trace metal, an environmental contaminant. They analyzed a great number of foods of animal and plant origin by a sensitive colorimetric method and found the element to be present in the parts per million range of concentration in nearly all of them. They analyzed, on the other hand, a great number of human organs from various areas of the world by a rather insensitive method of emission spectrography carried out after ashing at 450 C in the muffle furnace. By the latter method, they have found "zero" levels of tin in the newborn and in organs of natives of some foreign countries. From this it was concluded that "the evidence is convincing that measurable tin is not necessary for life or health." We investigated the behavior of organotin compounds under these conditions and found recoveries of 0%. It is obvious that under the conditions of their analysis of human material serious losses of tin must have occurred. Most organic tin derivatives evaporate below 200 C, and even inorganic tin salts have low boiling points. Stannic chloride evaporates at 114 C, stannous acetate at 240 C, and so on.

Since tin had been disqualified as a potentially essential element, we were very cautious with the discovery, first made in 1968, that it strongly promoted growth in the rat. A number of rigid postulates were established to ascertain that the claim for essentiality of tin was justified. These standards have also been applied to our studies on other elements. They are: *1*) The experiments should produce highly significant responses. *2*) They should be reproducible at will, and in series of tests over a lengthy interval of time. *3*) A dose-response curve should be established, and the minimum effective dose level of the element should be determined. *4*) Several compounds of the same element should be tested and compared in potency. In some cases, for example silicon, this was not readily possible. *5*) The effect should be physiological, i.e., it must be produced by amounts which are normally present in foods and tissues.

The results obtained with tin comply with these standards in all respects (32, 42). The growth rate is enhanced by nearly 60% if 1-2 ppm of tin in form of stannic sulfate are added to the amino acid diet (Table 2). To our knowledge, tin has never before been shown to exert any physiological role in animals or man. Even references to effects of tin in plants are found only sporadically in the literature. The element has many properties, however, which lend themselves to essential, unique functions in the organism (Table 3).

Tin belongs with carbon, silicon, germanium, and lead to the fourth main group of elements. It has many chemical and physical properties in common with the other members of this group. It is noteworthy that aside from the paramount role of carbon in living matter of

Figure 4. Effect of diet on glucose tolerance. *Steep curve*: normal diet, removal rate 4.1 ± 0.4% per minute. *Upper curve*: glucose tolerance factor deficient basal, removal rate 2.0 ± 0.4% per minute.

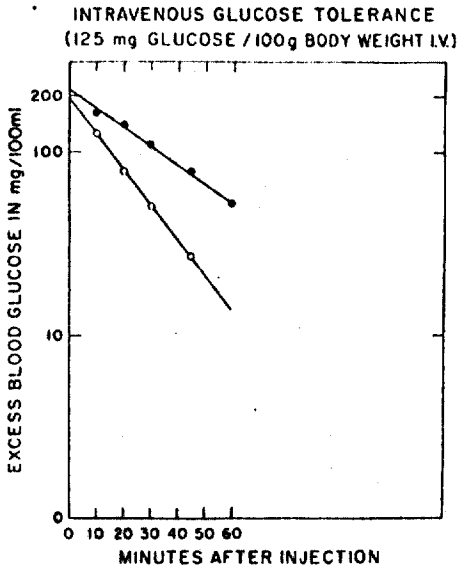


TABLE 2. Growth effects of tin in rats in trace element-controlled environment

Compounds	Dose level, μg Sn/100 g	No. of animals	Average daily weight gain, g	% Increase	*P* Value
Control		5	1.10 ± 0.05[a]		
Stannic sulfate, $Sn(SO_4)_2 \cdot 2H_2O$	50	8	1.37 ± 0.10	24	0.02
Stannic sulfate, $Sn(SO_4)_2 \cdot 2H_2O$	100	8	1.68 ± 0.10	53	0.001
Stannic sulfate, $Sn(SO_4)_2 \cdot 2H_2O$	200	8	1.75 ± 0.10	59	0.001

[a] Mean ± standard error.

our planet and that of silicon as structural building material in plants, none of the other elements of this group previously had been implicated in biological functions.

In its chemical properties, tin is similar to carbon in its tendency to form truly covalent linkages. A great number of organotin compounds have been synthesized. Many of them are of industrial or practical usefulness as catalysts. Others, especially the triethyl and tripropyl tin derivatives, are used as bacteriostatic and fungistatic agents. Even stannic chloride is largely covalent in its chemical nature. It has little ionic character. Other tin salts, however, are ionic by nature. In addition to its capacity to form covalent organic compounds, trivalent tin has a strong tendency to form coordination complexes with 4, 5, 6, and 8 ligands. Thus, it could well contribute to the tertiary structure of proteins or other biologically important macromolecules such as nucleic acids. Tin could function as the active site of metalloenzymes. The oxidation–reduction potential of $Sn^{2+} \rightleftharpoons Sn^{4+}$ is at −0.13 V. This is well within the range of physiological oxidation–reduction reactions. As a matter of fact, it is very close to the potential of the flavin enzymes.

It is thus understandable that tin has a biological function. As might be expected, different tin compounds have different biological activities. The organism is obviously capable of utilizing tin covalently bound to carbon since not only inorganic, but also organic tin compounds enhance growth. These compounds are lipid soluble. Commercial fats often are good sources of tin in small amounts, and a rather large proportion of tin in tissues is lipid extractable. The general assumption, therefore, that "trace metals are bound to protein" does not necessarily pertain to all of them. The biological chemistry of tin is unknown and needs to be investigated.

VANADIUM

Vanadium is required in much smaller amounts than tin. Approximately 0.1 ppm produces near optimal growth effects in rats under our experimental conditions (Table 4). Vanadium has been the subject of much attention in the past. It has been suspected of playing a physiological role even though proof of its essentiality was lacking (27). The element had previously been shown by Hopkins to stimulate feathering in growing chicks, but growth effects were not observed (12, 14). Studies on the requirement for vanadium for bone development in chicks have been carried out by Nielsen (23). Properties of vanadium pertaining to its biological role are summarized in Table 5. It is most likely that it functions as an oxidation-reduction catalyst in the organism.

FLUORINE

The element fluorine, as fluoride, has been considered as essential by some in the past because of its effects on dental caries and in the maintenance of a normal skeleton (11). Under our experimental conditions, it has been found to be essential for growth and general development. In contrast to most other elements recently established to be essential, a vast literature exists on the physiological and toxicological effects of fluoride. Prominent reviews are found in (8, 44, 46).

Efforts have been made previously to

TABLE 3. Tin

Chemistry	Atomic number 50, atomic weight 118.7 IVth main group of elements (with C, Si, Ge, and Pb) Valence $2^+ \rightleftharpoons 4^+$, ox.-red. potential −0.13 V Coordination complexes, 6, also 4, 5, and 8 ligands Covalent bonds with carbon; organo-tin compounds. Even stannic chloride largely covalent Catalyst in polymerization, transesterification, olefin condensation, etc.
Requirement	1.5–2.0 ppm in diet, as stannic sulfate, for growth and normal tooth development Various tin compounds differ in potency
Occurrence	Widespread in foods, feeds, and tissues Human kidney 0.23–0.70, liver 0.35–1.00, lung 0.44–1.20 ppm (wet weight)
Human intake	Estimated 3.6–17 mg or more per day, as compared to 5–10 μg for a rat
Biological effects	Possible oxidation–reduction catalyst

TABLE 5. Vanadium

Chemistry	Atomic number 23, atomic weight 50.9 Group V of the periodic table (with Nb and Ta) Early transition element of first series of metals, between Ti and Cr Valence states 5+, 4+, 3+ and 2+ Strong tendency to form coordination complexes
Requirement	0.1 ppm in the diet, as sodium orthovanadate Large differences in potency of various vanadium compounds and different oxidation states Ortho > meta > pyrovanadate "Physiological" V^{5+}, also V^{4+}, but lower oxidation states possible in organisms
Human intake	2 mg daily on "well-balanced diet", for 75 kg weight, as compared to 1–2 μg for a 75 g rat
Biological effects	Essential for *Aspergillus niger* and *Scenedesmus obliquus* Found in porphyrin complexes in fossil fuels, and in feathers Catalyses phospholipid oxidation by liver protein in vitro Catalyses nonenzymatic oxidation of catecholamines Inhibits cholesterol synthesis Lowers phospholipid and cholesterol in blood Inhibits caries (stimulation of mineralization) Functions most likely as oxidation-reduction catalyst

TABLE 4. Growth response of rats to varying levels of vanadium supplements (sodium orthovanadate, Na_3VO_4)[a]

Dose level, μg V/100 g	Unsupplemented controls		Supplemented animals		% Increase	*P* value
	No. of rats[b]	Average daily weight gain	No. of rats	Average daily weight gain		
1	7	1.05 ± 0.08[c]	7	1.27 ± 0.10	21	n.s.
5	16	1.04 ± 0.08	16	1.38 ± 0.08	33	<0.01
10	6	1.02 ± 0.14	7	1.38 ± 0.07	35	<0.05
25	14	0.87 ± 0.10	14	1.21 ± 0.09	41	<0.02
50	6	1.02 ± 0.14	7	1.49 ± 0.12	46	<0.02

[a] Pooled results of five successive experiments. [b] A total of 37 rats served as controls in five successive experiments. [c] Mean ± standard error.

determine whether the element is needed for growth, mostly with negative results. Since some of these studies were clearly [illegible] because of inadequate analytical [illegible]s or techniques, others, for instance those by Maurer and Day (16) and Doberenz et al. (5), applied rather sophisticated means. The cause for the discrepancy between their results and ours is not clear. It is well known, however, that the fluoride requirement is influenced by the levels of calcium, phosphorus, magnesium, and various other constituents of the diet. In our experiments, these were contained in adequate but balanced amounts in an amino acid diet which was completely defined. The fluoride supplement was added to the diet while most other investigators supplied it in the drinking water. The utilization of fluoride in water appears to differ from that in foods. Recent studies using our isolator technique indicate that silicates may also interfere with fluoride utilization; addition of 50 mg/100 ml of silicon in form of sodium metasilicate inhibits the fluoride effect on growth (20).

The level of fluoride required to produce optimal growth is approximately 2.5 ppm (Table 6). This amount is physiological [illegible] it occurs normally in foods and [illegible] including average human diets. Compared to the amount of fluorine found in the earth's crust, the dietary requirement for fluoride appears very small. Fluorine is quite a common element, constituting 0.065% of the earth's surface. Indeed, it is more abundant than chlorine. In the oceans, however, chloride is highly concentrated and fluoride is far less prominent, the level of seawater amounting to 1.5 ppm. It appears that the nutritional requirement for fluoride corresponds to the amount in the oceans and not that found on land. A similar case can be made for other trace elements.

Rats in the trace element sterile isolator fail to develop normal incisor pigmentation. "Bleached" incisors, indicating a disturbance of enamel development, or pigment disposition, or both, are observed regularly. Such changes have previously been related to deficiencies of vitamins E, D, and A, or to lack of Ca, P, Mg, and Fe. The latter is a constituent of the orange-brown pigment normally deposited in the upper layer of enamel. Significant improvements of tooth pigmentation are produced by fluoride supplements, as measured on a standardized color scale on the 26th day of the experiments (32). However, fluoride was only partially effective in restoring normal pigment deposition. Significant improvements were also produced by supplements of physiological amounts of tin, vanadium, and silicon. The beneficial effect of low, physiological levels of fluoride in the diet on the development of normal incisor pigmentation stands in sharp contrast to the effects of higher amounts of fluoride. When added to drinking water, higher levels cause a bleaching of normally pigmented incisors in rodents. This depigmentation may be related to the formation of the iron fluoride complex $[FeF_6]^{-3}$, which is particularly stable.

The metabolism of fluorine in mammals presents many criteria which support the concept that it is essential (Table 7). For details, see references (8, 44). The concentration of fluorine in blood plasma is homeostatically controlled at 0.1–0.2 ppm, even if large amounts of fluoride are given. Any excess is rapidly deposited as fluoroapatite in the bone, which acts as the chief depository, or excreted through the urine. Normal fluorine levels in teeth and bone range from 100 to 600 ppm, but then easily reach much higher values. Up to 7,000 ppm are considered within the normal range. Since bone fluoride levels increase with age, amounts exceeding 1,000 ppm (0.1%) in bone ash may be seen routinely even in low fluoride areas in individuals beyond 40 years of age (51). In the embryo and newborn, vertebrae and ribs contain 50–150 ppm of the element. Even in low fluoride areas, fetal bone ash contains 40–47 ppm of fluorine. The placenta plays an active role by accumulating fluorine and regulating its transfer to the fetus (10). Fluorine is supplied in rather constant amounts (0.1–0.2 ppm of wet weight) by milk. The normal fluorine levels in soft tissues, such as liver, heart, kidney, and brain, are approximately 2–5 ppm on a dry weight basis. These concentrations are maintained constant, except for kidney, even when high amounts of fluoride are supplied over extended periods of time (Table 8). Fluorine occurs in practically all foods and feeds, and food appears to be the main source of fluorine for animals and man. Drinking water also serves as a source of the element for the human organism, and fluoride levels in bone and teeth are well documented to be related to the amount of fluoride in water supplies.

TABLE 6. Growth effect of dietary fluoride in rats under trace element controlled conditions[a]

Supplemented fluorine,[b] μg F/100 g diet	No. of rats	Daily weight gain	% Increase over controls	*P* Value
0	19	1.04 ± 0.07[c]		
100	17	1.22 ± 0.09	17.3	
250	17	1.36 ± 0.09	30.8	<0.01
750	19	1.33 ± 0.07	27.9	<0.01

[a] Pooled results of three successive experiments. Duration of experiment, 28 days. [b] Supplied as KF. [c] Mean ± standard error.

TABLE 7. Fluorine

Chemistry	Atomic number 9, atomic weight 19 Highly reactive halogen, Group VIIa 0.065% of earth's crust (more abundant than Cl), but only 1–1.5 ppm in seawater May function as ligand to transition element or in relation to phosphate (fluoroapatite)
Requirement	2.5 ppm, as F^-, in diet for optimal growth and normal incisor pigmentation Potency of other F compounds possible
Occurrence	Abundant in food and feeds, also supplied by drinking water Blood plasma 0.1 ppm, homeostatically controlled Liver, heart, kidney, brain, etc. 2–5 ppm of dry weight Bone (storage organ) and teeth 100–600 ppm, easily reaching 1,000–7,000 ppm. Embryo and newborn (vertebrae and ribs) 50–150 ppm Transfer and accumulation of F^- by placenta
Human intake	Available daily intake of 4.4 mg in adult men, as compared to 25 μg in rat Normal animal diets supplying large excess, from 20–100 ppm
Biological effects	Growth and general development, dental caries prevention, maintenance of normal skeleton Stimulating growth in tissue culture Activating citrulline synthesis by liver, and enzymatic decomposition of nitromethane Activating adenyl cyclase (mediator of hormone effects)

TABLE 8. Growth effect of dietary silicon in rats on low silicon diet[a]

	No. of animals	Average daily weight gain, g	% Increase	P Value
Control	12[b]	1.51 ± 0.11[c]		
Si-supplemented	11	2.02 ± 0.06	33.8	<0.005

[a] 50 mg/100 ml Si, as $Na_2SiO_3 \cdot 9H_2O$. [b] Summary of 2 experiments. [c] Mean ± standard error.

In contrast to animal diets, which usually contain much higher amounts, normal human diets provide levels that are close to those found essential for support of growth in the rat, i.e., 1.5–2.5 ppm of fluorine on the dry weight basis. Figures for human fluorine intake from food vary. An available daily fluorine intake of 4.4 mg, including fluoride from fluoridated drinking water, was found for adult men in careful balance studies (48, 49). The skeleton of an average man of middle age may contain approximately 2–6 g of fluorine, based on a fluorine content of 200–600 ppm and 10 kg bone mass. It seems that the organism must handle fluoride supplies rather economically to accumulate and maintain this deposit.

In connection with these findings, it must be noted that Messer et al. (19) have recently described an impairment of fertility in female mice maintained on a low fluoride diet. The number of litters produced by first and second generation females was reduced, but growth or litter size was not affected. The condition was prevented by 50 ppm of fluoride in the drinking water. The investigators also found an anemia in pups from rats not supplemented with fluoride (18). It is not possible to draw definite conclusions from this study about the essentiality of fluoride since the level of fluoride applied far exceeds the physiological range of fluoride in water. Access to fluoride did not appear to be satisfactorily eliminated since fluorine levels in bone ash of second generation animals still contained 83.5 ppm of the element. One must consider the possibility, therefore, that the beneficial effect of fluoride on female fertility and on anemia in newborn mice could in this case be secondary to a dietary imbalance, for instance, an excess of magnesium in the basal ration applied.

Studies with various fluorine-containing compounds in our system have led to the conclusion that considerable differences exist with respect to their biological effects. Some compounds, like fluoride or fluoropyruvate, might be utilized in processes relating to growth or possibly iron metabolism. Others, such as hexafluorosilicate and monofluorophosphate, may not be readily available to the rat as sources of metabolically active fluoride even though they lead to fluorine deposition in bone (20). While the fluoride ion has been investigated thoroughly with respect to metabolism and toxicity, organically bound fluorine may also be of importance. In human blood serum, for instance, most of the fluorine is organically bound. It travels electrophoretically in the area of the albumin peak. The fluorine-containing compound is not a protein but a weak, lipid soluble organic acid (25, 52). In eggs, fluorine has been reported to be associated primarily with the lipid portion of the yolk (24).

Thus, some important basic questions remain to be answered, in spite of prior efforts and a large number of publications on the subject. Past investigations used bone and enamel fluoride levels and resistance against caries as criteria and concentrated on biogeochemical questions and fluoride toxicity. The fluoride ion shows an unexplained low index between the effective dose in the prevention of caries and undesirable effects on the enamel organ during tooth formation. The chemical nature of fluorine in food and feeds, in which it occurs often in large quantities, is still rather unexplored, and very little is known at this time about metabolism and biochemistry of fluorine in tissues at physiological levels, or its mode of action.

SILICON, AN ESSENTIAL CROSS-LINKING AGENT IN CONNECTIVE TISSUE

Since the all-plastic isolator system excludes contact with silicon, the second most abundant element in the biosphere, this element has been one of the first investigated after our system became operative in 1965. Significant growth responses were seen at that time with sodium metasilicate, but the amounts needed to elicit the effect were so high that we suspected trace element contaminants in the silicate to be responsible for this observation. After identifying tin, vanadium, and fluorine as elements needed for growth, the effects of silicon were reinvestigated in 1971. It was found that the initial observations were correct: silicon is indeed required for growth (Table 8), and if sodium metasilicate is used, relatively large quantities are needed to produce an optimum response (41). Metasilicate may be poorly retained because it is highly soluble, easily absorbed, and rapidly excreted. Screening of other silicon compounds has led to substances which are five to ten times as active per atom of silicon (to be published).

Significant growth effects amounting to 25–34% in growth rates are observed. The deficiency of silicon in growing rats documents itself not only in a retardation of growth, but also in disturbances in the development of bone structures (Fig. 5). Considerable differences in skull size and bone architecture are evident between littermates raised with and without silicon supplements. In addition, silicon beneficially affects the lack of incisor pigmentation seen in animals under our trace element sterile isolator conditions (21). As indicated, our work on the growth effects of silicon was independently and almost simultaneously confirmed by Carlisle (3) who worked with chicks on amino acid diets.

The main properties of silicon relating to its biological functions are summarized in Table 9. The element has qualities which make it appear likely that it may function as a structural rather than a catalytically active entity in metabolism. We have discovered a highly important structural function for silicon by showing that it is an integral building block, an important cross-linking agent in connective tissue. This, of course, does not eliminate the possibility that the element could be catalytically active at specific sites of intermediary metabolism. These findings provide an example of the potential impact of modern trace element research on biochemistry and medicine.

Both the distribution of silicon in the organism and the occurrence of deformities in silicon-deficient rats and chicks suggested that the element is specifically associated with the ground matrix and structural elements of connective tissue, cartilage, skin, and bone. Analyses in our laboratory confirmed the impression gained from a review of the literature that the organism contains silicon primarily in connective tissue such as cartilage, ligaments, and skin. Silicon levels in blood are very low, similar to those in the ocean. In parenchymal organs, such as liver, lung, muscle, and brain, 2–20 ppm are reported (results found prior to the introduction of plastic laboratory ware

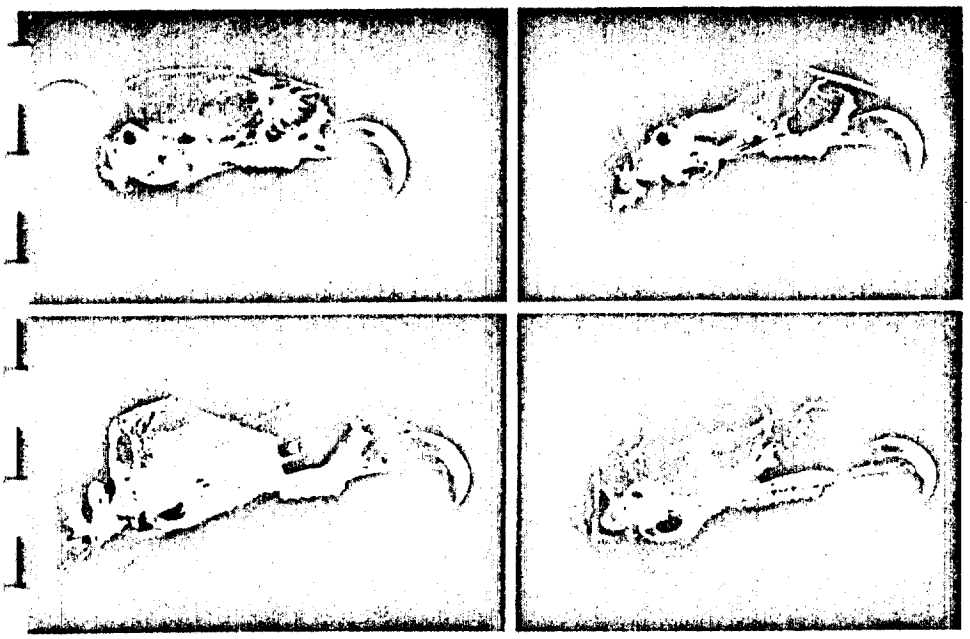


Figure 5. Skull deformation in silicon-deficient rats. *Upper:* without silicon. *Lower:* litter-mates on same diet supplemented with 50 mg/100 g silicon. (Grenzray pictures courtesy Dr. t. Sognnaes, School of Dentistry, UCLA.)

are much higher); the silicon content of connective tissue is usually much higher. Values amounting to 100 ppm or more are reported by careful analysts (9).

tensive fractionation experiments rtilage and other source materials, we found that the silicon is not present in free form as silicate or oligosilicate. It is bound to the organic elements of connective tissue. When trying to identify the binding site of silicon, we discovered that it is an integral constituent of several main acid mucopolysaccharides (34). This finding has led to the identification of silicon, in form of an organic derivative of silicic acid, as an important cross-linking agent in connective tissue.

A large number of acid mucopolysaccharides or related substances have been analyzed for bound silicon (Table 10). Among the specimens analyzed were a number of Standard Reference Samples, prepared to serve as standards for research on connective tissue polysaccharides. Since strong alkali or acid tend to liberate silicic acid, the amount of silicon found in various specimens may be dependent on the method of preparation. High levels of bound silicon, amounting to 330–554 ppm, were detected in purified hyaluronic acid from umbilical cord, chondroitin-4-sulfate, dermatan sulfate, and heparan sulfate. These amounts correspond to 1 atom of silicon per 60,000–85,000 molecular weight or 130–280 disaccharide repeating units. Recently high molecular hyaluronic acids have been found which contain up to 2,000 ppm of the element. Lesser amounts, 57–191 ppm, occur in chondroitin-6-sulfate, heparan, and keratan sulfate-2 from cartilage, while hyaluronic acids from vitreous humor and keratan sulfate-1 from cornea were silicon-free. High levels of bound silicon are also present in pectin (2,580 ppm) and alginic acid (451 ppm). Four polyglycans, glycogen, starch, dextran, and inulin, contained only negligible amounts of silicon, most probably as silicate. All had been manufactured under conditions that avoided exposure to extreme pH conditions. This does not exclude the possibility that starch from other plant species or other storage forms of carbohydrate from elsewhere contain silicon as a structural entity.

Numerous experiments were carried out to characterize the form in which silicon may be present (Table 11). Bound silicon is not dialyzable, does not react with ammonium molybdate, is not liberated by autoclaving or 8 M urea, and is stable against weak alkali and acid. Strong alkali and acid hydrolyze the silicon–polysaccharide bond. Free, direct-reacting, dialyzable silicate is obtained. Enzymatic hydrolysis of hyaluronic acid or pectin does not liberate silicic acid but leads to products of low molecular weight still containing silicon in bound form (34).

These data indicate that the silicon atom is bound over oxygen to the carbon skeleton of the mucopolysaccharides:

$$—Si—O—C— \qquad (I)$$

Silicon is thus covalently linked to the polysaccharides as a silanolate, an ether- or esterlike derivative of silicic acid. Orthosilicic acid, $Si(OH)_4$, could connect

TABLE 9. Silicon

Chemistry	Atomic number 14, atomic weight 28.1 Group IV with C, Ge, Sn, and Pb Tetravalent; chemistry determined by strong affinity to oxygen Si—O—Si (siloxane) very stable Si—C bond also stable Si—Si and Si—H bonds less so SiO_2 very stable Silicic acid: strong tendency to colloid formation (gel)
Requirement	50 mg/100 ml in the diet as $Na_2SiO_3 \cdot 9\ H_2O$ Other forms possibly more potent Necessary for growth and prevention of bone deformation
Occurrence	Second only to O_2 in natural abundance Present in all living beings Blood level approximately 2 ppm Liver, muscle, lung and brain 2–20 ppm Much higher in connective tissue, bone (region of active calcification), and skin
Human intake	Not determined. Much of intake possibly not biologically available Clay eaters (geophagy; pregnancy)
Biological effects	Structural, but possibly also "matrix" for formation of organic compounds Crosslinking agent, contributing to stability of acid mucopolysaccharides and other connective tissue components Forms stable ring structures or networks (Si—O—Si) Catalytic effects on peptides

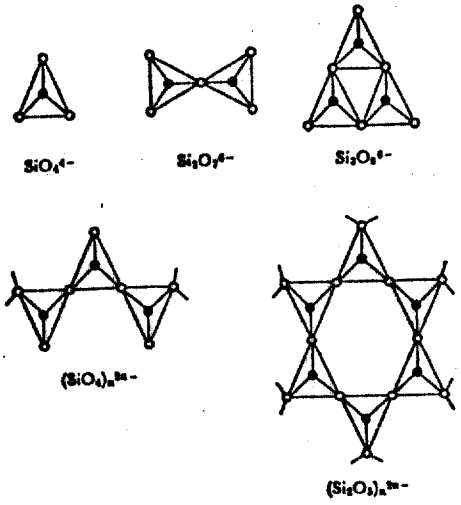
TABLE 10. Stability of silicon bonding in hyaluronic acid

Dialysis, 0.1 NaOH	stable
Autoclaving	stable
8 M urea, 60 C	stable
pH 12.4, r.t.	stable
pH 12.4, 100 C	hydrolyzed
pH 13.5, r.t.	hydrolyzed
pH 3.0, r.t.	stable
pH 1.2, r.t.	hydrolyzed

TABLE 11. Free, total, and bound silicon in glycosaminoglycans, polyuronides, and some glycans

Substance and source	Si, μg/g		
	Free	Total	Bound, total minus free
Glycosaminoglycans			
Hyaluronic acid			
human umbilical cord	25	354	329
human umbilical cord	1,533	1,892	359
bovine vitreous humor	980	949	(0)
Chondroitin-4-sulfate			
notocord of rock sturgeon	44	598	554
rat costal cartilage	30	361	331
Chondroitin-6-sulfate			
human umbilical cord	45	123	78
human cartilage	36	227	191
sturgeon cartilage	64	121	57
Dermatan sulfate			
hog mucosal tissue	46	548	502
Heparan sulfate			
beef lung	39	466	427
Heparin			
hog mucosal tissue	33	175	142
Keratan sulfate-1			
bovine cornea	31	37	(0)
Keratan sulfate-2			
human costal cartilage	37	105	68
Polyuronides			
Pectin			
citrus fruit	5	2,586	2,581
Alginic acid			
horsetail kelp	—	43	—
horsetail kelp	5	456	451
Polyglycans			
Glycogen			
rabbit liver	8	34	26
Starch			
corn	—	22	—
Dextran			
Leuconostoc mesenteroides	19	22	(0)
Inulin			
dahlia tubers	15	29	14

Figure 6. Oligo-silicate formations. Silicon atoms black, oxygen atoms white. An additional oxygen atom is hidden behind each silicon atom.



maximally four different binding sites to each other. Different molecules, or two binding sites within the same macromolecule would be connected over oxygen–silicon bridges.

$$R_1—O—Si—O—R_2 \qquad (2)$$

Since silicon atoms have a strong tendency to attach over oxygen to each other, two silicon containing molecules could be linked by an oxygen bridge, forming the group:

$$R_1—O—Si—O—Si—O—R_2 \qquad (3)$$

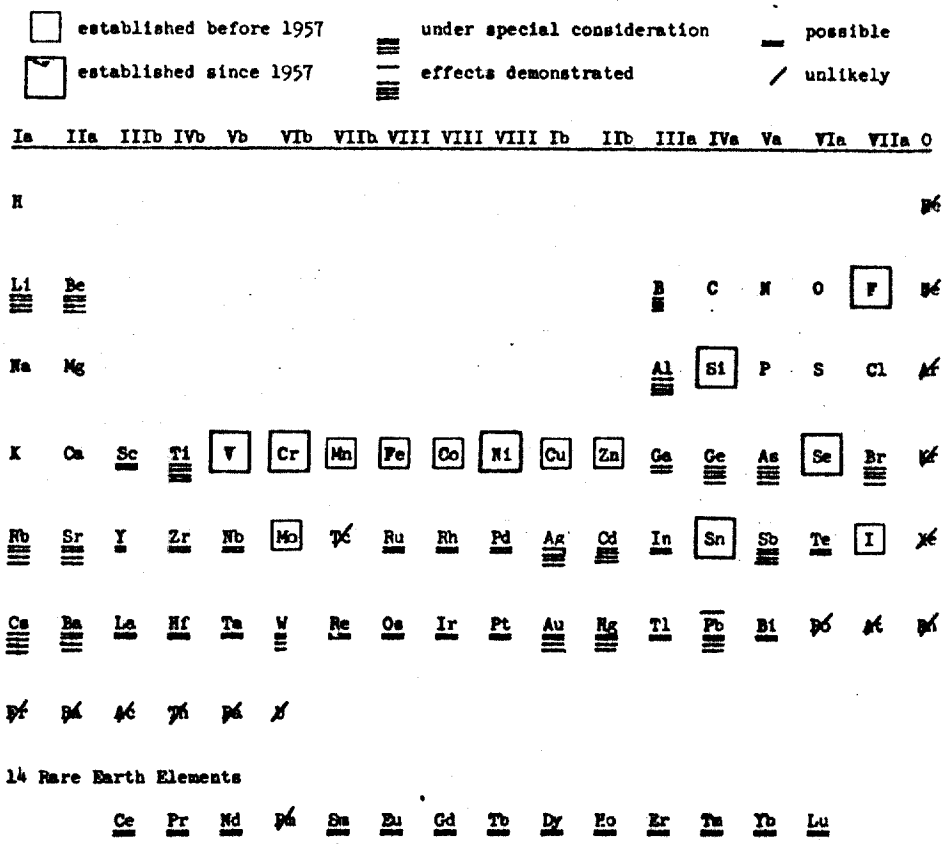
In this case, the two silicon atoms could carry four hydroxyl groups provided they are stereochemically prevented from associating with other silicon atoms. An alternative would be the binding of four additional radicals by way of oxygen bridges. In spite of its thermodynamic stability, the Si—O bridge contains a built-in "switch" mechanism. The Si—O bond has a strong ionic component and can, therefore, be transferred from one oxygen atom to another with only small changes in energy.

A large variety of structural, highly organized arrangements between bundles of mucopolysaccharides or proteins are possible in this way. Interlacing structures of this nature, based on the tendency of Si—O to form polymers or ring systems (Fig. 6), could hold elements of the ground substance in connective tissue or elements of membranes together in an organized fashion, contributing to the architecture and resilience of connective tissue and membranes. From a physiochemical point of view, this function of silicon makes eminently good sense. The Si—O—C bridge is thermodynamically very stable. The silicon atom is small; it has the same stereochemistry as carbon, but it is known to be more rigid. It neither bends its bond angles nor does it undergo stereochemical conversions as easily as carbon (47).

More recent studies have shown that the proteins in connective tissue, notably collagen, also contain bound silicate (to be published). The use of silicic acid as a cross-linking agent is not limited to the animal kingdom. Analysis of pectin and alginic acid shows that plants employ the same principle.

To our knowledge, the occurrence of bound silicate as a structural component in acid mucopolysaccharides has not been mentioned previously even though the literature on these compounds is very extensive. The finding opens up a number

Figure 7. Periodic system distribution of trace elements of known and potential importance for warmblooded animals. Status as of 1973.



of fascinating possibilities in biology, biochemistry, pathophysiology, medical research, and therapy.

OUTLOOK

If one delineates the roles of the 92 elements of the classical periodic system with respect to their significance for the organism (Fig. 7), some interesting facts evolve:

1) All the 11 elements which form the bulk of living matter have very low atomic weights. They belong to the lowest 20 elements of the periodic system.

2) There is some overlap between the 11 main elements and the 14 elements now recognized as essential trace elements, with fluorine and silicon being below 20 in atomic number.

3) None of the 39 elements beyond iodine (atomic number 53) have ever been shown to be of any physiological significance. However, some of these may eventually be proven to be essential. Lead, for instance, has many of the properties which one would postulate for an essential trace element. It is obvious from past experience that toxicity is no argument against potential essentiality.

Through the addition of vanadium [illegible]kel in the first series of transition [illegible]ts, a continuous set of 8 elements has been formed which all are essential for warm-blooded animals. From vanadium to zinc, the first series of transition elements, therefore, constitutes an area of special emphasis, a point of gravity in the periodic system where most essential trace element functions are located. This may have significance with respect to the development of life in prebiotic times. It is also interesting to note that plants have trace element requirements distinctly different from those of animals, while their organic biochemistry is quite similar to that of the animal world.

5) According to Fig. 7, there are 20 additional elements which must be taken into consideration as potential pretenders for trace element function in animals. This evaluation is based on properties which would be expected from an element that is essential. Such elements should be present in the biosphere, normally found in the organism and tissues, present in egg, milk, and the newborn, present in normal diets, and be maintained at con[illegible]s [illegible]vels in the bloodstream.

[illegible] most likely from these considerations that further essential elements will be discovered. Some of them may be easily accessible by currently available methods of experimentation. Others may require more advanced techniques of analysis, preparation of ultraclean dietary constituents, ultraclean techniques not only in the animal room, but throughout the experimental area including the analytical laboratory and the diet preparation facilities. It can be calculated that one would have to go down by four to five orders of magnitude, into the area of parts per trillion, before coming to the threshold where only one atom of a specific element would be present for one specific gene in the chromosomes of a cell. I could conceive this as the extreme lower limit of biological trace element function.

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Possible Relationships Between Trace Metals and Chronic Diseases

The extensive spectrographic analyses of human tissues for trace elements by Tipton *et al.*[1,2]* have provided a basis for what may be a new frontier in Medicine. Their data show several metals with unknown functions in all or most adult bodies in addition to those recognized as essential. Less extensive analyses by Koch *et al.*,[3] in New York, and Stitch,[4] in England, have tended to confirm their results. Furthermore, accumulation of several metals during life is suggested.[2,3,4]

The trace metals found in man may be divided into four groups: (1) metals considered to be essential for mammals, in that requirements for growth have been established, deficiency states discovered, or essentiality inferred as cofactors for certain purified metalloenzymes—manganese, cobalt, copper, zinc and molybdenum; (2) metals ubiquitous in plant and animal tissues with suspected metabolic functions but with essentialities unproven—rubidium, boron, barium, strontium, vanadium, chromium, nickel and aluminum; (3) metals without known functions believed to be contaminants—silver and lead; and (4) metals usually not detected in infants but apparently acquired and accumulated as environmental contaminants—cadmium, tin, titanium and, in some locations, bismuth, gold and gallium (rarely).

Specific organ and tissue accumulations of some metals have been demonstrated, while other metals apparently are distributed throughout most tissues in roughly equal concentrations. Those showing affinities for a specific tissue and accumulating in relatively high concentrations are cadmium in kidney and liver; aluminum,* vanadium and titanium in lung; barium and strontium in bone; tin in intestine.[41]

Of the Group II-A earths (Periodic Table, p. 362), which are concentrated in bone, magnesium was considered to be an exception until Tipton *et al.* demonstrated an increased concentration over other tissues.[41] Likewise, alkali metals penetrate cellular membranes following certain definite laws, lithium displacing sodium, and rubidium and cesium displacing potassium. Thyroid tissue concentrates all Group VII elements heavier than chlorine.[5] Presumably, specific tissues with uniform ligands form similar complexes with similar metals.

Spectrographic analyses of newborns and children[2-4] have shown that some metals first appear in childhood and accumulate during the next decades. While some of these metals may be inert or may occur in such traces as to be inactive, metabolic activities in vivo or specific enzymatic alterations in vitro have been shown for several, and overt toxicities for a few. Therefore, it is tempting to speculate that a metal accumulating in an organ from the modern environment could so modify an enzymatic reaction that eventual breakdown of a metabolic pathway would result in a chronic disease.[6] The appearance of age-linked diseases is consistent with this hypothesis. But speculations based on present knowledge are hazardous when conclusions are drawn therefrom, and in only a few chronic disorders can metals be implicated as causative factors. Speculation is justified if it points

* EDITOR'S NOTE: See also Tipton, page 27, and Butt *et al.*, page 43.

* EDITOR'S NOTE: There is some disagreement among investigators who have done spectrographic analyses for aluminum in human tissues. See the discussion by Tipton on page 41.

out directions for research, and this discussion will be so confined.

SOME TELEOLOGICAL THOUGHTS ON TRACE METALS

All necessary trace elements come from soil. Either plants require them for growth, or soil bacteria need them for fixation of atmospheric nitrogen. Therefore, it is virtually impossible for rare metals or those confined to local areas to be required for metabolic processes; only metals ubiquitous and available can be considered as essential. All trace metals essential for terrestrial animals come from plants. Carnivores obtain metals by eating herbivores. It is unlikely that evolving mammals acquired for metabolic needs a metal not found in plants or running water. (Marine animals may be an exception.) It is also unlikely that metals not in sea water are essential for mammals. Furthermore, it is unlikely (but not impossible), that a trace metal not present at birth is acquired later to fulfill an essential function.

These considerations exclude approximately three fifths of the Periodic Table of Elements (p. 362) from possible metabolic roles, leaving 40 elements for our biological thinking. Twelve are bulk elements* and 7 (B, Mn, Co, Cu, Zn, Mo, I) are known trace elements in plants or animals. Of the remaining 21, 4 (F, Si, Se, Br) are nonmetals not pertinent to this discussion. The remaining 17 metals have been found in human tissues, and most show metabolic activities. In addition, 3 (Be, W, Sb), not normally present, are biologically active as competitors.

The following postulate appears reasonable: Any element not present in plants is not essential for man. As a corollary, any element found in man but not in plants is an environmental contaminant.

From this viewpoint, of the alkali metals, Rb is ubiquitous, with traces of Li frequently present and Cs rarely present. The functions of Rb are unknown. Both earths, Sr and Ba, are ubiquitous,[1, 7] with suspected functions. In the first transition group, Ti, V, Cr and Ni are ubiquitous; in the second and the third, Ag and Au are not found usually in plants and may be classified as environmental contaminants. Of the remaining metals, Al is ubiquitous, while Sn, Cd, Hg, Pb and Bi are probable contaminants.*

Based on this reasoning, 7 metals not usually present in plants but found in human tissues are possible or probable contaminants from special environments.

METABOLIC ROLES OF TRACE METALS

We will not consider at length the various roles of the five essential trace elements. Metalloenzymes are being discovered at an increasing rate, and appreciation of their importance in human metabolism is widening. Many enzymes depend upon manganese,[10] especially several peptidases. At least one is cobalt dependent.[11] The copper oxidases are well known. Six mammalian zinc metalloproteins have been isolated, five by Vallee and his co-workers.[12] Other pyridine-nucleotide dependent enzymes, particularly dehydrogenases, have been suspected to require zinc for activity. There are two mammalian molybdenum enzymes, one containing iron-flavin and one iron-porphyrin, and there may be a nitrate reductase.[13, 17]

Other first transitional metals have metabolic or enzymatic activities in vivo or in vitro. Bernheim and Bernheim showed that titanium inhibited the oxidation of cysteine to the corresponding sulfonic acid.[14] Although present in all human lungs (from dust?) in increasing amounts with age,[1] little attention has been paid to its possible role.

Vanadium causes several actions, as shown by Curran, who has reviewed its relationship to cholesterol synthesis on page 216. This element and strontium have caused marked stimulation of mineralization of bone and teeth in animals and prevented caries.[17]

Vanadium salts are known to catalyze the oxidations of 5-hydroxytryptamine (serotonin), DL-5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, dopa, dopamine, L-epineph-

* Editor's Note: Although some writers do not include Fe with the "bulk" elements.

* One may be misled by analyses of plants exposed to commercial fertilizers; pasture herbage and fertilizer in England contained Ti, Cr, Ni, Sn, Pb, V and Al. The tissues of domestic animals are also suspect: tap water in the United States contained Cr, Ni, Pb and Ag; in Argentina, Al; in Germany, Cr, Pb and Al.[8] Purina Laboratory Chow contains 0.2 ppm. cadmium,[9a] enough to accumulate in kidney, as well as As, Pb, Al, Sn, Ni, Ti, Cr, Ba and V.[9b]

rine and L-norepinephrine in vitro, showing preference for hydroxylated compounds (copper is more active only on serotonin). Tyrosine is not affected.[18] In our hands, V^{+3} and V^{+4} were the only metallic activators of tryptamine oxidation by monoamine oxidase from guinea pig liver; this action was greatly enhanced. V^{+5} showed little activity.[19] These various metabolic actions strongly infer, but do not prove, the essentiality of vanadium for man.

Chromium increased hepatic cholesterol and fatty acid synthesis more than did manganese.[16] It appeared to promote the reaction between cytochrome c and succinic dehydrogenase.[13] Aside from obvious toxicity as chromates, little is known of its functions in the body.

Nickel, with others, activates arginase (which is Mn dependent), carboxylase and trypsin, and inhibits acid phosphatase.[13] It is apparently toxic only in industrial exposures, where it causes respiratory tract neoplasia and "nickel eczema."

Boron, essential for plants and present in man, has no known activity in mammals.

Aluminum promotes the reaction between succinic dehydrogenase and cytochrome c in vitro and has been suspected to be essential.[13] It forms chelates with pyridoxal amino acid Schiff bases, which act as model transaminases[20] (see Fig. 8, p. 185).[1] Indirect toxicity results from its inhibition of phosphate absorption in the gut.

There are two recent important findings. Wacker and Vallee[21] showed that ribonucleic acid (RNA) from plant and animal sources contained chromium, manganese and nickel in constant amounts and believed that the association was significant. (Zn, Cu, Sr, Ba, Ca, Mg, and Al also were found). Beef liver RNA contained 50 to 137 μg. Cr per gram. Specific inheritance of the ability to accumulate one metal or another according to eye or body pigment has been demonstrated by Kikkawa.[22] Drosophila mutants with white eyes absorbed nickel, while mutants with cinnabar-colored eyes absorbed cobalt from media containing both metals; the metal abnormal for the eye color eventually was toxic. Red goldfish absorbed molybdenum; white absorbed nickel from water, rejecting the other. Black mice accumulated cobalt in livers and lungs from drinking water, while white mice accumulated nickel, the abnormal metal being excreted in the urine. Rabbits appeared to behave similarly, according to color. These two findings, if applied to man, can have wide implications in genetics and point indirectly to specific functions of chromium and nickel in mammals.

SIMPLE METAL DEFICIENCIES

Soils and plants deficient in one or more trace elements are well known. Deficiencies can be transmitted to livestock grazing on grasses and, thereby, presumably to man. Many soils of the world are deficient in molybdenum, copper, boron, cobalt, manganese or zinc.[23]

In the United States, owing to wide distribution of fresh and processed foods, it is doubtful that simple deficiencies of the known essential trace metals exist in man to any extent. This statement does not hold for inadequate diets, but deficiencies have not been completely confirmed. Nothing is known of elements present in tissues with essentialities unproven but with metabolic activities.

Manganese deficiency has been suspected in disseminated lupus erythematosus (L.E.), although the relationship is not clear.[19, 24]

In adults, simple copper deficiency has not been demonstrated, although, in children, it has been suspected in certain anemias. Cobalt deficiency has not been demonstrated. Molybdenum deficiency also has not been described in humans, although abnormal purine metabolism may eventually be so linked.

Postalcoholic cirrhosis of the liver has been found to be associated with plasma and hepatic zinc deficiency by Vallee and his coworkers,[26]* with some reversal when zinc salts were fed. Laennec's (postalcoholic) cirrhosis has long been suspected of association with deficiency of one or more of the B vitamins; it occurs also in countries where alcohol is not used but where dietary deficiency stems from high carbohydrate intake, especially rice. Here, some speculation is permissible. Enzymes dependent upon both zinc and a vitamin as cofactors would be inactivated if either zinc or the vitamin, or both, were missing.

* EDITOR'S NOTE: Refer also to the hepatic zinc levels reported by Butt *et al.*, page 45.

One of these mechanisms could be operative in the production of skin lesions following $CaNa_2EDTA$ therapy, where lesions resembling acute vitamin B_6 deficiency occur despite supplemental B_6 therapy.[27]

SPECIFIC METAL DISPLACEMENTS AND CONDITIONED DEFICIENCIES

Several metals are carried in the blood on proteins, bound to sulfur or imidazole groups,[28] and undoubtedly complexes exist in tissues where metals are accumulated. Iron and cobalt exist in the porphyrin chelates heme and vitamin B_{12}; zinc and nickel also may form such chelates. Iron and copper are bound to flavins in several enzymes. Pyridoxal phosphate probably forms pyridoxal amino acid complexes as cofactors for several enzymes; such complexes are strong binding agents and, with some metals, act as transaminases. All B_6 enzymes may require metals as additional cofactors.[29] Manganous complexes in enzymes have been postulated.[11]

If essential metals form complexes, it is probable that "abnormal" or contaminating metals also do. Therefore, we should examine the laws of chelation[30] to see what might happen if an abnormal metal were carried to tissues. Would it compete for a ligand with an essential metal on a metalloenzyme? If so, which would be displaced? From the laws of chelation and atomic structure, there are reasons to believe that Cd^{++} and Hg^{++} will chelate with protein or coenzyme ligands that ordinarily chelate with Zn^{++}, and that Be^{++} and Mg^{++} also will compete in this way for donors. This probability holds true for all criteria. According to two criteria, it seems likely—and there is some experimental evidence—that Cu^{+}, Ag^{+} and Au^{+} will compete, as will Cu^{++} and Ni^{++}. Furthermore, Cr will compete with the two lower valences of Mo, and W with the three higher ones. Under certain conditions, the possibility exists that Al^{+3} and Cr^{+3} may interfere with Fe^{+3} and Co^{+3}.

We may define these interactions as *close competition* or specific metal displacement. There are examples in biological systems where one metal apparently displaces another and inactivates the system quite specifically, or, less commonly, enhances it. Be^{++} displaces Mg^{++} on certain magnesium-dependent enzymes, resulting in inactivation. W^{+6} depletes hepatic Mo^{+6} enzymes, xanthine oxidase and aldehyde oxidase.[17] Tyrosinase is reversibly inactivated by Ag^{+} and Au^{+}, the Cu^{+} being loosely bound.[32] The Rb and Cs inhibition of K-dependent enzymes has been discussed. Biological competitions of Zn and Cd, and Cu and Ni, have not yet been demonstrated clearly.

Less directly enzymatic, but equally valid, displacements have been shown for As on P systems, providing the toxicity of Lewisite gas, Te and Se on S, causing abnormalities of keratin structure, and the actions of Sr and Ba on bone, the former promoting and the latter inhibiting calcification.

Close competition by metals for ligands usually is a *vertical* series of displacements according to the Periodic Table and follows certain well-defined laws. From the organic models already investigated,[30] insight into the nature of the binding of metal to protein can be obtained to some small degree. One can also make certain predictions with some accuracy:

1. If an unknown metalloenzyme system is exposed to diminishing concentrations of many metals in various valence states, and if only one or two in the same periodic group or with the same characteristics for forming chelates inactivate that enzyme, one may be able to predict the natural metal. Experiments must be done with great care in order to avoid nonspecific sulfhydryl or imidazole binding (by using arsenic, for example). Thus, we found that only Cd^{++} and Hg^{++} inhibited dopa decarboxylase from guinea pig kidney.[19] Therefore, Zn^{++} may be suspected of being the metal bound to the pyridoxal amino acid Schiff base.

2. The valence state of the metal on a known metalloenzyme may be predicted by utilizing these laws. By taking advantage of the various valences of chromium and tungsten, interactions confined to one valence would suggest the valence of molybdenum.

3. One might obtain indirect evidence for the presence of other first transitional metals by such techniques. A niobium and tantalum inhibited enzyme might be expected to contain vanadium, a zirconium and hafnium inhibited one to contain titanium, a palladium and platinum inhibited one to contain nickel,

TABLE 1. CONCENTRATIONS OF CADMIUM AND ZINC IN KIDNEY AND LIVER IN CITY DWELLERS ACCORDING TO GEOGRAPHIC LOCATION (MG. PER GM. ASH)[2]

City	No. Subject	Kidney		Liver	
		Cd	Zn	Cd	Zn
Adults					
Tokyo, Kyoto	18	6.03 ± 0.8	6.2 ± 0.67	0.54 ± 0.09	7.64 ± 1.45
Hong Kong	10	5.62 ± 0.95	5.47 ± 0.4	0.67 ± 0.15	5.9 ± 0.57
Bangkok	10	4.91 ± 1.47	8.190 ± 1.36	0.47 ± 0.24	7.99 ± 1.98
Taipei	9	4.15 ± 1.16	19.4 ± 4.27	0.71 ± 0.17	18.6 ± 3.0
United States[7]	70	2.91 ± 0.11	4.17 ± 0.12	0.17 ± 0.01	3.63 ± 0.11
Oxford (England)	26	> 1.0	..	± 0.3	..
Bern	9	2.26 ± 0.48	4.33 ± 0.84	0.19 ± 0.03	5.66 ± 0.78
Delhi, Lucknow	16	2.12 ± 0.31	3.29 ± 0.33	0.2 ± 0.04	3.54 ± 0.2
Nigeria*	14	1.7 ± 0.15	4.29 ± 0.57	0.1 ± 0.03	6.95 ± 1.15
Ruanda Urundi*	11	0.71 ± 0.15	4.15 ± 0.71	0.07 ± 0.04	4.68 ± 0.62
Fetus to term					
U. S. S. R.†	6(?)	0.0037	..	0.0075	..
Oxford (England)‡	5	0.002 (1 only)	..	0.004 (1 only)	..
St. Louis§	16	0	..	0	..
U. S. 45 da. to 11 yr.	30	0.066	..	< 0.05	..

Values expressed as mean ± standard error.
* Non city dwellers. 8 adults showed no Cd.
† Ref. 40. Fetuses 4-9 mo. Cd present in heart, muscle, lung, skin, pancreas, spleen, brain.
‡ Ref. 15. Cd present in 4 of 7 from 7 mo. fetus to 9 days old, in thyroid, cartilage, muscle, heart, gallbladder, pancreas or lung. Cd present in kidney and other organs in 1 child 3 mo. old, absent in 2 at 1 yr.
§ Ref. 41. Infants 0-45 da. old. 25 infants 0-45 da. old showed no Cd; 2 showed small amounts.[2]

provided that solubilities were similar. These trios have similar binding properties.[30]

PRACTICAL CONSIDERATIONS OF CLOSE COMPETITIONS AND POSSIBLE CHRONIC DISEASES

It is impossible to apply these considerations to clinical states at this time. However, one can exclude several competitions. High concentrations of a metal in a tissue place it under suspicion if the concentration approaches or exceeds that of an essential metal with the same characteristics. Small concentrations widely distributed probably are not very significant. Metals accumulating from industrial exposures can be neglected in this discussion.

There are only two pertinent close competitions with first transitional metals—copper, silver and gold, and zinc and cadmium. Copper enzymes are plentiful, but silver in tissues is so ubiquitous and concentrations are so small[1] that it probably causes little or no inhibition. Gold is present in few tissues, except those from Dallas.[1] Its function is not known.

Cadmium and zinc in kidney and liver have been measured in cases from England,[4] the United States, Switzerland, Japan, Formosa, Hong Kong, Old Delhi, Lucknow, Bangkok, and areas in Africa, including one where natives were relatively unexposed to modern civilization[2] (Table 1).

If cadmium is an external contaminant and not essential, accumulating in kidney during life, and if it forms complexes readily with zinc enzymes, displacing zinc (as it may do), we must look for cadmium-induced diseases as outcomes of renal or possibly hepatic zinc deficiencies.

All experimental studies on cadmium have shown evidence of toxicity and renal and hepatic accumulation.[33] It is an inhibitor of several mammalian enzymes,[34-38] among them dopa decarboxylase,[19] and is thought to activate other enzymes.[39, 40]

Voinar's brief report (in translation from the Russian) summarizes a considerable volume of work without many actual data.[40] While showing the accumulation of cadmium

in tissues of four subjects with age (4, 18, 40, 64 years) by spectrographic techniques, he finds, by the dithizone method, values in most tissues of human *embryos* ranging from 0.3 to 1.6 mg. per 100 Gm. ash. These amounts are barely within the lower limits of detection by spectrographic methods (4 μg./Gm.,[42] 20 μg./Gm.[15] or 50 μg./Gm.[41]) and might have been found by Tipton,[1] Koch[3] or Stitch[4] in the newborn but usually were not.

Margoshes and Vallee have found a stable cadmium protein in (civilized) horse kidney;[43] cadmium also has been found in the kidneys of domestic beef, rabbits and hogs.[33] It may come mainly from exposure of water to galvanizing grades of zinc, which contain 1 per cent or more.[44] Inference for essentiality or contamination will await analyses of natural materials unexposed to tap water, fertilizers and processed foods.*

LOOSE COMPETITIONS AND THEIR SIGNIFICANCE

The best known enzymatic competitions may be called *loose* competitions. Usually these vary with atomic number and concern transitional metals of all three groups horizontally. Thus, a metal on a metalloenzyme may be displaced by another that activates, inactivates or substitutes for it just as one metal with a higher stability constant can displace another on a simple chelate of a compound such as EDTA.

These relationships have been studied in model systems and enzymes. In vitro, some transitional metals will substitute for an essential one on an enzyme, with change of activity, but the significance in terms of disease is largely unknown. The V, Cr and Mn activities on hepatic lipid synthesis may be an example.[16] In these cases, considerable excesses are required, while, with a vertical displacement, reactions occur more closely to an equimolar basis.

Little is known about the other metals found in human tissues: aluminum, tin, lead and bismuth. There are extensive data on lead toxicity: "permissible limits" showing no overt effects have been established for human beings; most tissues contain it in relatively large amounts.[41] Tin is toxic for animals only in large amounts; a considerable quantity is ingested by civilized human beings from food (as much as 420 mg. per day if canned food is eaten exclusively), and some is retained.[41] Monier-Williams[31] has speculated that some metals considered to be nontoxic may possibly combine with constituents of the blood cells or plasma and affect some of their functions, especially tin, which readily forms lakes with organic pigments. Bismuth is toxic to animals in relatively large amounts, causing renal damage; relatively little has been found in tissues.[41] Direct aluminum toxicity is not known, nor have deficiency states been produced in rats.

CHRONIC DISEASES POSSIBLY LINKED TO TRACE METAL IMBALANCES

Areas in which populations unexposed to modern civilization can be studied are fast disappearing, and chronic diseases are moving in. Therefore, it is hard to obtain demographic data on abnormal trace metals. Much basic experimental data is required before metals can be implicated as causes of specific diseases.

ATHEROSCLEROSIS

The work of Curran and his colleagues suggest a hypothesis that hypercholesteremia, and perhaps atherosclerosis, may be influenced by metals affecting cholesterol and fatty acid synthesis by liver (see p. 216). Human plasma cholesterol levels have been shown to fall during several days of intravenous $CaNa_2$ EDTA therapy, associated with marked zincuria and increased excretion of Cd, V, Pb and Mn.[46]

Abnormally high rates of cholesterol synthesis could result from chromium excess or vanadium deficiency. However, the concept of an etiological chromium excess must consider four factors: (1) chromium does not accumulate in human liver but in lung, although widely distributed in all tissues;[41] (2) Cr decreases markedly with age in the first two decades;[41] (3) the stability constants of Cr^{++} in known chelates are less than those

* There is also a definite amount of cadmium in certain vinyl plastics,[45] and in canned fruit juices, fresh spinach, canned oysters, dried milk[33] and bottled drinks.[19] Our local tap water has 0.4 μg./L.

of many transitional metals, although probably greater than those of vanadium; (4) urinary chromium levels were unchanged after intravenous $CaNa_2EDTA$.[46] The possibility of an etiological vanadium deficiency needs further investigation.

Cancer*

There have been several reports on the relationship of tissue metal levels to sarcomata and carcinomata.[47-49] Whether these changes are cause or effect is unknown. According to the hypothesis of Warburg,[50] tumor growth is initiated by a change from oxidative to anaerobic metabolism. As known oxidases are metalloenzymes, displacement or deficiency of metal causing oxidase inactivation could produce this change.

Rheumatoid Arthritis and Other "Collagen" Diseases

While most of the drugs affecting these diseases are metal-binding agents,[6, 19, 51]† no direct relationship has been established between metals and disease, except for the unexplained alleviative effect of gold complexes. This deserves investigation. "Hydralazine disease" may be related to a metal deficiency.[6, 24]

Allergies

Late toxic drug reactions are often produced by metal-binding drugs.[6] This curious coincidence deserves investigation.

Hypertension

A trace metal imbalance is deduced from the indirect evidence (a) that all antihypertensive agents not acting on nerves appear to be metal-binding agents, some 24 being active in rats and 6 in man;[52] (b) that divalent metal EDTA chelates with stability constants *less* than that of copper are acutely depressor in rat renal hypertension, not affecting pressures of normal rats;[53] (c) that human hypertensive urine contains increased amounts of Zn, Mn, V, Pb, Ni and Sn, and a fortyfold increase in Cd, with normal Mo, Ti, Cr and Ag;[10] (d) that the pressor peptides pherentasin, isolated from hypertensive blood, and angiotonin are inactivated by some metal-binding agents and certain metals, suggesting the presence of a metal (copper?) for required activity;[54] and (e) that pherentasin disappears from blood when patients are treated with oral hydralazine, a chelating agent.[55]

Others

There are many other conditions responding to strong metal-binding drugs, where metal imbalance can be inferred indirectly but without direct evidence. For example, scleroderma responds to Na_2EDTA.* Probably most antibiotics are metal-binding agents, as are many antithyroid drugs (for copper, especially), many analgesics, some tranquilizers and many other specific drugs.[6]†

SUMMARY

A critical analysis has been made of the trace metals found in American human tissues from the viewpoint of their possible metabolic activities and relationships to disease. Along with essential Mn, Co, Cu and Zn, one cannot exclude Mo, Rb, Sr, Ba, V, Cr, Ni and, possibly, Ti and Al, as having normal biological roles. These metals show various metabolic activities.

On the other hand, the "abnormal" metals Ag, Au, Cd, Sn, Pb and Bi appear to fulfill no normal biological role and probably are environmental contaminants. Cd and Sn increase with age. Based on the laws of chelation, specific metal displacements on metalloenzymes might be expected between Ca, Sr and Ba, between Zn, Cd and Hg, and between Cu, Ag and Au, just as Mo is displaced by W, and K is displaced by Rb and Cs. Loose competition of metal for metal according to atomic number occurs in the transition groups, and displacements can occur in the presence of excesses.

Chronic diseases possibly linked to trace metal imbalances are discussed.

* Editor's Note: Dr. Furst has a detailed discussion of the relationship of metals to cancer on pages 336 and 346.

† Editor's Note: Dr. Schubert discusses metal-binding and salicylate action on page 325.

* See note, Appendix, page 353.

† Editor's Note: Most of these have received more detailed comment in other presentations at this Symposium. See Index.

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Serum Cholesterol Levels in Rats Fed Thirteen Trace Elements [1]

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ABSTRACT To ascertain differences in serum cholesterol levels as possibly affected by trace elements in the drinking water, rats were given soluble salts of zirconium, vanadium, niobium, chromium, nickel, cadmium, germanium, tin, lead, arsenic, and antimony at 5 ppm and selenium and tellurium at 2 ppm from the time of weaning until 11 to 30 months of age. The feeding of chromium at 1 ppm was associated with suppressed levels in males but not in females; 5 ppm appeared to be required for this effect in females. The lowest serum cholesterol levels were observed in groups given niobium, chromium and nickel, the highest in groups fed tellurium, with all animals receiving 1 ppm chromium. Significant differences in values of the 2 sexes appeared for 7 elements. It is possible that chromium, nickel and niobium exert anti-cholesterogenic properties, whereas tellurium may be cholesterogenic.

Mean circulating cholesterol levels in human beings tend to rise with age. This tendency is exaggerated in persons living in industrialized countries and is less evident in more primitive societies. The possibility arose that some abnormal trace element to which civilized man is exposed and which accumulates in his tissues with age might influence the synthesis of cholesterol or interfere with its catabolism. Conversely, some modern nutritional practices might promote marginal deficiency of an essential trace element on which homeostasis of cholesterol depends.

Curran (1) showed that rat liver, in vitro and in vivo, synthesized cholesterol less well in the presence of vanadyl ions and better in the presence of trivalent chromium and manganous ions than liver unexposed to trace elements. Other trace metals in the first transitional series were relatively inert. Curran's rats were fed a commercial ration, which at that time was marginally deficient in chromium (2). The action of chromium feeding in stabilizing serum cholesterol at a low level in male rats has been demonstrated (3, 4); associated with this effect was virtual abolition of spontaneous aortic plaques and accumulation of aortic lipids (4).

Studies on the life-term effects of a number of trace elements given in low doses to rats and mice (5) have provided an opportunity to measure serum cholesterol and glucose levels. The present report concerns 13 trace elements fed to rats from the time of weaning. Differences in serum cholesterol associated with element and age are shown.

METHODS

The environmental conditions in the laboratory, the precautions taken to avoid metallic contamination, the low metal diet and the double-deionized drinking water used have been reported in detail (6, 7). The trace element content of the diet, which was composed of seed rye, powdered skim milk and corn oil, and the estimated total intakes, are shown in table 1. To the basic drinking water, which contained zinc (50 ppm), manganese (10 ppm), copper (5 ppm), cobalt (1 ppm), molybdenum (1 ppm) and chromium (1 ppm), was added one of the following at 5 ppm element; zirconium sulfate, sodium niobate, antimony potassium tartrate, sodium germanate, stannous chloride, sodium arsenite, vanadyl sulfate, nickelous acetate, cadmium acetate, lead acetate. Sodium

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TABLE 1

Trace element content of diet and drinking water and estimated daily intakes [1,2]

	Diet	Water	Total
	μg/g	μg/g	μg/100 g body wt/day
Zirconium	2.66	5	50.96
Vanadium	3.24	5	54.44
Niobium	1.62	5	44.72
Chromium	0.16	0	0.96
Chromium	0.16	1	7.96
Chromium	0.16	5	35.96
Nickel	0.44	5	37.64
Cadmium	0.14	5	35.70
Germanium	0.32	5	36.92
Tin	0.28	5	36.68
Lead	0.20	5	36.20
Arsenic	0.46	5	37.76
Antimony	nd [3]	5	35.0
Selenium	0.05	2	14.30
Tellurium	0.16	2	14.96

[1] Based on 6 g food and 7 g water ingested/100 g body weight/day.
[2] These intakes are approximate but are comparable. Gains in weight of young rats were similar, weights of adult animals remained fairly constant, and variations among groups were not observed. The intake of water was measured on control rats for one year; marked variations in other groups were not evident.
[3] Not detected.

selenate and sodium tellurite were added at 2 ppm element; sodium selenite at 3 ppm.[3] In addition, the basic water was given without chromium or with 5 ppm as the acetate.

Random-bred pregnant female rats were purchased from a supplier[4] and their offspring weaned at 21 to 23 days. Groups of 52 or more of each sex, four to a cage, were given one of the trace elements until natural death occurred. Each group was placed in separate wooden racks, and cages were not interchanged between groups.

In addition, 4 generations of rats were bred without exposure to chromium other than the small amount in the diet, as reported (8). The second and fourth generations were used in these experiments. Thirty brood females obtained from the supplier, which had been fed a diet containing adequate chromium, were also studied. Their offspring were not believed to be deficient in chromium according to analyses of tissues of similarly bred animals (9).

Blood was obtained from the tail of the warmed rat by cutting with a razor blade, and centrifuged. Serum cholesterol was measured by the method of Huang et al. (10), using a premixed reagent and a Berkeley Medical Instrument spectrophotometer;[5] a few were measured by the method of Abell et al. (11). Duplicate and replicate analyses agreed within 3%.

Analyses of the diet for trace elements were made by a number of standard methods, colorimetric for zirconium, vanadium, niobium, chromium, nickel, germanium, arsenic, tin, lead and selenium, and atomic absorption spectrophotometric for chromium, cadmium, antimony and tellurium.

RESULTS

Serum cholesterol levels in rats given various amounts of chromium are shown in table 2. Sex differences appeared. Males fed 1.0 to 5.0 ppm in drinking water had lower mean values than those given none in water. Females given 5.0 ppm in water or about 2 ppm in food and water had lower values than those given 1.0 ppm or less. The difference in male and female values at 1.0 ppm was significant ($P < 0.001$). According to these data, female rats apparently require more chromium than males in order that cholesterol in serum be suppressed. Fourth generation chromium-deficient young rats of both sexes had higher values than second generation young rats which were assumed to be less deficient.

In table 3 are the mean serum cholesterol levels of rats fed 13 trace elements in drinking water, arranged according to the values of males. Again sex differences appeared in 7 cases. Male values were higher in the groups given vanadium, chromium, selenium ($P < 0.025$), and in the controls without chromium ($P < 0.05$). Female values were higher in those given cadmium ($P < 0.05$), germanium and tin ($P < 0.001$). More or less consistently low values were noted in the groups of both

[3] In a previous publication on the effects of selenite and selenate on mice and rats (14), the concentration of selenium in the selenite given in water was erroneously reported as 2 ppm. The supplier of sodium selenite (Nutritional Biochemicals Corporation, Cleveland) stated in a letter that this product was $Na_2SeO_3 \cdot 5H_2O$. Nearly 2 years later the compound was found by D. V. Frost, in our laboratory, to be anhydrous, and was then so admitted to be by the supplier. As the hydrated form contains 45.7% selenium and the hydrated form 30%, the rats and mice given selenite actually received 3 ppm selenium.
[4] Blue Spruce Farms, Altamont, New York.
[5] Berkeley Medical Instruments, New England X-Ray and Electronic Equipment, Brookline, Massachusetts 02146.

TABLE 2

Serum cholesterol levels in rats given chromium

	Chromium	Males [1]			Females [1]		
		No.	Age		No.	Age	
	ppm		*days*	*mg/100 ml*		*days*	*mg/100 ml*
Young [2]							
F_2	0	11	204	86.0 ± 3.9 [3]	12	204	78.5 ± 2.5
F_4	0	8	115	114.0 ± 5.0 [4]	10	127	109.6 ± 2.7 [4]
Mature							
	0	12	761	122.9 ± 8.2	12	761	94.5 ± 11.2
	1	23	662	77.5 ± 2.1 [5]	19	660	116.0 ± 6.0
					7	689	108.6 ± 4.2
	2 ±				30	250 [6]	83.3 ± 1.7 [7]
	5	12	405	86.2 ± 3.2 [8]	12	405	72.1 ± 5.3 [8]
	5	12	510	77.0 ± 6.6 [8]	8	709	63.0 ± 4.2 [9]

[1] Male and female values differ significantly: 2.0 ppm group, $P < 0.025$; 1.0 ppm group, $P < 0.001$; 5.0 ppm groups, $P < 0.025$.
[2] F_2 and F_4 are rats bred from increasingly deficient mothers.
[3] SE.
[4] Differs from F_2 group, $P < 0.001$.
[5] Differs from mature group, no chromium, $P < 0.001$.
[6] Breeders purchased while pregnant, food containing chromium.
[7] Differs from mature groups, 1.0 ppm 660 and 689 days old, $P < 0.001$.
[8] Differs from 2.0 ppm group, $P < 0.01$.
[9] Differs from 2.0 ppm group, $P < 0.001$.

TABLE 3

Serum cholesterol levels in mature rats given various trace elements

	Males			P value [1]	Females			P value [1]
	No.	Age			No.	Age		
		days	*mg/100 ml*			*days*	*mg/100 ml*	
Germanium	12	557	63.4 ± 3.2 [2]	< 0.001	12	578	107.6 ± 5.9	ns [3]
Nickel	12	342	75.3 ± 2.6	ns	12	329	75.3 ± 3.8	< 0.001
Niobium	12	849	75.7 ± 2.7	ns	11	849	78.6 ± 4.8	< 0.001
Controls (Cr 1)	23	662	77.5 ± 2.1	—	19	660	116.0 ± 6.0	—
Tin	12	520	82.5 ± 3.4	ns	12	580	105.3 ± 3.5	ns
Chromium	12	405	86.2 ± 3.2	< 0.02	12	405	72.1 ± 5.3	< 0.001
Lead	14	695	86.6 ± 6.5	ns	12	760	103.7 ± 8.5 [4]	ns
Cadmium	10	750	88.6 ± 8.7 [4]	< 0.05	12	750	113.0 ± 9.0 [4]	ns
Zirconium	12	893	89.7 ± 5.6	< 0.01	12	893	100.7 ± 9.0	ns
Arsenic	12	804	91.0 ± 10.1	< 0.05	13	806	109.5 ± 7.4	ns
Vanadium	11	761	91.6 ± 5.1	< 0.005	10	761	67.9 ± 9.2	< 0.001
Antimony	10	790	97.6 ± 4.9	< 0.001	10	790	97.0 ± 5.6	< 0.01
Selenium (VI)	12	445	109.6 ± 6.1	< 0.001	12	445	90.4 ± 5.0	< 0.005
Selenium (IV)					12	445	90.2 ± 3.5	< 0.005
Tellurium	12	405	111.4 ± 3.0	< 0.001	12	452	110.4 ± 9.9	ns
Controls (no Cr)	12	761	122.9 ± 8.17 [4]	< 0.001	12	761	94.5 ± 11.19 [4]	< 0.05

[1] Significance of differences from controls.
[2] SE.
[3] Not significant.
[4] Method of Abell et al. (11), previously reported (4), for comparison.

sexes given niobium, chromium, and nickel, and high values in those fed tellurium.

Compared with the controls given 1.0 ppm chromium, values for males were especially elevated in groups fed tellurium, selenium, antimony and no chromium and slightly elevated in the groups fed zirconium and vanadium. Cholesterol levels in males given germanium were suppressed.

Female control levels were the highest of any group. Other elevated values (> 100 mg/100 ml) occurred in the groups given zirconium, cadmium, germanium, tin, lead, arsenic and tellurium. Low values (< 80 mg/100 ml) were found in the groups given vanadium, niobium, chromium and nickel.

Because two of these elements, chromium and vanadium, have been shown to

affect cholesterol metabolism in vitro and in vivo (1, 4), comparisons were made as to significant differences in circulating cholesterol between rats fed these metals and other elements (table 4). In relation to the chromium-fed group, levels were higher in male rats given antimony, selenium and tellurium and lower in those fed niobium, nickel and germanium. In females, mean values were higher in rats given all of the elements except niobium and nickel.

Comparisons of levels of male rats fed vanadium with those given other elements showed significantly elevated values only in rats fed selenium and tellurium. Significantly depressed values were observed in those given niobium, nickel and germanium. In females, all values, except those

TABLE 4

Significance of differences in serum cholesterol levels of rats given various trace elements compared with those given chromium and vanadium

	Males				Females			
	Chromium		Vanadium		Chromium		Vanadium	
	Difference [1]	P value [2]	Difference [1]	P value [2]	Difference [1]	P value [2]	Difference [1]	P value [2]
Controls, no Cr	+	< 0.001	+	< 0.001	+	< 0.05	+	< 0.05
Controls, 1 ppm Cr	−	< 0.02	−	< 0.005	+	< 0.001	+	< 0.001
Zirconium		ns [3]		ns	+	< 0.01	+	< 0.01
Vanadium		ns				ns		
Niobium	−	< 0.01	−	< 0.005		ns		ns
Nickel	−	< 0.01	−	< 0.005		ns		ns
Cadmium		ns		ns	+	< 0.001	+	< 0.001
Germanium	−	< 0.001	−	< 0.001	+	< 0.001	+	< 0.001
Tin	−	ns		ns	+	< 0.001	+	< 0.001
Lead		ns		ns	+	< 0.001	+	< 0.005
Arsenic		ns		ns	+	< 0.001	+	< 0.001
Antimony	+	< 0.02			+	< 0.01	+	< 0.02
Selenium	+	< 0.001	+	< 0.001	+	< 0.01	+	< 0.02
Tellurium	+	< 0.005	+	< 0.005	+	< 0.001	+	< 0.005

[1] Plus sign indicates that the value is larger; minus sign, smaller than that for chromium or vanadium.
[2] Significance of difference in the value from that of chromium or vanadium (see table 2).
[3] Not significant.

TABLE 5

Changes with age in serum cholesterol in rats fed zirconium, niobium, antimony, lead and cadmium

Element	Males			P value [1]	Females			P value [1]
	No.	Age			No.	Age		
		days	*mg/100 ml*			*days*	*mg/100 ml*	
Zirconium	12	270	69.0 ± 3.8 [2]		12	270	76.0 ± 1.8	
	12	893	89.7 ± 5.9	< 0.005	12	893	100.7 ± 9.0	< 0.01
Niobium	12	166	69.3 ± 3.4		12	228	81.1 ± 3.7	
	10	655	70.3 ± 2.89	ns [3]				
	12	849	75.7 ± 2.7	ns	11	849	78.6 ± 4.8	ns
Antimony	12	125	76.7 ± 2.4		12	161	86.3 ± 4.4	
	10	790	97.6 ± 4.9	< 0.001	10	790	92.6 ± 5.6	ns
Lead	11	300	72.3 ± 4.74					
	12	510	79.3 ± 6.48	ns	12	510	103.4 ± 7.12	
	14	695	86.6 ± 6.7	ns				
	12	750	74.0 ± 10.03	ns	12	750	103.7 ± 8.53	ns
Cadmium	10	300	76.1 ± 4.74					
	10	485	85.6 ± 2.3	< 0.05	10	485	97.0 ± 8.9	
	12	510	67.6 ± 2.81	ns	12	510	86.5 ± 9.80	ns
	12	750	88.6 ± 8.70	ns	12	750	113.0 ± 8.98	ns
Arsenic [4]	12	770	91.6 ± 10.5					
	12	804	91.0 ± 10.1	ns				

[1] Significance of difference from first value in each group.
[2] SE.
[3] Not significant.
[4] For comparison of repetitive analyses on different rats of same group.

of animals given niobium and nickel, were significantly elevated.

Changes in circulating cholesterol levels with age are shown in table 5. No increase was found in animals fed niobium, lead or cadmium, nor in females given antimony. Increases occurred in rats of both sexes fed zirconium and in males fed antimony. Data on chromium (table 1) show some decrease with age, especially in females.

DISCUSSION

The rat is a poor animal for the study of experimental hypercholesteremia, requiring special diets and the feeding of cholesterol and saturated fats to develop high serum levels. The present study concerns adequate diets and unsaturated fats (corn oil), and high serum levels were only noted occasionally. Nevertheless, trends and tendencies appeared which might turn out to be significant in more susceptible mammals.

On this basis, these data indicate that trivalent chromium given to rats in drinking water was associated with lowered serum cholesterol levels, as shown previously (4), that chromium deficiency in young rats was associated with elevated levels, and that female rats appeared to require larger doses for this effect to appear than males. The data show that, in general, higher levels occurred in male rats fed the metalloid or nonmetallic elements in the "A" groups of the Periodic Table — arsenic, antimony, selenium and tellurium — than in those fed metals in the transitional or "B" groups — vanadium, niobium, nickel and cadmium — or heavy metals in the "A" groups — tin and lead. Female rats, however, also had higher levels when they were given those elements situated in the right of the Periodic Table, beginning with cadmium, and lower levels when fed elements situated in the left or "B" groups.

To test this impression, the elements were arranged in order of position in the Periodic Table from left to right and assigned numbers from 2 (zirconium) to 27 (tellurium). Paired rank correlations with serum cholesterol levels were calculated. For males, $r = +0.486$ ($P \sim 0.05$); for females, $r = +0.622$ ($P < 0.01$). Although the list of elements studied is incomplete, those on the right of the Table were more likely to be associated with high serum cholesterol levels than were the transitional metals on the left. There was no significant correlation with atomic number. In the experience of this laboratory, which will be reported, the elements on the right of the Table were more likely to exert innate or overt toxicity in mice and rats than were the transitional metals.

The data on changes in serum cholesterol with age are incomplete. They suggest, however, that age-linked increases can occur in marginal chromium deficiency, in rats of both sexes fed zirconium and in males fed antimony. They suggest that such increases may not occur in rats given niobium, lead and cadmium.

When the groups fed tellurium were compared with the others, as in table 3, significant differences appeared largely in males. Probabilities (P) of the differences being due to chance of the order of <0.005 occurred in the zirconium, niobium, chromium, nickel, germanium, tin, lead and control groups, of < 0.01 in the cadmium group, of < 0.025 in the antimony group and < 0.05 in the arsenic group, all values being smaller. Only the selenium group did not so differ. In females, the vanadium, niobium, chromium, and nickel groups differed ($P < 0.005$), as possibly did the selenium group ($P < 0.05$), values being smaller than those of the tellurium group.

Because it was possible that differences in cholesterol levels might be related to factors other than the trace elements fed to the animals, attempts were made to avoid unknown influences as far as practicable. Males and females were strictly comparable. Several groups of animals were bred and observed simultaneously as follows: Controls (no chromium), chromium (5 ppm), cadmium and lead; controls (1 ppm chromium), arsenic, germanium and tin; lead (males), zirconium, niobium and antimony; chromium (5 ppm), vanadium, nickel, selenium and tellurium. Each experiment required 4 years for completion (5). As facilities were limited to 1,000 rats, additional groups were bred and started, usually in the fall or winter, when previous groups were partly depleted through death. Analyses

of sera were made during the summers on several groups at weekly intervals. The diet was as uniform as feasible, although annual variations in the trace element content of seed rye obtained from the same area each year were possible, owing to annual variations in rainfall. No consistent changes were found among the groups analyzed at each interval which could be attributed to unknown factors.

The values obtained by the method of Abell et al. (11) in the first series (4) were compared with those obtained by that of Huang et al. (10) which was an essentially similar but more simple method. Mature males fed lead in the first series (4) had 79.3 ± 6.5 mg cholesterol/100 ml; those in the second had 86.6 ± 6.5 mg /100 ml. Mature males fed chromium had 77.0 ± 6.6 mg/100 ml in the first series and 86.2 ± 3.2 mg/100 ml in the second. Apparently the 2 methods gave values in the same ranges for 2 sets of animals fed the same metals.

Because all these animals, except those in one control group, were given chromium, it is evident that if an element influenced serum cholesterol levels, the effect was either additive to or antagonistic to that of chromium. Additive effects in one sex might be postulated for niobium and nickel; antagonistic effects for tellurium, selenium, antimony, vanadium and zirconium.

Therefore, it is possible that a common property of nickel, niobium and chromium may be to influence serum cholesterol of rats at low levels, and that tellurium in some way may raise it. The lowest standard deviations, and therefore individual variations, were found among analyses of sera from rats fed these metals. Tissue levels of the various elements will be or have been reported (5). Application of these data to the chromium deficiency noted in samples from the adult population of the United States (9) and the excess of tellurium (12, 13) deserve study.

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Interactions of Trace Metals in Rat Tissues. Cadmium and Nickel with Zinc, Chromium, Copper, Manganese[1,2]

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ABSTRACT In order to ascertain what effect one essential trace metal might have on tissue concentrations of another, rats of the Long-Evans strain were born and raised in an environment controlled as to contaminating trace metals until natural death, and fed a diet of rye flour, dry skim milk and corn oil. One group of 60 was given plain deionized water for a year and then killed. Two groups of 100 or more equally divided as to sex were given a basal water containing added zinc (50 ppm), manganese (10 ppm), chromium (5 ppm), copper (5 ppm), cobalt (1 ppm) and molybdenum (1 ppm) as soluble salts, or this basal water without chromium. Two other groups were given cadmium (50 ppm) or nickel (5 ppm) in the chromium-containing basal water. Five organs from each rat were ashed and analyzed for zinc, copper, manganese, chromium and cadmium by atomic absorption spectrophotometry. Compared with the group given plain water, zinc was markedly increased in all organs of rats given the basal water, copper was increased in liver, lung and spleen, and chromium was increased in liver and lung. In the group given nickel and the basal water, zinc was increased in all organs but liver, copper was increased in spleen, manganese was increased in lung and kidney, and chromium was increased in liver and kidney, compared to the plain water group. Chromium was especially concentrated in spleen and heart, copper in heart and kidney and manganese in liver of rats given plain water. When rats were given basal water plus cadmium (50 ppm), there was a marked increase in zinc in all organs but spleen, and a very marked increase in cadmium in all organs from very low levels (0.03 ppm, wet weight when no cadmium was given). Consumption of basal water plus cadmium was also associated with some depression of heart chromium, an increase in kidney and liver manganese levels and an increase in renal copper. From observations on the effect of increasing the intake of trace metals via water on the tissue levels of the respective trace metals, the following conclusions are suggested: a) that zinc at a level of 22 μg/g diet and copper at 1.4 μg/g diet were adequate for growth but inadequate for filling all binding sites and stores, b) that manganese at 13 μg/g diet may be insufficient for repletion of splenic manganese stores, c) that chromium at 0.14 μg/g diet produces partial chromium deficiency, and d) that cadmium at 0.07 μg/g diet does not cause cadmium accumulation with age and is thus in balance. The data also demonstrate interactions of cadmium with zinc, manganese and copper, and interactions of nickel with copper and manganese in some organs. J. Nutr. *104*: 167–178, 1974.

INDEXING KEY WORDS mineral metabolism · trace minerals · metallic micronutrients

During the course of exposing rats for life to each of 14 trace metals and metalloids in low doses in drinking water, in an environment designed to exclude metallic contamination (1–3), we have collected thousands of tissues for analysis. The purpose of that investigation was to ascertain which metals accumulated in mammalian

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[1] See NAPS document #02277 for 15 pages of supplementary material. Order from ASIS/NAPS, c/o Microfiche Publications, 305 E. 46th St., New York, N. Y. 10017. Remit in advance for each NAPS accession number $1.50 for microfiche or $5.00 for photocopies. Make checks payable to Microfiche Publications.

[2] Supported by Public Health Service Research Grant ES 00095-12A1, Ciba-Geigy Corporation and Cooper Laboratories, Inc.

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tissues and which did not, and to evaluate recondite toxicity in terms of growth rates, survival, longevity and chronic disease. Although in several cases accumulation in rat tissues was demonstrated (4, 5), we had not studied the effects of one metal bound to tissues on another (6, 7).

In the cases of the essential trace metals chromium, manganese, iron, copper and zinc, one can assume that a certain number of binding sites are specific and available for each metal, and that these sites are filled when the animal is consuming nutritionally adequate or luxus amounts. In man, no accumulation of essential trace metals with age has been found (8–15), attesting to excellent homeostatic mechanisms. In rats, we have found no marked accumulations with age. On the other hand, when intakes are marginal or low, an assumption appears valid that some binding sites are either unfilled, or that the enzymes on which the metal is a cofactor are reduced in amounts when tissue concentrations are also low.

This report has two phases. In the first phase, the effects of luxus amounts of chromium, manganese, copper and zinc added to drinking water of rats for life were compared with those of metals naturally in the diet and not added to water, in respect to tissue concentrations. In the second phase, the effects of cadmium and nickel added to drinking water on tissue concentrations of these metals were evaluated. Nickel was chosen because it is apparently inert in rats and mice; cadmium, a toxic metal, was chosen because it accumulates in certain tissues (16, 17), and in the human kidney it accumulates with age as does zinc (10).

MATERIALS AND METHODS

Rats of the Long-Evans (BLU:(LE)) strain were born in our laboratory of random-bred females obtained pregnant from the supplier.[4] At weaning time litter mates were divided, four to a cage, into groups of 50 or more of each sex. They remained in cages of the same group on the same rack until natural death from 2 to 4 years later. They were handled as little as possible, except for weighing and cleaning cages. Cages were of solid acrylic plastic with stainless steel covers. The laboratory was specially built to avoid metallic contamination. Electrostatically precipitated forest air was supplied under slight positive pressure. The location, on a remote hill, and the environmental conditions, have been described in previous publications (1–3).

The diet was relatively low in most trace elements (18). It was composed of whole seed rye flour (60%), dried skim milk (30%), corn oil (9%), iodized salt (1%), with added iron and vitamins. Intakes of essential trace metals per 100 g body weight per day[5] are calculated in table I.

Drinking water obtained from a forest spring was doubly deionized to a resistance of 5 million ohms. Trace metals were below the limits of detection. One group of rats was given this water for a year, and then killed for analysis of tissues. The composition of the water given to the other groups and the daily intakes of trace metals are shown in table 1. The basal water contained chromium, manganese, cobalt, copper, zinc and molybdenum (5) as simple complexes. One group of rats received basal water from which the chromium had been omitted. One received basal water plus 5 ppm nickel and one received basal water plus 50 ppm cadmium.

There were two control series. The first, designated basal water-3, was used in the comparison of plain water and nickel-supplemented rats. The second, designated basal-4, was used in the studies on cadmium and on chromium. Intake of water was calculated from a year's measurement (19) and of food estimated from consumption.[5]

Heart, lungs, liver, kidneys and spleen of each rat dying naturally were removed at autopsy. Portions were taken for microscopic sections and the remainder frozen and stored in polyethylene bottles. Tissues were later ashed in muffle furnaces at 400° in silica dishes after drying to constant weight at 110°. The ash was acidified with distilled nitric acid, dissolved in 25 or 50 ml deionized water and stored at room temperature in polyethylene bottles until analyzed up to 5 years later.

Absorption of trace metals by polyethylene was investigated at length. Solu-

[4] Blue Spruce Farms, Altamont, N. Y.

[5] Food consumption for 194 adult rats was measured for a month and the intake calculated. The inevitable wastage was estimated at 20%.

TABLE 1

Daily intakes by rats of ~~trace metals~~ from food and water, μg/100 g body weight

Metal	Food		Basal water[3]		Total intake, food & water	
	Conc.[1]	Intake[2]	Conc.[4]	Intake[4]	P. water[6]	Basal water[3] (basal-3 & basal-4)
	μg/g	μg	μg/g	μg	μg	μg
Chromium	0.14	0.84	5	35	0.8	35.8[7]
Manganese	12.7	7.62	10	70	7.6	77.6
Copper	1.36	8.16	5	35	8.2	43.2
Zinc	22.3	133.80	50	350	133.8	483.8
Nickel	0.44	2.64	0[5]	0[5]	2.6	2.6[8]
Cadmium	0.07	0.42	0[5]	0[5]	0.4	0.4[9]
Cobalt	0.4	2.40	1	7	2.4	9.4
Molybdenum	0.25	1.50	1	7	1.5	8.5
Selenium	0.05	0.30	0	0	0.3	0.3
Vanadium	3.24	19.44	0	0	19.4	19.4

[1] Analysis by atomic absorption spectrophotometry (8–11), except molybdenum (12) and vanadium (13) by colorimetry and selenium (14) by photofluorimetry. [2] Calculated on estimate consumption of food by adult rats at 6 g/100 g body weight/day. [3] Basal water providing luxus amounts of trace metals. [4] Added to water. [5] Calculated on measured consumption of water by adult rats at 7 ml/100 g/day (19). This amount may vary individually and by season. [6] Plain water doubly deionized, assumed to contain no trace metal ions. [7] When the basal water minus Cr was given, total chromium intake was 0.8. [8] When the basal water plus 5 ppm nickel was given, nickel intake from water was 35 μg and the total nickel intake was 38 μg. [9] When the basal water plus 50 ppm cadmium was given, cadmium intake from water was 350 μg and the total cadmium intake was 350 μg.

tions of different strengths either frozen or standing at room temperatures were analyzed monthly. Bottles in which solutions of ash had been stored for several years were analyzed by neutron activation analyses.[6] Trace metals in small amounts enter the interstices of polyethylene rather rapidly, but these apparently become saturated. A solution of low concentration will therefore show larger percentage changes than one of high concentration. For tissue ash with its large matrix, the loss from absorption has been considered negligible.

Organs from animals of the same sex and similar ages were pooled after thawing in order to obtain samples of large enough size. The number of animals pooled was partly governed by the number dying at any interval. There was one pool each of 12, 11 and 10 animals, two pools of 9, four of 8 and 7, eight of 6 and 33 of 5 or less. Subsequently, data for statistical evaluation were weighted according to the number of animals pooled. Weighted averages were compared by Student's *t* test.

Analyses were carried out by means of atomic absorption spectrophotometry.[7] Reproducibility of the method at various concentrations was usually good.[8] Corrections for background absorption and light scatter were made as required, at nearby nonabsorbing lines. For cadmium measured at the 2288 Å line, a nonabsorbing lead line at 2204 Å was used. Corrections were also applied for reagent blanks, as required, and for a tissue matrix which produced light scatter. All measurements were made in duplicate or triplicate.

A three-slot Boling burner with air–acetylene flame was used for chromium, cadmium, manganese, copper and nickel. For zinc, a nitrous oxide burner with air–acetylene flame was rotated 45° to the optical axis in order to reduce sensitivity. When low values were found, analyses were made by the method of additions.

[6] Dr. David M. Linekin, The Massachusetts General Hospital, Boston.

[7] Perkin-Elmer 303, The Perkin-Elmer Corp., Norwalk, Conn.

[8] Relative standard deviations for 5 to 15 replicate determinations in the ranges of actual concentrations encountered were as follows:

Cr	0.50 ppm	±1.7%	*Cd*	0.8 ppm	±1.9%
	0.33 ppm	±2.0%		0.4 ppm	±1.7%
	0.22 ppm	±3.6%		0.2 ppm	±2.4%
	0.11 ppm	±5.1%		0.1 ppm	±4.7%
	0.025 ppm	±9.3%		0.5 ppm	±5.8%
	0.01 ppm	±25.6%		0.01 ppm	±14.8%
				0.005 ppm	±17.1%
Cu	5.9 ppm	±1.7%	*Mn*	0.8 ppm	±1.5%
	2.3 ppm	±2.5%		0.1 ppm	±1.7%
	0.7 ppm	±2.8%		0.05 ppm	±4.9%
	0.1 ppm	±4.3%		0.01 ppm	±12.4%
	0.5 ppm	±4.7%			
Zn	4 ppm	±0.4%	*Ni*	2.0 ppm	±2.6%
	1.6 ppm	±0.5%		0.2 ppm	±6.5%
	0.8 ppm	±1.2%		0.14 ppm	±11.2%
	0.4 ppm	±1.9%		0.08 ppm	±12.8%
	0.2 ppm	±2.5%			
	0.1 ppm	±5.0%			

The practical limits of detection for the metals in solution of ash and in standard solutions were: copper, chromium, manganese, cadmium 0.01 μg/ml; nickel 0.05 μg/ml. Limits of detection of zinc were not reached, because of its relatively high concentration in all samples.

In order to evaluate trends when scatter of individual data was wide, means of means were calculated and their differences tested by Student's *t*. For major trends, chi-square analysis was also used. In the tables, a dash indicates $P > 0.05$. Trends were also evaluated by the sign test, the numbers of pairs of values which differ in the same direction (20). For ascertaining accumulation with age, linear regression analysis and the correlation coefficient (r) were used.

RESULTS

Accumulation with age. Although homeostatic mechanisms control the concentrations of most of the essential trace metals in the tissues of man and other mammals, there is a possibility that dietary levels were either inadequate or so large as to cause accumulation with age in these rats. Therefore, the individual data on all rats of both sexes given the basal water (basal-3, basal-4, no chromium and nickel supplemented) were tabulated, examined and plotted according to age at death (not shown), in order to detect age-linked trends.

No age-linked changes appeared in any organ for chromium or manganese. Scatter was wide for zinc and copper in heart, lung, kidney, and spleen, without apparent and significant trends. Hepatic copper, however, increased with age of death; linear regression analysis showed a slope of +4.64 μg/g of liver, dry weight/100 days and a correlation coefficient (r) of +0.51 ($P < 0.001$). Hepatic copper of younger animals dying at less than 600 days old (mean age 499 days) was 13.2 ± 2.81 μg/g and of animals older than 900 days (mean age 993 days) was 29.5 ± 7.55 μg/g; the difference in the means, however, was not significant by Student's *t* test.

A similar analysis for zinc showed a slope of +6.45 μg/g of liver/100 days and a correlation coefficient of +0.269 (not significant). Mean hepatic zinc of animals less than 600 days old was 91 ± 19.0 μg/g and of those more than 900 days old was 107 ± 21.5 μg/g; the difference was not significant and it appeared that zinc did not accumulate with age to any great extent.

In order to ascertain whether the rats given plain water were "normal" in respect to zinc and copper for their age, their mean hepatic concentrations (table 2) were compared to the control animals of two different age groups given the basal-3 water. The means of hepatic copper and zinc in young control rats dying at a mean age of 499 days did not differ significantly from the plain water group, which was killed at 1 year of age, although the controls had more zinc. The means of the older rats (mean age of natural death 933 days) did differ in zinc ($P < 0.05$) and copper ($P < 0.025$) from this plain water group. From these comparisons one cannot predict what the concentrations of zinc might have been should the plain water group have survived to old age but the relatively low values in each organ (table 2) and the low intake suggest that a comparison with control rats might be valid provided some reservations be made. In respect to copper (table 2), concentrations in the plain water group were significantly higher than those of the basal-3 group in male hearts and kidneys, and in kidneys of both sexes combined, and in no cases were obviously low, suggesting that the dietary intake was adequate for most of the tissues, and that a comparison is reasonably valid for all organs but liver. The latter comparison is not valid because liver copper increased with age.

In rats fed 50 ppm cadmium, cadmium accumulated with age in kidney, liver and spleen, but not in heart and lung. In kidney, from 454 to 1185 days of age, the slope of the regression line was +28 μg cadmium/g dry weight kidney/100 days; owing to the small number of points, the coefficient of correlation (+0.70) was not significant. In liver, the slope was 14 μg/g/100 days and the coefficient of correlation was +0.84 ($P < 0.05$). No accumulation occurred in rats not fed cadmium, the renal concentrations at all ages varying less than 18%.

TABLE 2

Trace metals in organs of rats given plain water, basal water and basal water plus nickel, dry weight, both sexes combined[1]

Organ	Plain water	P[2]	Basal water-3	P[2]	Nickel-supplemented	P[2]
	μg/g		μg/g		μg/g	
			Zinc[4]			
No. rats/no. samples	(23/12)		(22/6)		(64/10)	
Liver	64 ±4.4	<0.0005	142 ±12.8	—	94 ±20.2	—
Lung	45 ±1.5	<0.0005	80 ± 5.4	<0.05	60 ± 5.7	<0.005
Heart	61 ±3.2	<0.0005	117 ±11.0	—	99 ±14.0	<0.005
Kidney	60 ±2.1	<0.0005	108 ± 9.2	—	128 ±19.8	<0.001
Spleen	70 ±3.4	<0.0005	124 ±11.7	—	100 ±10.2	<0.005
Intake, Zn, μg/g diet +μg/g water	22.3 +0		22.3 +50		22.3 +50	
Ni, μg/g diet+μg/g water	0.44+0		0.44+0		0.44+5	
			Copper[5]			
No. rats/no. samples	(23/12)		(22/7)		(63/10)	
Liver	12.7 ±0.61	<0.025	22.8 ± 4.88	—	20.7 ± 7.09	—
Lung	10.3 ±0.44	<0.001	15.2 ± 1.28	<0.01	9.8 ± 0.97	—
Heart	30.4 ±1.98	—	22.7 ± 5.20	—	32.8 ± 7.30	—
Kidney	33.5 ±1.38	<0.05	22.1 ± 6.58	—	27.0 ± 5.76	—
Spleen	10.1 ±0.75	<0.001	24.2 ± 2.75	~0.025	16.3 ± 2.24	<0.01
Intake, Cu, μg/g diet +μg/g water	1.36+0		1.36+ 5		1.36+ 5	
Ni, μg/g diet+μg/g water	0.44+0		0.44+ 0		0.44+ 5	
			Manganese[6]			
No. rats/no. samples	(23/12)		(24/9)		(63/10)	
Liver	4.8 ±0.40	—	4.5 ± 1.04	—	4.0 ± 0.87	—
Lung	1.0 ±0.05	<0.005	1.8 ± 0.33	—	1.8 ± 0.28	<0.005
Heart	1.9 ±0.16	~0.05	2.7 ± 0.46	—	3.1 ± 0.73	—
Kidney	2.3 ±0.11	—	2.2 ± 0.52	<0.025	4.2 ± 0.79	<0.01
Spleen	1.9 ±0.22	<0.025	3.2 ± 0.59	<0.025	1.8 ± 0.40	—
Intake, Mn, μg/g diet +μg/g water	12.7 ±0		12.7 ±0		12.7 ±0	
Ni, μg/g diet+μg/g water	0.44±0		0.44+0		0.44±5	
			Chromium[7]			
No. rats/no. samples	(23/12)		(29/6)		(66/11)	
Liver	0.19±0.015	<0.001	0.51± 0.078	—	0.41± 0.061	<0.005
Lung	0.47±0.664	<0.01	2.03± 0.738	—	1.04± 0.325	—
Heart	1.27±0.235	<0.005	0.61± 0.160	<0.025	1.86± 0.406	—
Kidney	0.57±0.142	—	0.94± 0.270	—	1.48± 0.247	<0.01
Spleen	2.02±0.357	—	1.11± 0.355	<0.02	2.97± 0.679	—
Intake, Cr, μg/g diet +μg/g water	0.14+0		0.14+5		0.14+5	
Intake, Ni, μg/g diet +μg/g water	0.44+0		0.44+0		0.44+5	
Median age, days	360		862		880	

[1] Male and female values have been deposited as tables 2a, 2b, 2c and 2d with the American Society for Information Science, National Auxiliary Publications Service, New York, and may be obtained therefrom (see text footnote 1). [2] P is significance of the difference in the means of contiguous groups by Student's t. [3] Significance of difference between nickel-fed and plain water groups. Tissues were pooled in groups of two to ten. All values are weighted. Relative mean values are shown, ±SEM. [4] *Zinc*: By chi-square analysis, the numbers of mean values of zinc which were increased in the basal water group differed from those increased in the nickel group (P <0.0005). By the sign test, the plain water group differed in zinc from both the basal water and the nickel-fed group, P <0.01. Female hearts had more zinc than males, P <0.005. [5] *Copper*: Plain water and basal-3 female livers had more copper than males, P <0.025 and <0.01, respectively. By chi-square analysis, the numbers of means increased in the basal-3 group differed from those increased in the nickel group, P <0.05. By the sign test, males and females of the nickel group differed from the plain water group, P <0.05. [6] *Manganese*: Plain water female lungs had more manganese than did those of males, P <0.025. *Chromium*: Livers and kidneys of plain water group females had more chromium than did those of males, P <0.025 and <0.05, respectively. By chi-square analyses the numbers of male and female means increasing in the chromium-fed group compared to the no-chromium groups differed, P <0.0001. By the sign test, both non-chromium groups differed from the chromium-fed group, P <0.01.

TABLE 3

Chromium in organs of rats given plain water, basal water without chromium, and basal water with chromium, dry weight, both sexes, combined[1]

Organ	Plain water	P[2]	Basal water minus chromium	P[2]	Basal water-4 (5 ppm chromium)	P[2]
	μg/g		μg/g		μg/g	
No. rats/no. samples	(23/12)		(31/6)		(66/11)	
Liver	0.19±0.015	—	0.15±0.053	—	0.49±0.197	—
Lung	0.47±0.664	—	0.20±0.031	<0.05	2.14±0.735	<0.025
Heart	1.27±0.235	<0.005	0.48±0.031	<0.05	2.30±0.673	—
Kidney	0.57±0.142	—	0.51±0.233	<0.025	2.47±0.555	<0.005
Spleen	2.02±0.357	—	1.33±0.466	—	3.01±1.090	—
Chromium intake, μg/g diet +μg/g water	0.14+0		0.14+0		0.14+5	
Median age, days	360		838		863	

[1] Male and female values have been deposited as table 3 with the American Society for Information Science, National Auxiliary Publications Service, New York, and may be obtained therefrom (see text footnote 1). [2] P is significance of differences between contiguous groups, by Student's t. [3] Significance of difference between plain water and basal water-4. Numbers in parentheses are numbers of rats in each group. Tissues were pooled in lots of two to six; all values were weighted. Means±SEM. By chi-square analysis, the number of means increasing in the chromium-fed group compared to the no-chromium groups differed significantly ($P<0.0001$). By the sign test, both no-chromium groups differed from the chromium-fed group, $P<0.01$.

Comparison of plain water and basal water in respect to trace metals in tissues. Rats given plain water grew normally, appeared healthy and did not differ significantly in body weight at any 3-month interval from rats given the basal water.[3]

In table 2 are given the concentrations of zinc in five organs of rats receiving only the zinc in their food and of those receiving more than 3 times as much zinc in food and water. Zinc was markedly increased in all organs, being more than doubled in liver. In order to verify these results, data from a group fed a noncumulative metal, nickel, at 5 ppm, were included. They also differed from those of the plain water group in all organs but liver. Nickel appeared to suppress the concentration of zinc in male livers and slightly suppress it in lungs of both sexes. If these rats were comparable in trace metal content, in spite of differences in age, the data suggest that the diet was marginal in respect to tissue zinc, although adequate for growth.

The situation with copper was dissimilar (table 2). Rats obtaining only the small amount of copper in food alone had more copper in heart (males only) and kidney (both sexes) than did rats given 4.6 times that amount in water and food. These data suggest that the diet was adequate in copper. Nickel might possibly affect copper deposition in tissues by competition (21); this effect appeared in lung and spleen. Only in spleen did nickel-fed rats differ in copper from the plain water group.

Apparently the diet was marginal in manganese, for rats consuming nearly twice as much as the diet contained had more in heart, lung and spleen. It was increased in kidney and decreased in spleen in the group fed nickel (table 2). When the mean values in the five organs in male and female rats of each of the three groups were calculated and compared, they did not differ significantly from each other.

Three groups were used to study deposition of chromium in tissues, one given plain water, one given the basal water without chromium and one given the basal water which contained 5 ppm chromium (basal water-4). This experiment attempted to evaluate a) any possible effects of the manganese, cobalt, copper, zinc and molybdenum in the basal water on retention of dietary chromium, and b) whether or not chromium in drinking water was absorbed. The group given the basal water minus chromium had less chromium in heart than did the plain water group, and a tendency to smaller concentrations, although not significantly so, in lung and spleen (table 3). When 5 ppm chromium was given, concentrations were consistently higher than in tissues of rats dependent on diet alone for chromium. The large standard deviations pre-

[3] Unpublished data.

TABLE 4

Trace metals in organs of rats fed cadmium at 50 ppm compared to controls given basal water, dry weight, both sexes combined[1]

Organ	Basal-4	P[2]	Cadmium-supplemented	Basal-4	P[2]	Cadmium-supplemented
	μg/g		μg/g	μg/g		μg/g
		Zinc			Cadmium	
No. rats/no. samples	(56/7)		(44/9)	(56/7)		(44/9)
Liver	49±10.8	<0.001	165 ±15.9	0.12±0.035	<0.001	88 ±7.0
Lung	62 ± 4.4	<0.01	92 ± 8.8	0.13±0.003	<0.001	19 ±4.8
Heart	76 ± 5.0	<0.005	97 ± 2.0	0.10±0.008	<0.001	17 ±1.4
Kidney	60 ± 4.9	<0.001	141 ± 7.6	0.11±0.007	<0.001	277 ±30.1
Spleen	102 ± 7.3	—	104 ± 5.7	0.27±0.083	<0.001	29 ±8.8
Intake, Zn, μg/g diet+μg/g water	22.3 +50		22.3 +50	22.3+50		22.3+50
Cd, μg/g diet + μg/g water	0.07+0		0.07+50	0.07+0		0.07+50
		Manganese			Chromium	
No. rats/no. samples	(66/7)		(44/9)	(66/11)		(44/9)
Liver	1.91± 0.488	<0.005	4.51± 0.496	0.49±0.197	—	0.67±0.320
Lung	1.56± 0.156	—	1.86± 0.298	2.14±0.795	—	0.58±0.135
Heart	1.87± 0.097	—	2.67± 0.409	2.30±0.673	<0.05	0.68±0.105
Kidney	1.55± 0.187	<0.025	2.80± 0.397	2.47±0.555	—	1.31±0.371
Spleen	3.45± 0.459	—	4.22± 1.196	3.01±1.090	—	3.08±1.551
Intake, Mn, μg/g diet+μg/g water	12.7+10		12.7+10			
Intake, Cr, μg/g diet+μg/g water				0.14±5		0.14+5

Organ	Basal water-4 μg/g	P[2]	Cadmium-supplemented μg/g
		Copper	
No. rats/no. samples	(56/7)		(44/9)
Liver	10.7±2.42	—	14.3±2.55
Lung	13.3±1.17	—	17.7±2.00
Heart	28.0±1.53	—	30.6±1.59
Kidney	19.7±1.40	<0.001	42 ±2.49
Spleen	20.3±2.85	—	18.3±2.27
Intake, Cu, μg/g diet +μg/g water	1.36+5		1.36+5
Median age, days	863		941

[1] Male and female values have been deposited as tables 4a, 4b, 4c with the American Society for Information Science, National Auxiliary Publications Service, New York, and may be obtained therefrom (see text footnote 1). [2] Significance of difference between means of contiguous groups, by Student's t. Numbers in parentheses are numbers of rats in each group. Tissues were pooled in groups of two to six, all mean values being weighted. Means±SEM. By the sign test, the five organs of both sexes differed both in zinc and cadmium, $P<0.01$, and in manganese, $P<0.005$. Chromium in 8 of 10 pairs of male and female organs showed lower values in cadmium-fed than in basal-4 controls, $P\sim0.055$. Copper by the sign test in 10 organs was larger in the cadmium-fed than in the controls, $P<0.05$. Female values were larger than male for copper in basal-4 spleens and cadmium-fed kidneys.

cluded significant differences between livers and spleens. This diet has been found to be marginal in chromium according to other physiological experiments on glucose (22) and cholesterol (23).

A group, not shown in tables, given basal water with 1 ppm chromium was compared with that given 5 ppm. There was less chromium in liver ($P < 0.005$) and lung ($P < 0.05$) in rats of both sexes combined when 1 ppm was given than when 5 ppm was given. Compared to rats given the basal water without chromium, the 1 ppm group had about the same concentration in

liver, somewhat more in lung, and considerably more in heart, kidney and spleen. Therefore, 1 ppm chromium in water provided more in four tissues than did no chromium, and less in two tissues than did 5 ppm.

Effect of cadmium on essential trace metals. In order to ascertain effects of an accumulative trace metal, cadmium, on essential trace metals, one group fed 50 ppm cadmium in basal water was compared with its controls given basal water. Table 4 shows the marked effect of cadmium on zinc deposition in tissues (except spleen), as well as the relatively enormous retention of cadmium in cadmium-fed animals. Zinc in liver was 3.3 times, in lung 1.5 times, in heart 1.3 times and in kidney 2.3 times the comparable value of the controls. There was no significant change in spleen. These data suggest that zinc binding sites are synthesized in response to cadmium,[10] probably by an increase of metallothionein. The data also show that our diet contained cadmium at a level which did not accumulate in kidney and liver, which on a wet weight basis had only 0.03 ± 0.002 μg/ and 0.03 ± 0.008 μg/g cadmium respectively, at the end of a life time.[11]

The feeding and accumulation of cadmium appeared to suppress chromium levels in heart significantly (table 4), also in male kidneys. Manganese levels, however, were increased in liver and kidney in cadmium-fed rats compared with controls, and the overall trend was significantly higher than in the controls. Copper concentrations more than doubled in kidney and were increased in male lungs, but were relatively unchanged in other organs (table 4), and the trend was not significant.

Sex differences in tissue concentrations of metals. In a few instances, concentrations of a metal in an organ differed significantly between males and females. In every case female levels were higher than male: zinc in heart and copper in liver and kidney of rats of the basal-3 group, manganese in lung and spleen, copper in liver and chromium in liver and kidney of the plain water group, copper in kidney of the cadmium-fed group, and copper in spleen in the basal-4 group. There were no significant differences between the sexes in rats given basal water without chromium or in the basal-4 or cadmium-fed group in respect to chromium, manganese and zinc.

Organ predilections of essential trace metals. By examining the groups of rats given plain water and low dietary intakes of trace metals, organ predilections for one or more metals can be seen (table 2), possibly an index of metabolic requirement. The heart concentrated chromium and copper, the kidneys copper, the liver manganese and the spleen chromium where dietary intakes supplied the sole sources. These organ affinities largely disappeared when basal water was given.

Cadmium went mainly to liver and kidney when it was fed to rats, although it was deposited in all of the other organs (table 4). On a molar basis the five organs accumulated the following concentrations of cadmium plus zinc (μmoles/g dry tissue): kidney 3.75, liver 2.52, lung 0.63, heart 0.44, spleen 0.29. Kidney accumulated more cadmium than zinc (ratio Cd:Zn 1.93) as did spleen, whereas liver (ratio 0.44) lung (ratio 0.39) and heart (ratio 0.52) accumulated less. The concentrations of zinc accumulated in response to cadmium feeding were (μmoles/g of dry tissue): liver 1.78, kidney 1.28, lung 0.46, heart 0.29 and spleen 0.03. If the concentrations in tissues reflect the synthesis of cadmium and zinc binding proteins,[9] it is clear that the kidney and liver have a greater ability to make them in response to cadmium than do the other organs.

DISCUSSION

Most studies on metal displacement in tissues have involved large concentrations of one or another metal added to a normal or abnormal diet. Whanger and Weswig (24), for example, fed rats from 1,000 to 8,000 ppm zinc and produced displacement of hepatic copper in proportion to the dose. Hill et al. (25) studied interactions of cadmium with copper, zinc and iron in chicks, using concentrations of 25 to 400 ppm cadmium, 200 ppm zinc and 10 to 50 ppm copper; tissue concentrations were

[10] Shaikh, Z. A. 1972 The fate of cadmium in the mammalian organism. (Dissertation for Ph.D., Dalhousie University.)

[11] Low dietary cadmium has been confirmed on samples of diets analyzed in duplicate by us and by Dr. William Fulkerson of Oak Ridge National Laboratory by stable isotope dilution, spark source, mass spectroscopy.

not measured. The present experiments are concerned with concentrations normally found in food. The basal water given to rats and the diet contained somewhat lower levels than those provided by standard rat chows.

A dietary intake of an essential trace metal which is adequate for growth is not necessarily one adequate for optimal function. In view of the efficient homeostatic mechanisms for the essential trace metals zinc, manganese, chromium and nickel and the less efficient one for copper (in liver), it is possible that the observed increase in the tissue level of a metal in response to increased dietary intakes may reflect low tissue stores, or may indicate that binding sites for the metal are only partly filled, or may indicate a requirement for some function not otherwise indicated by the performance of the animal. In animal husbandry, according to Underwood (26), it is well recognized that performance of animals improves when luxus amounts of zinc, manganese and copper are fed. It is doubtful that homeostasis was exceeded by the doses of essential trace metals used in the present study.

From these experiments, it is clear that the tissues of the five organs of rats analyzed were incapable of depositing as much zinc at a dietary intake of 22 ppm as when the intake was raised by adding 50 ppm in water. It is also apparent that 1.4 ppm copper in food was not enough to bring all tissues to similar levels. Tissue levels were increased by adding 5 ppm in water; the lower level was adequate for heart and kidney, but not for lung, liver and spleen. These tissues were not tested for functional derangements in zinc and copper metabolism except for growth of animals.

Higher levels of copper in male hearts and kidneys of rats of both sexes given plain water than in rats given the basal water require some explanation. Zinc and copper interact in vivo (26). The plain water group was ingesting marginal or low levels of zinc in the diet. When animals were given adequate zinc, it is possible that this zinc suppressed copper deposition in heart and kidney.

The concentration of manganese in the diet appeared to be inadequate for tissue deposition, except in liver and kidney, for raising the intake from about 13 ppm in diet by adding 10 ppm in water significantly elevated tissue manganese in lung, heart and spleen. The diet was clearly deficient in chromium, which was low in all tissues but spleen, mean values in four organs increasing 3- to 10-fold when 5 ppm was given in water (table 3). In these comparisons the plain water group was 1 year old and the basal water group was older. Low levels of chromium in these rats were associated with disturbances in glucose (22) and cholesterol (23) metabolism.

Cadmium consumed in water caused marked accumulation in kidney and liver, as expected. Apparently binding sites for cadmium were reasonably well saturated. Under these conditions concentrations of zinc more than doubled in kidney and increased more than threefold in liver. Spleen appeared to be unaffected by cadmium in respect to zinc, although it retained cadmium to about the same degree as lung and heart. These hepatic and renal concentrations are within the ranges found in human beings (10).

Molar ratios of mean cadmium to mean zinc concentrations in cadmium-fed rats were as follows: heart 0.10, lung 0.12, spleen 0.16, liver 0.79, kidney 1.15. The ratio of the concentrations of hepatic to renal cadmium was 0.32 in these rats given 50 ppm in basal water for life, 0.15 in rats given 5 ppm in basal water for 17 months (16) and 0.27 in rats given 5 ppm in basal water for life (mean age 1,083 days) (17). The lower dose produced levels comparable to those found with the higher dose. Thus, the higher dose resulted in no significantly greater proportion being bound to liver. When calculated on a weight basis the oldest six or eight rats fed 50 ppm cadmium in water for life retained about 96 to 125 μg in kidneys and about 250 to 470 μg in liver, whereas rats fed 5 ppm in water for life retained 190 μg and 455 μg, respectively (17). Therefore, 5 ppm cadmium appears to be a level adequate for eventual saturation of tissues, and 50 ppm does not result in a greater accumulation in a life time. These series were not run concurrently.

Large amounts of cadmium can cause renal damage and result in a relative loss of renal cadmium (27–29). That this toxic level may have been approached is suggested by somewhat lower renal levels found in the six oldest female rats aged 1,185 days than in eight females aged 830 days, values being 252 μg/g and 324 μg/g, respectively. Hepatic levels were higher, however, being 115 μg/g and 72 μg/g, respectively. These kidneys on microscopic examination showed lesions characteristic of arterial hypertension, but with little overt cellular damage (30). In male rats, renal and hepatic concentrations increased regularly with age, at a rate of 44 and 17.5 μg cadmium/g dry tissue/100 days, respectively.

Conclusions as to saturation of binding sites for cadmium are based on the observations that feeding 50 ppm cadmium did not increase tissue levels of kidney and liver above that produced by 5 ppm cadmium; that the oldest female rats had lower renal levels than did younger animals; and that renal cadmium increased regularly with age up to a point, but no further in female rats. Presumably, binding sites for zinc were equally well saturated as they increased under the influence of cadmium, but the evidence for zinc is indirect. Although these experiments were not concurrent, there is no reason to believe that the action of cadmium should differ to a large extent in two different groups of rats treated similarly.

In the former series (17), we have reported, but did not emphasize, an increase in hepatic and renal zinc in rats fed 5 ppm cadmium. Hepatic contents of zinc were 2.7 times and renal contents 2.1 times as much as in the controls given no cadmium. The present experiments with another group of rats are confirmatory. This same phenomenon occurs in the human kidney (10).

Z. A. Shaikh[10] has shown that metallothionein, a zinc and cadmium binding protein first described by Kagi and Vallee (31), is synthesized in mammalian tissues in response to the feeding or injection of cadmium, but not to any other heavy metal studied, including zinc, mercury, copper and lead. Liver and kidney are the most active synthesizers of this protein, although it can be made in other tissues. Metallothionein has a fixed number of metal binding sites which can be occupied by zinc and cadmium; cadmium has a greater affinity for it than zinc. This protein has a high cystine content, the metals binding to sulfur in cysteinyl residues. It can bind a number of metals: 0.5% mercury and 0.3% copper were found in human metallothionein (32).

Shaikh and Lucis (33) have found at least two fractions in the soluble portion of rat, mouse and human liver, one of which bound some (12.6%) of the zinc and most (75%) of the cadmium. The cadmium-binding protein of rat liver and human kidney, with a molecular weight of 11,000–12,000, could be fractionated into three components, two of which contained 58.5% of the cadmium bound. The second, or zinc-binding protein had a larger molecular weight, and zinc, unlike cadmium, was mobile and exchangeable with dietary zinc. Therefore, from these results metallothionein appears to be made up of at least four proteins, three binding cadmium strongly and one zinc loosely. All proteins probably bound zinc, cadmium and mercury to some extent.

The displacement of zinc by cadmium on metal-binding protein and the subsequent synthesis of more protein with more binding sites probably accounts for the accumulations of both cadmium and zinc found in these rats. On a molar basis, there was more zinc than cadmium in all organs but kidney, where the reverse was true. This renal situation is accompanied by arterial hypertension (17); all of these rats were hypertensive (30).

The feeding of supplemental cadmium appeared to suppress chromium levels in all male and three female organs, although the differences were significant only in heart. Manganese, on the other hand, was elevated in liver and kidney when cadmium was fed, as was copper in kidney. The mechanism of these effects is obscure.

Analyses of rats fed supplemental nickel for life have shown that little or no tissue accumulation occurred.[12] In fact, there was actually somewhat less nickel in the organs of nickel-fed rats than in the organs of rats

[12] Personal observations.

given no nickel. The basal water had little effect on tissue levels of nickel compared to plain water. A slight effect of nickel on manganese, copper and zinc in one organ or another was found, depression of concentration being usual. The mechanism is also obscure, although nickel and copper may interact.

Except for the rats given plain water which were sacrificed, the life spans and longevities of these rats were similar. Whereas it is possible that the prolonged act of dying may alter the concentrations of essential trace metals in cells, we have no evidence to suggest that this may be so; if it is, it must have occurred in the control as well as in the treated groups. Comparison of short-term rats given plain water and killed at 1 year of age with rats given basal water with or without nickel and allowed to die naturally (mean age 933 days) is useful in the case of manganese and suggestive but not definitive in the cases of zinc and copper.

Almost all of our analyses on rat tissues were made on animals dying naturally. The purpose of these experiments was to evaluate effects on survival, longevity, tumors, blood constituents and pathological lesions. Therefore, tissue levels of elements found may or may not represent the true levels attained during life. One series of rats were given lead, nickel and cadmium and killed at 17 months of age (16). Tissue levels of the essential trace metals were in general comparable to those of animals dying of old age except for changes induced by cadmium.

Interactions of essential trace metals are well known (7, 24, 25). Effects of copper on zinc, cadmium on zinc, zinc on cadmium, copper on molybdenum and cadmium on copper have been described in animals, usually in relation to relatively massive doses (26). Hill and Matrone (7) have shown that 10 ppm copper protected survival in chicks fed 50 to 300 ppm zinc, and that cadmium was antagonistic to copper. By a number of other experiments they proved that "elements whose physical and chemical properties are enough alike, i.e. whose electronic structure is similar enough, will act antagonistically to each other biologically." From our experiments, an accumulative metal such as cadmium also may interact with other essential metals which are dissimilar in structure. Cadmium is uniquely handled in the body by sequestration, and its turnover is the lowest of any metal studied (34).

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Effect of Chromium, Cadmium and Other Trace Metals on the Growth and Survival of Mice[1]

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ABSTRACT Seven hundred weanling white mice were fed a diet of rye, corn oil and dried skim milk containing no detectable cadmium and only small amounts of chromium, titanium, lead and nickel. They were housed in quarters from which most contaminating metals were excluded. Groups of 100 or more animals were given 5 ppm of these metals in drinking water as soluble salts. Trivalent chromium increased the growth of both sexes and lessened the mortality of male animals up to 17 months of age. Tetravalent titanium increased growth, but did not significantly affect mortality. Divalent lead, nickel and cadmium had no marked effect on growth, mature weights nor mortalities up to 18 months of age, but after that, cadmium and lead significantly increased death rates in males when tissue levels were less than or equal to those of adult human beings. Mice "cadmium-free" by microanalytical methods (< 0.02 μg/g, wet tissue) grew normally and did not differ in any obvious way from those given the metal, except for prolonged survival of males. Chromium appears to act as an accessory trace metal for growth and survival while cadmium is not essential in these terms, having an innate "toxicity" similar to lead.

Several "abnormal" trace metals have been found to accumulate with age in the tissues of man (1, 2), these include cadmium (3), lead (4), titanium (5) and tin. Six considered "essential" as having important metabolic functions (manganese, iron, cobalt, copper, zinc, molybdenum), do not accumulate, despite relatively large exposures in food and water. Three other transitional metals also do not increase in concentration with age, except in lung, but their biological significances are not known; vanadium, chromium (6) and nickel (7).

The purpose of this communication, the first of several on the subject, is to report the effects on growth and survival of 5 "abnormal" trace metals given to weanling mice fed a diet adequate for growth and reproduction. The metals were present in the diet in low concentrations or were absent, whereas the "essential" ones were provided in adequate amounts. "Abnormal" metals were given in concentrations designed to reproduce in so far as possible the tissue concentrations found in man (2). Environmental contamination was kept at a minimum. One metal, trivalent chromium, appeared to show biological activity on both these parameters, while lead and cadmium affected only survival.

METHODS

About 700 weanling white mice of the Charles River CD strain were housed in acrylic plastic cages with stainless steel covers (8). Six mice occupied each cage. Kiln-dried wood chips were used for bedding. Water bottles were of polyethylene with rubber stoppers and stainless steel or Pyrex drinking tubes (depending on the metal given). Stoppers were protected from nibbling by mice.

The animal quarters, constructed of wood, were situated at the top of a remote hill 500 m high at the end of a mile-long (1.609 km) dirt road, in order to avoid airborne contaminants, especially those from motor vehicle exhausts (4). Material used in the building was analyzed for the trace metals studied, and contamination prevented by triple coats of plastic varnish.[3] Cage racks were of white pine;

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[2] Requests for reprints should be sent to 75 Linden Street, Brattleboro, Vermont.

[3] Fabulon, Pierce and Stevens Chemical Corporation, Buffalo, New York.

all nail heads were covered with plastic wood. A minimum of varnished metal objects was allowed in the quarters and no galvanized metal was used inside or outside. Quarters were heated by butane gas vented externally and were dehumidified. Environmental temperatures were kept between 21 and 28° C. Air inlets, near a forest 100 feet (30 m) from the road, consisted of two electrostatic precipitators continuously operated; negligible quantities of dust accumulated in them yearly. Maximum precautions were taken against introducing metallic contaminants from the outside, and shoes were removed when entering the animal room. All procedures were performed in a separate room.

The diet consisted of whole ground seed rye,[4] 60%; powdered skim milk, 30%; corn oil, 9%; and sodium chloride, 1%. The rye was ground into flour at a local mill. To each kilogram was added: 100 mg ferrous sulfate; 5,000 IU vitamin A; 1,000 IU vitamin D; 10 mg Ca pantothenate; 50 mg niacin; and 1.0 mg pyridoxine hydrochloride. These amounts, plus others present in rye and milk, were similar to the vitamin contents of standard commercial diets. Concentrations of "abnormal" trace metals are shown in table 1. Food was prepared twice a week. Diet was given ad libitum.

Soft water was supplied from a forest spring through 210 feet (65 m) of polyethylene pipe, passed through a bulk water softener, a bulk demineralizer[5] and a laboratory demineralizer.[6] It contained no detectable metal and showed a resistance of more than one million ohms. To it was added in ppm metal: Zinc acetate, 50; cupric acetate, 5; manganous citrate, 10; cobaltous chloride, one; and sodium molybdate, one; these amounts being comparable to those found in commercial diets when converted to a mean water intake of 4 g/day.[1] This solution was known as "basic water." To it was added one of the following at 5 ppm metal: Chromic acetate, potassium titanium oxalate, nickelous acetate, cadmium acetate, or lead acetate. The final solutions were clear and colorless.

Water bottles were weighed to the nearest gram and tabulated continuously to measure the total intakes of metals. The mice were weaned 21 to 23 days after birth and immediately presented with the appropriate water solution, which remained identical for their lifetimes. They were weighed in groups of 6 at weekly intervals for 2 months and thereafter every month: they were handled as little as possible and no therapeutic measures were used. Cages, washed in demineralized water, were not interchanged from one group to another. Unnecessary noise and traffic in the quarters was restricted. Pilot studies with metal-containing and deionized water revealed no aversion to the basic water. During the period of the experiment no epidemics occurred in the animal quarters.

Data were analyzed statistically to determine the significance of differences between two means, using Students' *t* test, and for abnormal distribution, using chi square. Tissues of mice dying of "natural" causes were analyzed by microanalytical chemical methods previously reported (3–7).

TABLE 1

Concentrations of "abnormal" trace metals in mouse diets and bedding

Metal	Commercial diets	Special diet[1]	Bedding (softwood chips)
	μg/g wet wt	*μg/g wet wt*	*μg/g wet wt*
Chromium	ND[2]– 2.3[3]	0.10	0.11
Titanium	ND –11.7[3]	0.03	2.31
Nickel	0.4 – 3.0[3]	0.40	0.32
Cadmium	0.1 – 0.2	ND	0.05
Lead	0.4 – 1.9[3]	0.19	1.83

[1] The intake in food per day per mouse was approximately 4 times the amounts shown.
[2] ND indicates not detected (< 0.005 μg/g).
[3] Obtained by Keenan, see footnote 7 in text.

[4] Untreated seed rye, one lot for a year's supply, was obtained from Eastern States Farmers Exchange and stored in a tightly closed space provided with dehumidification. No gross spoilage occurred.

[5] Culligan Water Institute, Northbrook, Illinois.

[6] Barnstead Still and Sterilizer Company, Boston.

[7] According to Keenan, commercial diets contain the following "essential" metals by spectrographic or chemical methods (ppm): Zinc, 58.8 to 66.7; copper, 7.1 to 11.7; manganese, 6.3 to 20.7; cobalt, < 0.4 to < 08; molybdenum, < 0.5 to 5; iron, 130 to 600. A diet similar to ours devised by Schneider, using whole wheat, showed: Zinc, 50.1; copper, 3.4; manganese, 6.0; molybdenum, not detected; and iron, < 0.5 ppm. (Keenan, R. G., U. S. Public Health Service 1958 Occupational Health Field Headquarters, Cincinnati, personal communication). Therefore, the intake of trace metals on our regimen was somewhat high for zinc but was well within the ranges of other metals found in commercial diets considered adequate for nutrition and without mineral imbalances.

RESULTS

Growth and mature weights. The mean weights of the mice given the several metals are shown in tables 2 and 3. No significant differences from the controls appeared in those receiving lead, cadmium and nickel. Males and females given chromium and titanium, however, gained weight more rapidly and when mature were heavier than their controls. Although significant differences were not evident at 2 months of age, the growth curves diverged at 80 to 100 days of age (figs. 1–5). These two metals increased mean body weight of males about 14% at 6 months and 22% at 12 months of age; they likewise affected female mice approximately 9 to 16% at both intervals.

Mortalities. Under the conditions of the experiment, all female mice were healthier than males in terms of survival. Tables 4 and 5 illustrate survival at various periods. No significant differences in mortality occurred among the female groups. Males given chromium had a lower mortality at one year of age than did the controls ($P < 0.001$), which continued to 16 months ($P < 0.02$) and 17 months ($P < 0.05$) but not later. Therefore, chromium appeared to affect male mortality in the first half of life.

Cadmium and lead, on the other hand, caused increased mortalities appearing in the latter half of life. The administration of cadmium was associated with significant differences at 21 months ($P < 0.001$) and 24 months ($P < 0.01$) of age, as compared with their controls, 98.0 and 81.1%, respectively, having died. Similarly more males given lead were dead at 21 months ($P < 0.05$). These increased mortalities were not apparent at 16 or 18 months. Neither titanium nor nickel obviously affected mortality at any interval up to 18 months of age.

TABLE 2

Mean weights of male mice given various trace metals (5 ppm in drinking water)

Metal	No. of animals	Body weight at indicated ages		
		60 days	180 days	360 days
		g	*g*	*g*
Control	62	35.6 ± 0.38[1]	45.2 ± 1.09[1]	45.1 ± 1.61[1]
Chromium	54	36.3 ± 0.89	51.6 ± 1.19	55.4 ± 1.15
P[2]			< 0.005	< 0.001
Titanium	54	36.7 ± 1.84	52.4 ± 1.40	54.8 ± 1.36
P			< 0.005	< 0.001
Nickel	50	34.9 ± 1.27	45.6 ± 2.05	45.8 ± 2.35
Cadmium	65	35.4 ± 0.62	46.0 ± 1.57	51.5 ± 1.77
Lead	52	33.5 ± 1.56	46.7 ± 1.97	48.8 ± 2.18

[1] Mean weight ± SE of mean.
[2] Probability of difference being due to chance by Student's *t* test. Only significant *P* values are shown.

TABLE 3

Mean weights of female mice given various trace metals (5 ppm in drinking water)

Metal	No. of animals	Body weight at indicated ages		
		60 days	180 days	360 days
		g	*g*	*g*
Control	88	29.4 ± 0.38[1]	41.0 ± 0.73[1]	46.4 ± 1.20[1]
Chromium	54	30.7 ± 0.62	45.0 ± 1.32	50.7 ± 1.64
P[2]			< 0.025	~ 0.03
Titanium	53	31.3 ± 1.08	46.2 ± 1.19	53.9 ± 1.86
P			< 0.005	< 0.005
Nickel	54	29.8 ± 1.45	39.7 ± 2.05	43.7 ± 1.63
Cadmium	60	28.6 ± 0.46	43.1 ± 1.00	49.5 ± 1.12
Lead	52	27.9 ± 1.22	43.2 ± 2.52	48.6 ± 2.46

[1] Mean weight ± SE of mean.
[2] *P* indicates probability of difference being due to chance by Student's *t* test. Only significant *P* values are shown.

Significant differences between groups are shown in table 5. Males given chromium outlived those fed cadmium, lead and nickel. Females given lead outlived those receiving cadmium.

When the half-lives of the male groups (time when 50% were dead) were compared, the various metals appeared in the following order of lethal effects: Nickel > lead > cadmium > titanium = control >> chromium. The first 3 metals decreased the half-lives of their groups by 1 to 2 months from that of the controls while chromium increased it by 100 days (19.6%).

Nutritional adequacy of diet. Growth curves provided by the manufacturer[8] of a commercial diet showed that male mice 60 days old weighed about 27 g and females 24 g. Although the strain of mice fed this diet may have differed from ours, our control animals at 6 weeks of age were about equal in weight to those fed the commercial diet at 8 weeks, and were about 15 and 28% heavier at 2 months. None of the metals depressed growth or mature weights. The rate of growth of animals given cadmium, lead and nickel was almost identical to that of controls. In this sense the diet appeared adequate. There was, however, a somewhat excessive mortality confined to males (tables 4 and 5), 20% of the controls having died at 9 months and 31% at 12 months of

[8] Rockland Mouse Diet, Rockland Farms, New City, New York.

TABLE 4

Mortality of mice given various trace metals

Metal given	No. animals at 2 months of age	% Surviving, months				Half-life
		6	12	18	21	
						days
Male						
Controls	61	96.7	68.8	47.3	35.9	510
Chromium	54	100.0	92.6	61.1	38.8	609
P[1]			<0.001			
Titanium	54	98.1	85.2	42.1	27.8	513
P			<0.05			
Nickel	50	100.0	66.0	38.0	30.0	449
Cadmium	65	83.1	60.0	32.2	8.0	476
P		<0.02			<0.001	
Lead	52	96.2	67.3	28.8	19.2	463
P	—				<0.05	
Total	336					
Female						
Controls	88	97.7	93.2	72.7	53.4	662
Chromium	54	98.1	98.1	72.2	48.2	639
Titanium	53	96.2	88.7	78.6	49.1	624
Nickel	54	100.0	92.4	71.7	57.4	673
Cadmium	60	98.3	90.0	66.7	50.0	636
Lead	52	100.0	92.3	78.8	69.2	704
Total	361					

[1] P indicates probability of the differences from controls being due to chance by χ^2 analysis.
[2] Series incomplete.

TABLE 5

Significant differences between groups by χ^2 analysis, P

	Months	Cadmium	Lead	Nickel
Male				
Chromium	12	<0.0005	<0.001	<0.001
	18	<0.005	<0.001	<0.0005
	21	<0.0005	~0.025	
Female				
Cadmium	21		<0.05	

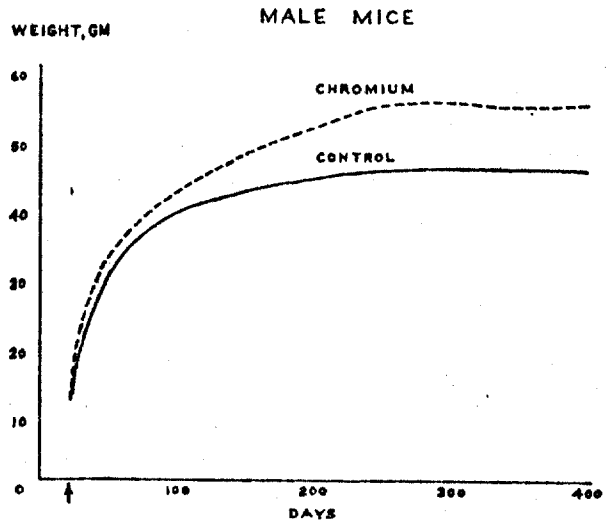

Figure 1

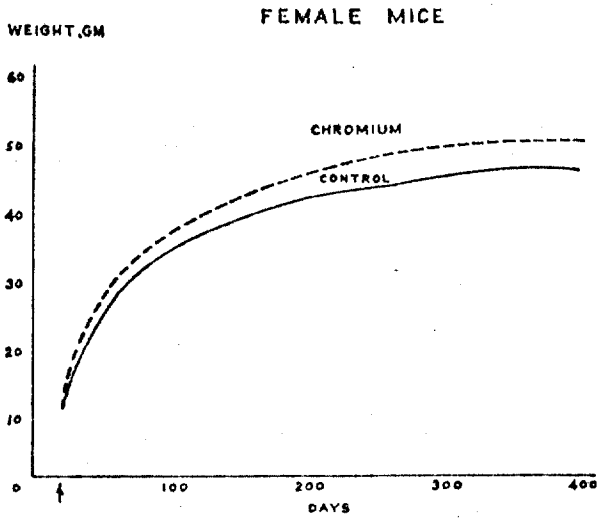

Figure 2

Figs. 1 and 2. Curves of growth of white mice given trivalent chromium at 5 ppm in drinking water from the time of weaning (shown by arrow). The continuous line indicates the mean weights of the controls, the interrupted line those of the treated animals. The abscissa shows days of age. Differences are statistically significant at 180 and 360 days of age (see tables 2 and 3).

age. This early mortality was shared by the groups given cadmium, nickel and lead, but not by those fed chromium and titanium. The survival of all female groups for the duration of the experiment was reasonable and in them the regimen showed no innate toxicity.

Absorption of trace metals. Analyses of several organs for each of the trace metals given to mice revealed that concentrations were increased, whereas less was usually found in tissues of the other 5 groups serving as internal controls. Detailed results showing rates of accumula-

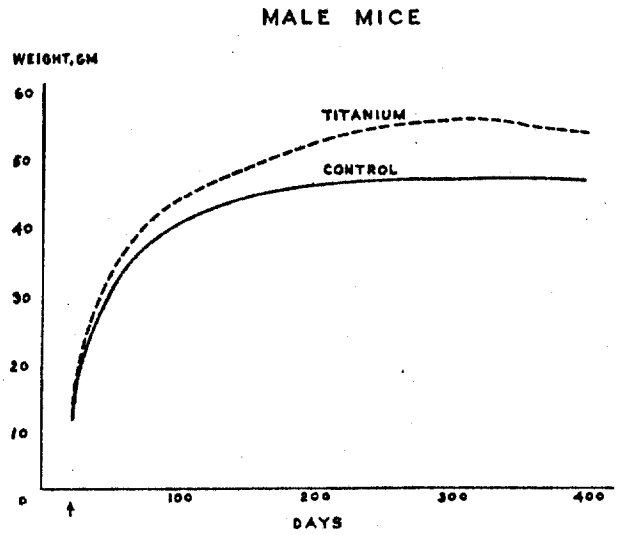

Figure 3

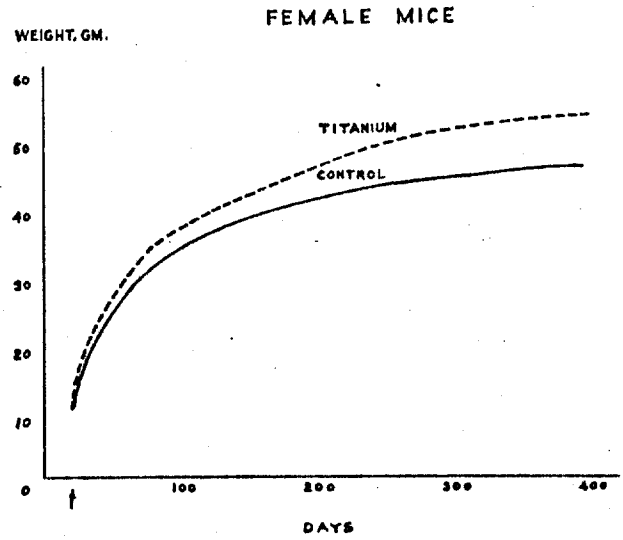

Figure 4

Figs. 3 and 4 Curves of growth of white mice given tetravalent titanium at 5 ppm in drinking water. Notations same as in figure 1. Differences are statistically significant at 180 and 360 days of age (see tables 2 and 3).

tion will be reported in a subsequent communication, but a brief summary is indicated in table 6. No cadmium (< 0.02 μg/g, wet weight) was detected in more than 50 kidneys and livers from the control and the other 4 groups not given this metal. These animals can be considered virtually "cadmium-free." Renal and he patic cadmium were regularly found i mice given it, but not in the high concen trations usually seen in adult Americar human beings (1). Little chromium ap peared in tissues from 5 groups; whil concentrations exceeded those of adul

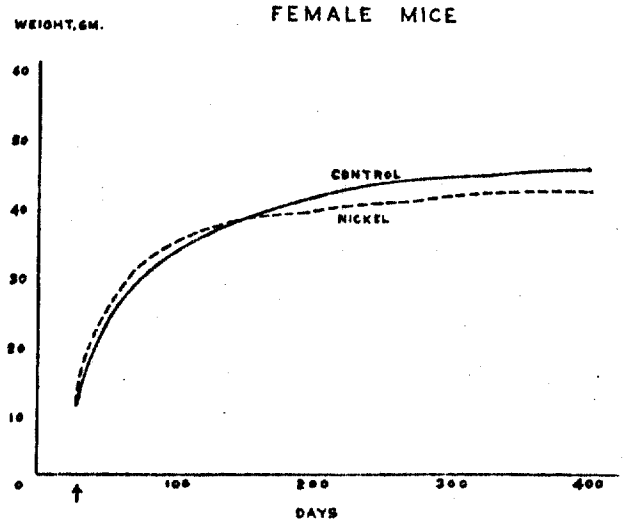

Fig. 5 Curves of growth of white mice given divalent nickel at 5 ppm in drinking water from the time of weaning. Notations are same as in figure 1. Differences were not significant.

TABLE 6

Abnormal trace metals in tissues of mice and man[1]

Group	Organ[2]	Mice		Adult man[3]	
		Not given metal	Given metal	Mean	Range
		µg/g wet wt	*µg/g wet wt*	*µg/g wet wt*	*µg/g wet wt*
Chromium	liver	0.0 –0.1	0.87– 1.28	0.02	<0.001– 0.3
Titanium	lung	2.5 –4.0	7.7 –18.1	2.8	0.1 –11.0
Cadmium	kidney	0.0	2.9 – 6.14	32.0	11.0 –66.0
Lead	liver	0.05–0.71	0.67– 3.3	2.0	0.5 – 3.2
Nickel	kidney	0.08–0.59	0.9 – 3.7	0.09	<0.05 – 0.9

[1] Data based on analyses of animals dying at 360 to 480 days of age.
[2] Organ of high concentration.
[3] From Tipton (1).

man many fold in animals receiving it. Nickel was considerably increased in mice given it, whereas their controls showed lesser amounts.

We were unable to prevent accumulation of lead and titanium by animals receiving little of these metals in the diet, probably because of the large amounts present in bedding. Control animals had pulmonary concentrations of titanium in the range of those of adult human beings and accumulated several times as much when given the metal orally. Mice given lead, on the other hand, showed hepatic and renal levels comparable to those of adult man, with lesser amounts in the controls.

DISCUSSION

The purpose of these experiments was to duplicate in mice the tissue concentrations of metals found in man and to observe any obvious effects. We were partly successful. Thus, "cadmium-free" mice were grown and compared with mice having renal concentrations up to one-fourth of that of man. Mice with relatively low levels of hepatic lead were compared with animals having concentrations approximately similar to the human. On the other hand, control mice with concentrations of chromium, titanium and nickel comparable to those of adult human beings were paired with animals accumulating larger quantities.

Cadmium-fed mice grew normally, with almost identical growth curves to those "cadmium-free." The striking difference between the 2 male groups was the late mortality in that given the metal. This difference was significant at 21 and 24 months. Females did not show this trend. The data indicate that cadmium is not essential for the growth of mice; on the contrary, it shortened the life span of males at renal and hepatic concentrations considerably less than those found in adult human beings. Therefore, cadmium can be considered a metal with innate toxicity in terms of the survival of male mice.

The effects of lead were similar, but less strikingly so with male mice, whereas females tolerated it better than any other metal. A late mortality appeared when hepatic concentrations were in the human range. These sex differences in results are unexplained.

The action of chromium on growth and survival differed considerably. Growth rates were enhanced in both sexes. Furthermore mortality up to 17 months of age was partly inhibited in male animals, who also outlived their counterparts given cadmium, lead and nickel. Therefore, chromium appeared to act as an accessory trace metal in terms of growth and of male survival.

It is unlikely that the enhancement of growth by chromium was the result of an action on intestinal bacteria such as occurs with certain antibiotics. The daily dose was too small, about 20 μg (or < 1 μg/g body weight). There is no evidence that chromium accumulates in the lumen of the large intestine. Trivalent chromium has a low order of toxicity for most living things (6), much less than some of the metals given in water (copper, cadmium, lead).[8] Furthermore, a similar effect with titanium was probably not caused by a favorable action on intestinal flora, for titanium has little or no toxicity to living organisms (5). The animals, however, had access to their feces, and this explanation, although improbable, must be considered.

Titanium at high tissue concentrations did not appear to affect mortality of males, the half-lives of this group and of the controls being similar. Evidence for its action as an accessory trace metal does not include effect on survival. The mechanism of action is unknown. Nickel did not manifest toxicity at any specific interval, although the half-life of the male group was shorter than any other.

More than 200 analyses of various foods were made to obtain a diet cadmium-free and low in the other metals used in the experiment. Our diet represents the best compromise, other than the use of synthetic foods, some of which are also contaminated by metals. The cost is low. Softwood chips were used for bedding despite the lead and titanium present. A number of other materials were analyzed and tested, but none was free from cadmium, lead and chromium and had absorptive qualities. Stainless steel wire cage floors were considered, but were found to donate traces of nickel and chromium to slightly acid solutions. Obviously, the environmental exposures of the regimen were poor for studying titanium and only fair for lead, facts which did not become apparent until tissues were analyzed. On the other hand they were reasonably good for chromium and nickel and excellent for cadmium.

The work of Schwarz and Mertz (10) has indicated that trivalent chromium may be an accessory essential trace metal for rats, preventing the low glucose tolerance induced by deficient diets and, with insulin, promoting *in vitro* the synthesis of glucose from acetate (11). The observations here reported tend to support their idea of the essentiality of chromium for mice, not especially for "normal" growth and development, but possibly for general health or resistance to disease. Parallel effects on these parameters for male rats will be reported subsequently (12). The effect of chromium, cadmium and lead on survival of male mice make these trace metals pertinent to study with other mammals, especially at tissue levels similar to those of man.

The following conclusions may be drawn from these experiments on mice: 1) Cadmium is not "essential" in terms of growth and survival, cadmium-free mice being successfully raised. On the

[8] In fact, chromium ions stimulated the growth of azobacteria (9).

contrary, small doses sufficient to provide renal accumulations about one-fourth of those of adult American human beings caused significantly increased mortality in males. Cadmium, therefore, exhibits an innate toxicity in terms of survival; 2) titanium acts as an accessory growth factor on mature weights when tissue concentrations are high, but does not affect survival; 3) nickel in these doses affects neither mortality nor growth; 4) lead, at hepatic concentrations equal to those of man, is associated with significantly increased male mortality and exhibits an innate toxicity similar to cadmium, although not affecting growth; 5) chromium acts as an accessory growth factor and inhibits male mortality during the first half of life. Thus, chromium resembles an "essential" trace metal in these terms.

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Fluctuations of the Fasting Glycemic Level and the Hypoglycemic Action of Nickel and Cobalt Salts

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Source: Boll. Soc. Ital. di Biol. Sper. 2:183-84, February, 1927.

In a report presented at the Academie des Sciences, Bertrand and Machebeuf have stated that extremely small amounts of nickel and cobalt salts are able to reinforce (increase) the action of insulin, and more recently have reported that these salts, by themselves, display a hypoglycemic action.

New evidence obtained by us in regard to the above statement made by these two French authors have led us to confirm this observation; namely, in studies carried out on normal and diabetic subjects, following the subcutaneous injection of 1-2 mg nickel or cobalt chloride, we have actually observed a reduction of glycemia.

In addition, control tests performed on the same subjects used in previous studies, involving the determination of the fasting glycemic level at various time intervals between 8 A.M. and 1 P.M., have shown a spontaneous and progressive reduction of hypoglycemia, which is not inferior to the one noted after injection of the above-mentioned metal salts.

Such a reduction in both normal and diabetic fasting subjects varies from 15 to 20% of the initial level.

In diabetics, there is no relation between the fluctuations of the glycemic level (with or without Ni and Co) and the variation of the glycosuria, which remains at a constant level in the urine samples collected from time to time.

We believe that it is advisable to report the results of these experiments, not in order to deny the hypoglycemic action attributed to nickel and

cobalt salts by Bertrand and Machebeuf, but rather to emphasize the spontaneous variations of fasting glycemia.

This question has not been examined in a sufficiently thorough manner in studies on glycemia, which have increased during the past five years after the discovery of insulin and which have been made possible by the introduction of micromethods for dosage measurement. Only recently, slight fluctuations (7-11%) have been reported by a Danish author (K.M. Hansen). But until then, such fluctuations were disregarded and fasting glycemia was considered as a constant factor for each normal individual, as a result of the studies carried out by Montsenatal, Clausen and Hiller.

Boll. Soc. Ital. di Biol. Sper.
2: 183-184, (1927)

SULLE VARIAZIONI DEL TASSO GLICEMICO A DIGIUNO E SULLA AZIONE IPOGLICEMIZZANTE DEI SALI DI NICHELIO E DI COBALTO.

Di F. SERIO e V. BONGIOVANNI.

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In una comunicazione all' *Académie des Sciences* BERTRAND e MACHEBEUF hanno riferito che piccolissime quantità di sali di nichelio e di cobalto sono in grado di rinforzare l'azione dell'insulina, e più recentemente hanno comunicato che detti sali, anche da soli, esplicano un' azione ipoglicemizzante.

La riprova da noi fatta dell' asserto dei due Aa. francesi ci ha condotto a confermarne le osservazioni, in quanto che, nelle ricerche eseguite in soggetti normali e diabetici, dopo l'iniezione sottocutanea di 1-2 mg di cloruro di nichelio o di cobalto abbiamo realmente constatato una diminuzione della glicemia.

Senonchè le prove di controllo, istituite negli stessi soggetti utilizzati per le ricerche precedenti, e cioè la determinazione del tasso glicemico a digiuno a diverse riprese, dalle ore 8alle 13, ci hanno dimostrato una diminuzione spontanea progressiva della glicemia, non inferiore a quella constatata dopo l' iniezione dei sali mettallici sopra menzionati.

Tale diminuzione negli individui a digiuno, sia normali, sia diabetici, oscilla fra il 15 e il 20 % del valore iniziale.

Nei diabetici alle oscillazioni del tasso glicemico (con o senza Ni e Co) non corrisponde alcuna variazione della glicosuria, che si mantiene, nelle urine raccolte di ora in ora, ad un livello costante.

Abbiamo creduto opportuno comunicare i risultati di queste esperienze, più che per negare l'azione ipoglicemizzante, attribuita da BERTRAND e MACHEBEUF ai sali di nichelio e cobalto, per mettere in rilievo le variazioni spontanee della glicemia a digiuno.

Gli studi sulla glicemia, moltiplicatisi nell' ultimo quinquennio, dopo la scoperta dell' insulina, e resi possibili dall' introduzione dei micrometodi di dosaggio, non hanno sufficientemente approfondito questa quistione. Solo recentemente (1923) un' autrice danese (K. M. HANSEN) ha segnalato lievi variazioni (7-11 %).

Ma fino allora tali variazioni vennero trascurate e la glicemia a digiuno considerata, come risultava dalle ricerche di MONSENATAL, CLAUSEN, HILLER, come un dato constante per ogni individuo normale.

J. Nutr. 95(4) 541-6 (1968)

Distribution and Excretion of Nickel-63 Administered Intravenously to Rats

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ABSTRACT The distribution and excretion of ^{63}Ni administered intravenously was studied using 64 Sprague-Dawley female rats. Small quantities of tissues (50–75 mg) were acid-digested and the isotope was counted using a liquid scintillation system. The major portion (61%) of a single intravenous injection of ^{63}Ni was excreted via the urine within 72 hours, with a lesser amount appearing in the feces (5.9%). In addition, the ^{63}Ni activity in the whole blood and plasma had completely disappeared by 48 hours with no accumulation in the red blood cells. The relative distribution of the ^{63}Ni per gram of fresh tissue was as follows: kidney > adrenal > ovary > lung > heart > eye > thymus > pancreas > spleen > liver > epidermis > gastrointestinal tract > muscle > incisor > femur = brain > adipose. However, in all tissues analyzed the ^{63}Ni decreased rapidly, and after 72 hours only the kidney contained significant amounts of ^{63}Ni. The ^{63}Ni distribution was compared with the blood volume of specific tissues. Correlation coefficients were 0.79, 0.76, 0.82 and 0.68 for intervals of 15 minutes, 2 hours, 6 hours, and 16 hours, respectively, following intravenous injection of the isotope. These correlations were statistically significant and suggest that the distribution of the ^{63}Ni was directly dependent upon the relative blood volume of the specific tissues analyzed.

This laboratory has initiated a program to investigate the importance of various trace elements heretofore not shown to be essential to life. Nickel was one of the elements chosen to be studied first for the following reasons. It consistently occurs in plant and animal tissues including the newborn (1). It is reported to activate various enzyme systems including arginase (2) carboxylase (3) acetyl coenzyme A synthetase (4) and trypsin (5). Nickel is present in RNA from various sources (6) and may play a part in maintaining the configuration of the protein molecules of crystalline complexes of ribonuclease (7). Finally, this element is of clinical interest since it is reported to be greatly elevated in serums from patients with myocardial infarction (8) and has been implicated as a pulmonary carcinogen in tobacco smoke (9). However, few studies have dealt with the retention or excretion of nickel in man or animals. The only investigation found that used radioactive nickel (^{63}Ni) was that by Wase et al. (10) in 1954, who studied the distribution and excretion of ^{63}Ni using a high level, 102 μg ^{63}Ni/mouse, administered intraperitoneally. The purpose of the present paper is to report the distribution and excretion of ^{63}Ni given intravenously to rats in trace amounts. A simple method for sample preparation and counting ^{63}Ni in biological samples with the use of liquid scintillation is also described.

EXPERIMENTAL

The sample preparation and counting procedure adapts the method described by Mahin and Lofbert (11) for counting tritium ^{14}C and ^{35}S in biological tissue. Briefly, the method uses acid digestion of small quantities of tissues at a low temperature. Up to 70 mg of fresh tissue or 200 mg of blood or urine were digested directly in the scintillation counting vials using 0.2 ml of 70% perchloric acid and 0.4 ml of 30% hydrogen peroxide. The vials were tightly capped and heated at 70° in a drying oven until the solution cleared, indicating that oxidation was complete. After cooling, 6 ml of ethylene glycol monethyl ether (Cellosolve) were added. In addition, 10 ml of the toluene[1] phosphor solution containing 6 g of 2,5 diphenyloxa-

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[1] Spectroquality reagent grade toluene. Matheson Coleman and Bell, East Rutherford, New Jersey.

zole (PPO)/liter of toluene were added. Before counting, the samples were allowed to equilibrate for 2 hours. For counting, a Beckman liquid scintillation counter was used with an adjustable iso-set module. The lower window was closed, with the upper window reading 3.5. The gain was adjusted to give an external standard counting ratio of 1.300 for the least quenched sample. A quenching curve was prepared using 1 μCi ^{63}Ni treated in the same manner as described for the tissue, with the exception that increasing quantities of bromcresol green indicator were added to the 2 vials after heating to give varying intensities of yellow color. The resulting quench curve (external standard ratio plotted against counting efficiency) was a straight line. Counting efficiency was 42% for the least quenched samples. All counts were adjusted to an external standard ratio of 1.300. Samples were counted to a 3% statistical counting error.

Radioactive nickel as $^{63}NiCl_2$ [2] in 1 N HCl was diluted with normal saline to give a stock solution of 10 μCi ^{63}Ni/ml. Either 0.25 ml or 0.5 ml of this stock solution was injected per animal, representing 2.5 μCi or 5 μCi of ^{63}Ni. Since the specific activity of the nickel was 3.4 mCi/mg the doses were equivalent to 0.74 μg or 1.47 μg ^{63}Ni.

Female rats of the Sprague-Dawley strain averaging 218 ± 26 g were used. They were fed laboratory ration [3] and tap water ad libitum.

For the excretion studies 5 rats were maintained in individual plastic metabolism cages.[4] The animals were adapted to the cage for 3 days before injection of the isotope. Each animal received 5 μCi of ^{63}Ni intravenously via the saphenous vein after light anesthesia with ether. Total urinary and fecal output were collected at intervals of 2, 4, 6, 8, 24, 48 and 72 hours.

The distribution studies were divided into 2 experiments. Experiment 1 used 5 animals per group which were intravenously injected with 2.5 μCi ^{63}Ni. They were held in individual suspended wire cages and killed at intervals of 0.25, 1, 2, 4, 8, 16, 24, 48 and 72 hours. After the proper interval the animals were exsanguinated under ether anesthesia by heart puncture to remove as much blood as possible from the tissues. Samples to be analyzed were excised, weighed, and frozen. Before analysis the tissues were minced and well-mixed so that a representative aliquot could be obtained. The samples for counting analyses in experiment 1 included whole blood, plasma, kidney, lung, spleen, liver and femur.

In experiment 2, four animals were used per group, injected similarly with 5.0 μCi ^{63}Ni. They were killed at 0.25, 2, 6, 16 and 72 hours. In addition to those samples analyzed in experiment 1, the following specimens were studied for ^{63}Ni distribution: blood cells, adrenal, ovary, heart, eye, thymus, pancreas, skin, brain, adipose, muscle, incisor and gastrointestinal tract. The carcasses of those animals killed at 72 hours were digested and an aliquot was counted for ^{63}Ni activity. Before the blood cells were counted, they were washed to remove plasma. This was accomplished by adding physiological saline (2 times the volume of cells) to the cells, mixing well, centrifuging at 2000 rpm for 10 minutes and decanting the saline. This procedure was repeated 3 times. Both the diet and drinking water were analyzed for nickel by atomic absorption spectrophotometry.[5] The diet was ashed by heating to 550° for 18 hours. Minimal quantities of concentrated hydrochloric acid were added. The reference standards for the diet analysis contained hydrochloric acid as the diluent. The drinking water (tap) was analyzed after concentrating 50-fold by boiling. Deionized water (nickel level non-detectable) was used as the solvent for the reference standards.

RESULTS

The accumulated excretion rate of ^{63}Ni via the urine and feces is shown in figure 1. Over 60% of the injected dose of ^{63}Ni was excreted by the urine within 72 hours. In contrast, less than 6% was found in the feces for the same duration. The data are presented as hourly urinary (fig. 2) and fecal (fig. 3) excretion of ^{63}Ni. The results show that the excretion rate was highest for the urine 2 hours following in-

[2] New England Nuclear Corporation, Boston.
[3] Lab Blox, Allied Mills, Inc., Chicago.
[4] Model 110, Maryland Plastics, Inc., 9 East 37th Street, New York.
[5] Model 303, Perkin-Elmer Corporation, Norwalk, Connecticut.

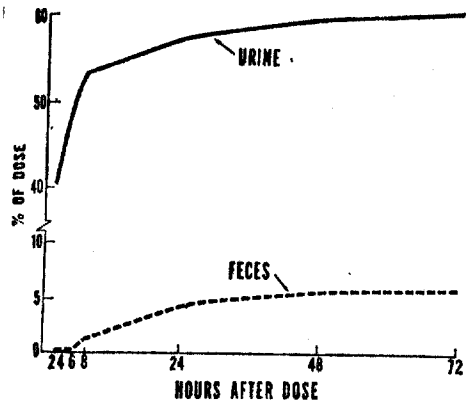

Fig. 1 Accumulated excretion of ^{63}Ni via the urine and feces following a single intravenous dose.

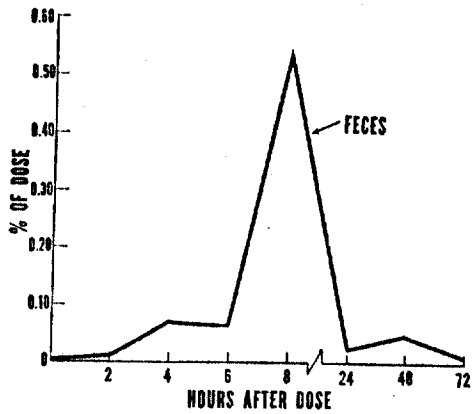

Fig. 3 Average hourly fecal excretion of ^{63}Ni following a single intravenous dose.

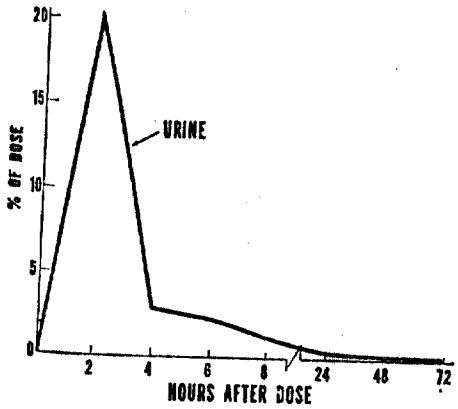

Fig. 2 Average hourly urinary excretion of ^{63}Ni following a single intravenous dose.

jection. In contrast, fecal excretion did not peak until 8 hours.

The results of the distribution study of experiment 1 are presented in table 1. The amount of ^{63}Ni retained per gram of tissue varied widely among the tissues analyzed. However, with the exception of the kidney, no significant amount of activity remained after 72 hours. In addition, by 48 hours all activity had disappeared from the whole blood and plasma (fig. 4).

The results of the more complete distribution study (exp. 2) are given in table 2. The greatest retention rate again occurred in the kidney. At 15 minutes after injection, the distribution of ^{63}Ni was as follows: kidney > adrenal > ovary > lung > heart > eye > thymus > pancreas >

TABLE 1

Distribution of ^{63}Ni in selected tissues at timed intervals after single intravenous dose [1]

Time after dose	Kidney	Lung	Spleen	Liver	Femur
hours			*% dose/g fresh tissue*		
0.25	6.54 ± 0.50	0.97 ± 0.06	0.40 ± 0.30	0.36 ± 0.03	0.24 ± 0.02
1	5.47 ± 0.38	0.66 ± 0.03	0.22 ± 0.01	0.24 ± 0.01	0.16 ± 0.01
2	4.46 ± 0.36	0.42 ± 0.03	0.14 ± 0.01	0.14 ± 0.01	0.08 ± 0.01
4	2.36 ± 0.16	0.32 ± 0.01	0.10 ± 0.00	0.12 ± 0.01	0.04 ± 0.00
8	1.57 ± 0.16	0.18 ± 0.01	0.05 ± 0.00	0.06 ± 0.01	0.01 ± 0.00
16	0.95 ± 0.06	0.11 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	nd [2]
24	0.92 ± 0.11 [3]	0.11 ± 0.01 [3]	0.07 ± 0.01 [3]	0.02 ± 0.00 [3]	nd [3]
48	0.52 ± 0.04	0.03 ± 0.00	0.01 ± 0.00	nd	0.01 ± 0.00
72	0.27 ± 0.02	0.02 ± 0.00	0.01 ± 0.00	nd	0.01 ± 0.00

[1] Mean ± SE, 5 animals/group.
[2] Non-detectable.
[3] Four animals used for 24-hour interval.

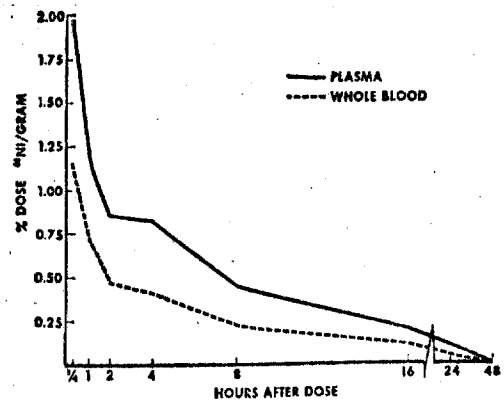

Fig. 4 Disappearance of ^{63}Ni from blood and plasma after single intravenous dose.

spleen > liver > epidermis > gastrointestinal tract with contents > muscle > incisor > femur = brain > adipose. In all specimens the ^{63}Ni decreased rapidly. As in experiment 1, 72 hours after injection only the kidney contained significant amounts of ^{63}Ni. The disappearance of the isotope from whole blood and plasma agreed closely with the results of experiment 1 with no activity present after 72 hours. In addition, no significant amount of ^{63}Ni activity was found in the blood cellular components when they were counted directly in this experiment.

DISCUSSION

The literature has been contradictory concerning nickel excretion. For example Drinker et al. (12) reported in 1924 that in human subjects using nickel cooking utensils, the majority of ingested nickel was excreted in the feces with little in the urine. In contrast, Kent and McCance (13) observed more nickel excreted in the urine than the feces, using 2 men. When intravenous nickel was given, the output of nickel increased in the urine but not in the feces. Using mice injected intraperitoneally, Wase et al. (10) noted the greatest excretion of ^{63}Ni in the feces. In a balance study on 4 dogs, Tedeschi and Sunderman (14) reported 90% of dietary ingested nickel excreted via the feces and 6% in the urine. However, following inhalation of nickel carbonyl, there was a sharp increase in urinary nickel, with fecal nickel remaining constant. The latter study and the experiments of Kent and McCance (13) indicate clearly that the method of administration directly affects the route of excretion for nickel. That is, using the data of Tedeschi and Sunderman (14), no more than 10% of ingested

TABLE 2

Distribution of ^{63}Ni in selected tissues at time-intervals after single intravenous dose [1]

	Hours after dose				
	0.25	2	6	16	72
	% dose/g fresh tissue				
Kidney	2.49 ± 0.54	2.57 ± 1.24	0.59 ± 0.23	0.20 ± 0.06	0.11 ± 0.06
Adrenal	0.92 ± 0.14	0.28 ± 0.03	0.15 ± 0.01	0.12 ± 0.02	0.03 ± 0.01
Ovary	0.90 ± 0.33	0.23 ± 0.06	0.11 ± 0.02	0.09 ± 0.01	nd [2]
Lung	0.81 ± 0.04	0.29 ± 0.04	0.14 ± 0.03	0.09 ± 0.01	0.01 ± 0.00
Heart	0.64 ± 0.04	0.21 ± 0.02	0.11 ± 0.01	0.07 ± 0.01	nd
Eye	0.56 ± 0.06	0.19 ± 0.03	0.13 ± 0.04	0.08 ± 0.03	0.01 ± 0.00
Thymus	0.55 ± 0.03	0.15 ± 0.02	0.12 ± 0.02	0.04 ± 0.01	nd
Pancreas	0.54 ± 0.05	0.16 ± 0.03	0.12 ± 0.03	0.08 ± 0.01	nd
Spleen	0.48 ± 0.03	0.13 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.01 ± 0.00
Liver	0.40 ± 0.03	0.13 ± 0.02	0.08 ± 0.01	0.05 ± 0.01	nd
Skin	0.38 ± 0.08	0.20 ± 0.05	0.07 ± 0.01	0.04 ± 0.01	nd
G.I. tract	0.33 ± 0.04	0.21 ± 0.04	0.11 ± 0.03	0.10 ± 0.01	nd
Muscle	0.29 ± 0.07	0.11 ± 0.05	0.04 ± 0.01	0.03 ± 0.05	nd
Teeth	0.21 ± 0.04	0.08 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.01 ± 0.00
Femur	0.15 ± 0.02	0.05 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	nd
Brain	0.15 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.02	nd
Adipose	0.14 ± 0.04	0.04 ± 0.01	0.02 ± 0.00	0.01 ± 0.01	nd
Carcass	—	—	—	—	nd

[1] Mean ± SE, four animals/group.
[2] Non-detectable.

nickel is absorbed. Therefore the majority of orally administered nickel would be expected to appear in the feces. However, our data show that *intravenous* nickel is cleared quickly via the urine, with only small quantities occurring in the feces (figs. 1, 2 and 3). As a result of the role of the kidney in nickel clearance, the high radioactivity of ^{63}Ni in this organ is probably due to urine retained within the organ.

In experiment 1, it appeared that all of the radioactivity of whole blood could be accounted for by the plasma fraction. That is, the activity of the plasma portion of one gram of blood was equal to the total activity for a similar quantity of whole blood. By counting the cellular fraction directly, it was confirmed in experiment 2 that the blood cells did not contain significant ^{63}Ni. Thus, these data are in disagreement with the studies of Wase et al. (10) who found that the red blood cells contained approximately one-half the activity of plasma in mice. However, during the duration of our experiment, ^{63}Ni did not accumulate in the blood cells as has been reported for zinc (15), selenium (16) and chromium (17).

The relatively high activity of both the adrenal and ovary is of interest since these organs are controlled hormonally. However, nickel was not actively retained. No previous studies were found in the literature regarding normal concentration of nickel (radioactivity or non-radioactive) for these tissues.

In experiment 1, of the tissues analyzed, the lung had the second highest ^{63}Ni per gram of tissue and in experiment 2, it ranked fourth, exceeded only by the kidney, adrenal, and ovary. In the study of Wase et al. (10), the lung had the greatest retention of ^{63}Ni when compared with the other organs analyzed. The authors suggested a high complex constant for Ni^{++} with lung protein. In addition, Schroeder et al. (1) reported that this element occurred more frequently in the lung of humans than in the kidney, heart, larynx, bone, trachea or aorta.

Since the isotope quickly disappeared from both the blood and organs, it was reasoned that the distribution of ^{63}Ni in the various tissues studied may be directly related to the blood volume of these tissues, i.e., blood volume as microliters per gram of tissue. Everett et al. (18) have reported the blood volume of various tissue and organs in female Sprague-Dawley rats of a weight range similar to that used in our study. Therefore, using the data of Everett et al. (18), the relative blood volume and distribution of ^{63}Ni in various organs and tissue were compared (table 3). As shown, a high correlation existed between ^{63}Ni distribution and tissue blood volume at 15 minutes, 2, 6, and 16 hours following a

TABLE 3

Comparison of ^{63}Ni distribution with tissue blood volume

Tissue [1]	^{63}Ni distribution [2]				Blood vol [3]
	15 min	2 hr	6 hr	16 hr	
	% dose/g fresh tissue				*µliters/g*
Adrenal	0.92	0.28	0.15	0.12	268
Ovary	0.90	0.23	0.11	0.09	268
Lung	0.81	0.29	0.14	0.09	505
Heart	0.64	0.21	0.11	0.07	238
Spleen	0.48	0.13	0.11	0.09	134
Liver	0.40	0.13	0.08	0.05	265
Skin	0.38	0.20	0.07	0.04	18.2
Muscle	0.29	0.11	0.04	0.03	22.9
Femur	0.15	0.05	0.03	0.01	32.8
Brain	0.15	0.04	0.04	0.05	30.1
Correlation coefficient	0.79	0.76	0.82	0.68	
	$P < 0.01$	$P < 0.05$	$P < 0.01$	$P < 0.05$	

[1] Kidney was omitted due to radioactive urine within it.
[2] No comparison could be made for the 72-hour interval because ^{63}Ni was non-detectable in several tissues (see table 2).
[3] Blood volume data from Everett et al. (18).

single injection of ^{63}Ni. These data strongly suggest that the distribution of ^{63}Ni was directly related to blood volume of the specific organ or tissue studied.

Because nickel has been reported to be present in ribonucleic acid (RNA) from various sources (6, 19), the level of ^{63}Ni was compared with the concentration of RNA in selected tissues. The RNA concentrations reported by Winick (20) for rat lung, heart, liver and spleen showed no significant correlation with the distribution of ^{63}Ni in these organs.

It is recognized that the animals in this experiment were not receiving a diet devoid of nickel. That is, analysis of the laboratory ration by atomic absorption spectrophotometry showed that the diet contained 3.3 μg of Ni/g. In addition, 2 μg of Ni/liter was found in the drinking (tap) water. Should a nickel-free diet be fed, it is conceivable that the excretion and distribution of injected ^{63}Ni would be quite different than reported here. Specifically, the injected ^{63}Ni of our experiments may be handled by the animal as excess nickel since an adequate quantity was being supplied by the diet. Current investigations to develop a nickel-deficient diet are in progress.

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Effect of Co^{++}, Ni^{++} and Zn^{++} on Corticoid Excretion by the Guinea Pig.* (25736)

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The guinea pig is useful for study of factors which influence ACTH release(1,2,3) since urinary corticoids are readily measurable in this species. The effect of Co^{++} was investigated because it has been suggested that events leading to establishment of polycythemia by this substance follow from production of a relatively anoxic state(4). It was observed that increased corticoid excretion followed intraperitoneal (but not subcutaneous or intramuscular) administration of Co^{++}. Increased corticoid excretion was also induced by intraperitoneal injection of Ni^{++} and Zn^{++}.

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Methods. The salts employed were $FeCl_2 \cdot 4H_2O$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $HgCl_2$, $ZnCl_2$, $PbCl_2$, $CuCl_2 \cdot 2H_2O$. 300-350 g animals of either sex were injected intraperitoneally with various divalent ions, in 5 ml of saline; control animals received 5 ml saline as well. Co^{++} was also administered along with 2.5 mg morphine sulfate/100 g according to procedure previously described(2). Urine was collected for 6 hours(3). Urinary corticoids were determined by methods of Silber and Porter(5). When Co^{++} was administered subcutaneously or intramuscularly it was administered in 0.25 ml saline. To compare toxicity of these ions Co^{++}, Ni^{++}, Fe^{++}, Cu^{++} and Zn^{++} were administered twice daily at

TABLE I. Effect of Divalent Ions on Urinary Corticoid Excretion. 4 μM/100 g. 6 hr collection period.

Group	No.	Corticoids	Group	No.	Corticoids
A. Subcut. route			*C. Intraper. route*		
Control	6	127 ± 19*	Control	18	103 ± 14
Co^{++}	6	112 ± 28	Co^{++}	11	204 ± 31§
B. Intramusc. route			Ni^{++}	18	190 ± 25§
			Fe^{++}	12	109 ± 12
Control	6	108 ± 17	Fe^{++}†	18	141 ± 14
Co^{++}	6	121 ± 20	Cu^{++}	18	112 ± 22
			Pb^{++}	18	118 ± 17
			Zn^{++}	18	199 ± 28§
			Hg^{++}‡	14	90 ± 19

* Stand. error.
† 16 μM/100 g.
‡ 2 μM/100 g, when 4 μM/100 g was administered, all animals died while in collection cages.
§ p value <.01.

doses of 4 μM/100 g to each of 10 animals.

Results. Co^{++} administered intraperitoneally (but not subcutaneously or intramuscularly) induced an increased urinary corticoid excretion. It was determined with use of graded doses of Co^{++}, that a dose of 4 μM/100 g consistently doubled the 6-hour excretion value. Accordingly, this dose was employed for other ions (Table I). The response to Co^{++}, Ni^{++} and Zn^{++} was similar. Cu^{++} and Pb^{++} induced a moderate, but insignificant increase in corticoid excretion (1.1-1.2 ×) Fe^{++} did not produce any effect although 40% increase was observed if dose was increased 4-fold. Hg^{++} at this dose level proved lethal. When administered at dose level of 2 μM/100 g (non-fatal) corticoid excretion was 10% below controls. Diarrhea was present in all but one of animals which received this dose of Hg^{++}. Several animals which received Cu^{++} died after collection was completed. No toxicity was manifest with other ions following a single injection. A red pigment was observed in urine of Co^{++} treated animals but not in others.

In the toxicity study Ni^{++} and Cu^{++} caused death of all within 5 days. At this time only one animal receiving Co^{++}, 2 receiving Fe^{++} and 3 receiving Zn^{++} had died. After 8 days, 5 receiving Co^{++}, and 3 receiving Fe^{++} and Zn^{++} remained. Gross inspection revealed that survivors from Co^{++} had peritonitis with ascites and obvious hepatic and splenic pathology. Fibrinous exudation was present. The peritoneal cavity of Zn^{++} and Fe^{++} treated animals appeared normal except for 2 mm zone of hepatic necrosis in one of the former, and a peritoneal adhesion to liver, in one of the latter. None of the 10 control animals, which received intraperitoneal injections of saline, exhibited any pathology.

Inspection of peritoneal cavity of guinea pigs 6 hours after a single injection of Co^{++} suggested presence of slight hyperemia.

Co^{++} was administered along with morphine to determine whether it acted at some site before or after morphine sensitive areas in the brain, in the central nervous system, hypothalamus, anterior pituitary, adrenal complex, involved in increased adrenal steroid production. There was a marked reduction in adrenal response (Table II).

Discussion. The 3 ions Co^{++}, Ni^{++} and Zn^{++} have approximately the same ability to increase corticoid excretion on an equimolecular basis. There appears to be no other property of these ions, not possessed by one or more of the other ions tested, which might explain these findings. Peritoneal reaction was not observed following Zn^{++}. The red pigment appeared only after Co^{++} administration. Ni^{++}(4) and Zn^{++} are not known to be capable of inducing erythropoiesis. Perusal of the literature in regard to the effects of various ions on enzymatic activity fails to reveal those which are common to Co^{++}, Ni^{++} and Zn^{++}, but not to other ions which failed to cause increased corticoid excretion.

The fact that Co^{++} is not effective in inducing increased corticoid excretion when administered subcutaneously and intravenously at dose of 4 μM/100 g, suggests that for Co^{++}, at least, the site of action may be found in an organ served by the portal system. For example, a relatively large dose of Co^{++} entering the pancreas might induce alpha cell

TABLE II. Effect of Morphine on Corticoid Excretion Following Co^{++} Administration.

Group	No.	Corticoids
Control	11	99 ± 7‡
M*	11	45 ± 4
Co^{++}†	12	203 ± 24
M* + Co^{++}†	12	93 ± 14

* 2.5 mg morphine sulfate/100 g was administered according to(2).
† 4 μM/100 g.
‡ Stand. error.

damage of the pancreatic islet cells(6). Ni^{++}, but not Zn^{++}, acts likewise in the rabbit(7). It is also known that Co^{++}, Ni^{++} and Zn^{++}, but not other ions tested, react with insulin(8). However, it is not clear how these mechanisms would lead to increased corticoid excretion.

Morphine caused a marked reduction in adrenal response to Co^{++} suggesting that it acts prior to morphine sensitive area in the brain. Because of possibility of complex formation between Co^{++} and morphine, interpretation of the finding must be accepted with caution.

Summary. Several divalent ions were administered intraperitoneally to guinea pigs, at a dose of 4 μM/100 g body weight. Urinary corticoid excretion was doubled by Co^{++}, Ni^{++} and Zn^{++}. Fe^{++}, Cu^{++}, Hg^{++}, and Pb^{++}, produced little or no effect upon corticoid excretion.

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Traces of Nickel in Beers and Brewing

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Introduction

The influence of trace metals on the properties of beer has long occupied the attention of our laboratories. This is evidenced by papers which we have published at different times on the development of simple analytical procedures for determining trace metals in beer. Other papers similarly have discussed the effects of certain of these metals in brewing.

The present paper concerns nickel and will describe a simplified procedure for determining traces of this element in beer and brewing materials and its effects. Our laboratories' concern with the effects of nickel goes back many years to a study of the influence of a series of metals which included nickel in catalyzing oxidation reactions in beer[1]. This interest was, however, heightened when, in our studies of the problem of wild beer some years ago[2], we pointed out that traces of nickel as well as iron and other metals greatly accelerated tendencies of beers to develop gushing characteristics.

At that time a survey of the literature indicated the need for a practical, but at the same time sensitive and precise, method for the determination of nickel. One which would be sufficiently precise to determine amounts of the order of hundredths of a part per million seemed necessary. Such a method was accordingly developed and is now being published[3].

Our interest in nickel recently was revived when a brewery consulted us about a fermentation problem which seemed, in their case, to arise as a result of the use of nickel equipment in the brewhouse. They had noticed a somewhat more sluggish fermentation in worts produced in nickel equipment than in companion brews from conventional equipment.

Our general interest in trace metals in the past has centered primarily on effects on shelf-life, on gushing, foam, and similar properties of the finished product. There are, however, other effects which various trace metals can exert. In particular, effects on yeast and fermentation have long been a subject for consideration by fermentation chemists.

With the aid of the analytical method for nickel, already available, we were able accurately to follow variations in nickel content during processing at the above brewery and at the same time experimentally to confirm the possibility of effects on fermentations under certain conditions. The details of this study form the basis for the present paper.

In presenting these data we do not at all imply that nickel-containing equipment is unsuitable for use in the brewery. Much depends, of course, on the particular nature of the equipment. There is no perfect metal which can serve as a material of construction for all brewing equipment. For each use there are some advantages to each metal and also some disadvantages. Other metals such as copper and iron, which may be shown to have inhibitory effects on yeast growth or fermentation, or which are detrimental to beer stability, have a long and time-honored usage in the brewing industry. Certain nickel alloy equipment no doubt also has a definite place. On the other hand, pure nickel is indicated by the literature, and by the experience and tests to be here reported, as not as well suited for use, at least in the brewhouse. It is of importance in the use of any metal that precautions of proper design and maintenance be taken to prevent undue pickup of the metal.

Review of Literature

There are but few references to nickel in the brewing literature. Hagues[4] in 1931 discussed metallic contamination of beer in English breweries. He conducted an experiment in which a fermentation was carried out in a glass beaker in the presence of a piece of brightly polished nickel tubing. Fermentations were also conducted in small-scale fermenting vessels made of nickel and Monel metal. The surface-volume relationships of these small vessels were compared with regular brewery vessels and as a result, it was assumed that, based on their measurements, the analytical values for nickel pickup for the beer and yeast in these laboratory fermentations could be divided by a factor of 8.7 to obtain the expected results in a brewery-size vessel. Such data indicated pickup of nickel from pure nickel fermenters in the range of 1.7 to 2.4 parts per million in beer and from Monel vessels, much less — i.e., 0.3 to 0.6 part per million. He therefore concluded that pure nickel would be undesirable for the construction of fermenters whereas Monel would be satisfactory. Small quantities of nickel gave the beer a "rough" flavor and produced a haze in a short time. He also mentioned that "the prolonged effect of this metal on yeast is unknown."

Mason[5] in 1940 reported on loss of weight due to corrosion of metal test assemblies in wort and beer at various stages of brewing under operating conditions. Considerable differences are apparent in his data as between loss in weight of pure nickel and Monel on the one hand and the nickel alloy Inconel on the other. Moreover, of special interest in view of the case history to be reported in the present paper is the fact that corrosion for all three metals was appreciably greater at brewhouse stages than in fermentation or storage. This appears to be due largely to the temperature differences in view especially of his results for different sections of the Baudelot cooler, although other factors may also be involved in the apparent differences between the effects of wort and fermented beer on the test pieces.

Although his tests regarding tolerance of fermented beer or ale to nickel salts present in solution were limited to observations only of haze, color, and flavor, Mason concluded from his data that Inconel is practically completely resistant to beer and that even nickel and Monel are sufficiently resistant to represent suitable materials for various pieces of brewing equipment, Monel being superior to nickel.

The paper by Essery[6] in 1945 on the estimation of traces of metals in wort included a method for nickel. Being essentially an analytical paper, it contained no data relating to the effects of nickel.

Doran[7] in 1946 reported on seven year's experience with the use of nickel-containing equipment under practical brewery conditions. He stated that after seven year's use the inside nickel surface of the brew kettle was in excellent condition. This appears to have been the only large piece of equipment in which nickel surfaces were in contact with the product.

A paper by Andrews and Harrison[8] appeared in 1954 which proposed a colorimetric method employing alpha-furildioxime for the determination of nickel. This paper, which is devoted mostly to the analytical method, gives a few values for nickel content of beers and brewing materials. These values are in the range of nickel results found by us.

Method for Colorimetric Determination of Traces of Nickel

The general procedure as finally worked out[3] may be outlined as follows: The sample is dry-ashed and the ash is taken up in dilute hydrochloric acid and extracted with a carbon tetrachloride solution of dithizone to remove the interference of copper and iron. The aqueous layer is treated with dimethylglyoxime and bromine and then made alkaline, developing the brown-to-red nickel color. The intensity of the color, which is directly related to the concentration in the

range 0.0-0.5 part per million, is then read in a photometer. After the ashing step the reactions are conducted in a stoppered 15-ml. graduated centrifuge tube and then transferred to a 10-ml. volumetric flask for color development.

Method

Reagents

Hydrochloric acid — 1 + 1 and 1 + 9.
Dithizone (diphenyl thiocarbazone) — 0.05% in carbon tetrachloride. Keep refrigerated in brown bottle.
Dimethylglyoxime — 1% in 95% ethyl alcohol.
Bromine water — a saturated solution of bromine in distilled water.
Ammonium hydroxide, conc. — (28% ammonia)
Standard nickel solution — nickel ammonium sulfate,
$NiSO_4 \cdot (NH_4)_2 \cdot 6H_2O$ – 6.730 gm. per liter.
1 ml. = 1 mg. nickel.

Calibration of Photometer

To graduated 15-ml. centrifuge tubes, add 1 ml. 1 + 9 hydrochloric acid and aliquots of freshly prepared appropriately diluted standard nickel solution keeping total volume under 6 ml. Cover the range from 0.0 to 0.5 mg. nickel in suitable increments. Dilute to the 6-ml. mark with distilled water, add 4 ml. dithizone solution, and continue as under *Procedure* below.

The intensity of color developed is proportional to the concentration of nickel in the above range so if the response of the colorimeter or photometer employed is linear, a straight line calibration plot should be obtained and an average calibration factor converting scale readings to parts per million may be calculated. If the response of the photometer is non-linear, a graphic method with a calibration curve may be used for the calculations.

Procedure

Evaporate 100 ml. of wort or "degassed" beer to dryness in a silica dish previously cleaned by boiling with 1 + 1 hydrochloric acid. Char with a Bunsen flame, then ash in a muffle furnace at 500-550° C until ash is white . (In the case of dry materials such as malt, weigh out a suitable charge, such as 10 grams, and char and ash as above.) To the ash add 2 ml. of 1 + 1 hydrochloric acid and 2 ml. of distilled water, taking care to wash down the sides of the dish. Evaporate to dryness on a boiling water bath, and while dish is still hot, take up residue with 1 ml. of 1 + 9 hydrochloric acid and 2 ml. of distilled water. Carefully transfer the acid solution to a graduated 15-ml. centrifuge tube (graduated in 0.1 ml. divisions). Wash out the silica dish with small increments of water, transferring to tube until the 6-ml. mark is reached.

Add 4 ml. of dithizone solution — cork tube tightly and shake vigorously for 30 seconds and allow layers to separate. Repeat shaking four times. If color of dithizone turns brownish, draw off spent dithizone and add a fresh 4 ml. portion. Repeat shakeouts and dithizone additions until no change in original dithizone color occurs. Using a 5-ml. volumetric pipet, carefully remove 5 ml. of the aqueous (top) layer and transfer to a 10-ml. volumetric flask. Add 0.5 ml. dimethylglyoxime solution, mix, and then add 1.0 ml. saturated bromine water. Mix and let stand 10 minutes. (The solution should be brownish due to the excess bromine. If not, add more bromine water.) Then add 0.5 ml. ammonium hydroxide, mix thoroughly, and dilute to the 10 ml. mark with distilled water. Transfer to centrifuge tube and centrifuge for three minutes at approximately 2500 rpm. Read the supernatant liquid (within ¼ hour) in a photoelectric colorimeter, using a green filter and a suitable cell. Set the "zero" reading of the instrument with water. The authors have used a 13 mm cell and a filter

photometer with a composite glass filter having a maximum transmission at 500 millimicrons.

A reagent blank is run by mixing 1 ml. 1 + 9 hydrochloric acid and 5 ml. distilled water in a graduated centrifuge tube and proceeding from the step where dithizone is added.

The results are calculated in the usual manner for a colorimetric procedure. If the calibration data is a linear function, multiply the factor (converting photometer readings to parts per million nickel) by the difference obtained by subtracting the blank photometer reading from the sample photometer reading. If a curvilinear function is obtained for the calibration, then pick off the values for the sample and blank from a curve constructed on graph paper relating photometer readings to parts per million nickel. Subtract the blank results from the sample value to obtain the parts per million of nickel to be reported.

For beers containing nickel in excess of 0.5 part per million, reduce the charge of sample used for ashing. Select a charge such that the color intensity of the solution read in the photometer falls within the value of the calibration curve and gives readings of acceptable precision.

Nickel Content of Brewing Raw Materials

Of the raw materials entering into a brew, water is in the largest proportion with the malt and adjuncts next. Table I gives the results on a series of scattered samples of American brewing waters varying in total solids content from 66 to 570 parts per million. Nickel was found to be absent in all samples within the sensitivity of the method which is about 0.01 part per million. The water, in general, probably contributes negligible amounts of nickel, if any, to the finished product.

TABLE I
Nickel in Brewing Waters

Source	Total Solids	Nickel-ppm
New Jersey	66	0.00
Maryland	101	0.00
Ohio	201	0.00
Wisconsin	131	0.00
Texas	570	0.00
California	---	0.00

Table II gives the results on a typical series of malt, adjuncts, and hops. In the case of the malts, the nickel content varied from 0.09 to 0.90 part per million. In all the malts we have examined to date, we have never encountered any having nickel in excess of 1 part per million; generally, the nickel content is much below this level. A malt with even 0.9 part per million nickel would contribute little more than about 0.1 part per million to the mash.

The adjuncts usually have been found to have very low nickel contents except in the case of an occasional sample of corn flakes. This slightly higher level encountered on some corn flakes possibly represents a slight pickup from nickel equipment during the processing of the material.

On the few samples of rices examined, the level of nickel has been similar to that found for malt.

The nickel content of hops has been found to be appreciably higher than some of the other raw materials, values being up to 3 parts per million in the hop samples examined. However, because the total amount of hops used per barrel is so small compared with malt and adjuncts, it is obvious that the actual contribution of nickel to the brew from the hops is negligible. Hops containing 3 parts per million nickel when used at a rate of 0.5 pound per barrel would ad

TABLE II
Nickel in Brewing Materials

Sample	ppm Nickel
Malt A	0.50
Malt B	0.24
Malt C	0.13
Malt D	0.64
Malt E	0.90
Malt F	0.20
Malt G	0.09
Corn Grits A	0.10
Corn Grits B	0.31
Refined Grits A	0.05
Refined Grits B	0.09
Corn Flakes A	0.16
Corn Flakes B	1.77
Corn Flakes C	0.05
Corn Flakes D	0.10
Rice A	0.66
Rice B	0.35
Brewers' Soy Flakes	0.02
Hops A	3.00
Hops B	1.11
Hops C	1.75
Hops D	0.87

to the nickel content of the wort about 0.006 part per million assuming that it was all soluble.

Based on the above values, the nickel content of worts should generally be no more than about 0.1 part per million and it is usually below this figure.

Nickel Content of Commercial Beers

A series of beer samples was examined representing commercial products of breweries in the United States and Canada and some foreign countries. The nickel content of these samples is shown in Tables III and IV.

The first group of samples in Table III represents beers having nickel contents from about 0.01 to 0.04 part per million and is representative of the bulk of beer samples examined. The second group of results in Table III represents beers from breweries reported to contain some nickel equipment. The results in the latter group indicate that while nickel content of many of the samples is low, they are not all in the consistently low range noted for the first group. Those samples showing nickel levels in the higher range appear to represent pickup of the metal in the brewery. In this cursory survey no attempt was made to correlate the level of nickel found with the extent or kind of equipment involved.

The results in Table IV for beers brewed in foreign countries show that here again, for the samples investigated, the nickel contents are quite low.

Effect of Nickel on Fermentation

As stated, we were recently called upon to investigate a problem at a brewery which had installed nickel equipment in the brewhouse a few years earlier. This gave us an opportunity to study the effects of nickel on fermentation.

Following installation of this equipment, brews had been made both in the

TABLE III
Typical Nickel Contents of Beers

Brewery	Location	Nickel-ppm
1	U.S.—east	0.04
2	U.S.—east	0.01
3	U.S.—east	0.03
4	U.S.—midwest	0.04
5	U.S.—midwest	0.03
6	U.S.—midwest	0.02
7	U.S.—south	0.02
8	U.S.—west	0.03
9	U.S.—west	0.03
10	U.S.—southwest	0.04
11	Canada	0.04
Beers from breweries stated to use nickel or nickel-alloy equipment		
12	U.S.—east	0.17
13	U.S.—east	0.10
14	U.S.—east	0.04
15	U.S.—east	0.02
16	U.S.—midwest	0.01
17	U.S.—midwest	0.07
18	U.S.—midwest	0.04
19	Canada	1.80
20	Canada	0.44

TABLE IV
Nickel Contents of Some Foreign Beers

Brewery	Location	Nickel-ppm
1	Cuba	0.01
2	Denmark	0.01
3	England	0.01
4	Malta	0.02
5	Mexico	0.03
6	Norway	0.01
7	Venezuela	0.06

nickel equipment and in the older, conventional equipment. It had been found that the fermentations of the worts made in the nickel equipment were appreciably more sluggish than for the regular brews. The temperature rise was also stated to be lower. To obtain a normal fermentation it was found necessary, as a regular practice, to use the two sets of equipment alternately and to add to nickel brews some actively fermenting wort from conventional equipment on the third day of fermentation.

The nickel equipment at this brewery comprised a cereal cooker, mash tub, lauter tub, and kettle. All were clad with pure nickel on the surfaces exposed to the brews. The conventional equipment on the other hand consisted of iron except for the kettle which was, as usual, copper.

Having developed a suitably precise analytical procedure for the determination of nickel, the first step in our study of the problem was the determination of the nickel content of a series of wort samples from this brewery representative of the two types of equipment. We were able to confirm a marked difference in nickel reflecting pickup from the equipment used. Nickel contents of 0.03 and 0.06 part per million were found for two kettle worts made in the conventional equipment as against 0.96 and 2.2 parts per million for two worts prepared in the nickel equipment.

Since the beers were customarily mixed during fermentation, samples of finished beer representative of the two types of equipment could not be obtained

separately. However, examination of samples of finished, blended beer showed 0.25 part per million of nickel to be present. This, of course, represents an appreciable level of nickel and the sample would definitely be grouped with the higher nickel beers in Table III.

Laboratory Fermentations

In all studies of effects of so-called yeast poisons in trace concentrations, it is necessary to take into consideration a wide variety of factors which by influencing cell metabolism may obscure the effects under investigation. Thus, the composition of the wort, the initial yeast cell concentration (pitching rate), fermentation temperature, aeration, and the surface-volume relationships of the fermentation vessels are all of the utmost importance in this connection. Retardation effects of fermentation therefore may or may not be exhibited from trace quantities of certain metals depending upon the extent to which cell protective factors in the media, cell concentration, or stimulation of reproduction, may offset the particular injurious effect.

Thus, as a matter of practical experience, it is well-known that rousing or aeration, or use of higher pitching rates or temperatures, are all sometimes resorted to when, for any reason, brewery fermentations becomes sluggish. In the same way, the addition of relatively small amounts of soya flakes is also known to stimulate yeast fermentations and this is now fairly common practice in certain conditions.

The possible influence of such factors was apparent in the preliminary stages of our experiments on this problem. We found for example that laboratory fermentations of worts from several local breweries to which varied amounts of nickel had been added did not behave uniformly. The wort from one brewery exhibited the fermentation retarding effect of small amounts of nickel while wort from another brewery containing the same amount of added nickel fermented normally. In this case, as our subsequent experiments will indicate, the differences could no doubt be ascribed to the use of a small amount of soy in the case of the latter brewery.

Similarly, differences were observed along the same lines in small-scale laboratory fermentations, depending upon the degree of cell respiration permitted by aeration and stirring during the fermentation period. Thus, in laboratory flasks or bottles a definite retarding effect on fermentation due to 5 parts per million of nickel was observed if the flasks were undisturbed, whereas fermentation was normal if the flasks were shaken.

Based on these preliminary studies, further experimentation was carried out in such a way as to permit the control of factors like aeration, air exposure, and initial yeast concentration. Two types of fermentation experiments were carried out.

a) On the relatively small quantities of wort samples as received from the brewery in question — small-scale laboratory fermentations were conducted in long glass-tube fermenters to be described.

b) Additional fermentation tests in 4-gallon pilot-scale fermenters were carried out on worts brewed for the purpose in our experimental brewing laboratory.

Laboratory Fermentations on Brewery Worts

Worts were obtained from the brewery as prepared respectively in the nickel and in the conventional equipment. These worts containing respectively 0.96 and 0.06 part per million of nickel were set up in the glass-tube fermenters and pitched at several pitching levels using a lager yeast as obtained from a local brewery. Fermentations were conducted at 10° C (50° F).

The laboratory fermenters consist of 4-foot long glass tubes of 1⅞" diameter with a capacity of about two liters. The yeast levels employed for pitching were

equivalent to 1.0, 0.5, and 0.25 pound of liquid yeast per barrel. Figures 1 and 2 show the course of the laboratory fermentation for the 0.25 and 1.0 pound per barrel levels. It will be seen that the fermentation of the wort prepared in the

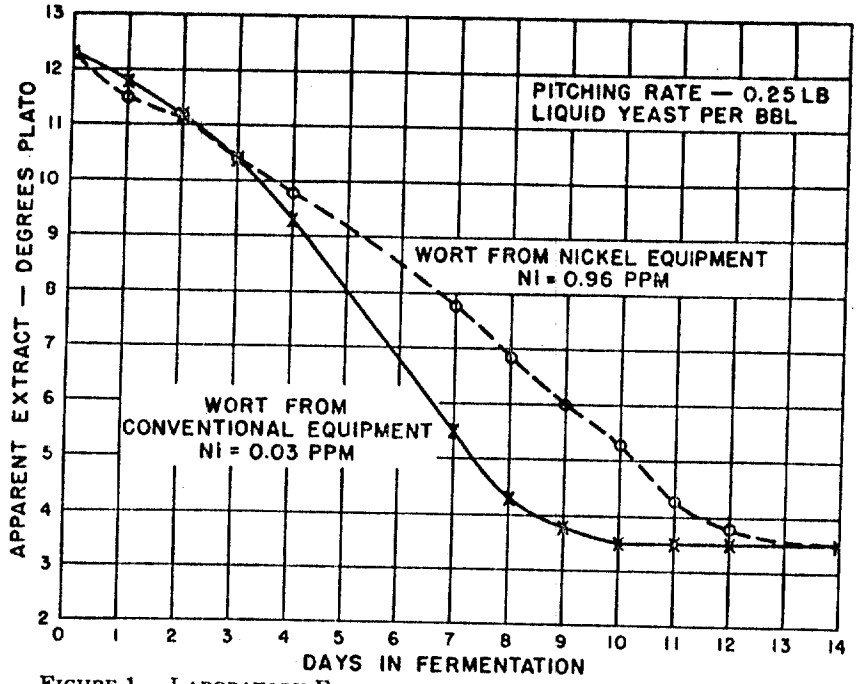

FIGURE 1. LABORATORY FERMENTATION AT 50°F OF BREWERY WORTS

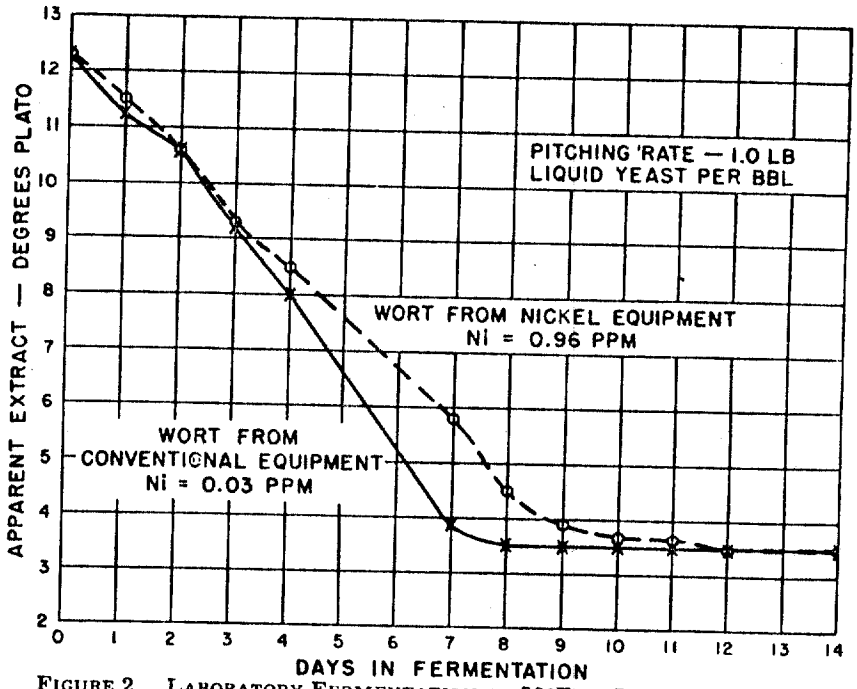

FIGURE 2. LABORATORY FERMENTATION AT 50°F OF BREWERY WORTS

nickel equipment starts slowing up after about the third day. After about seven days for the higher yeast levels and ten days for the lower, the fermentations are nearly completed for the wort from the conventional equipment. On the other hand, the corresponding nickel worts still have three or four days to go before fermentation is complete. As would be expected, this effect of nickel is more pronounced on the fermentation conducted at the low pitching rate. The fermentation course for the run pitched at 0.5 pound per barrel was intermediate. The results obtained on these small fermentations thus seem to follow the pattern of the brewery fermentations as reported to us.

A similar laboratory fermentation experiment was conducted on a sample of this brewery's wort made in the nickel equipment which contained 2.2 parts per million of nickel again in comparison with the control wort. In this experiment, one portion of the nickel wort was boiled with 0.25 pound of soy flakes per barrel of wort. After boiling, this wort was filtered before pitching, the comparison worts being similarly treated but without soy. All fermentations were pitched at the low level of 0.25 pound liquid yeast per barrel. The results of this test are shown in Figure 3. It is seen that the treatment with soy completely overcomes the inhibitory influence of the nickel on the fermentation.

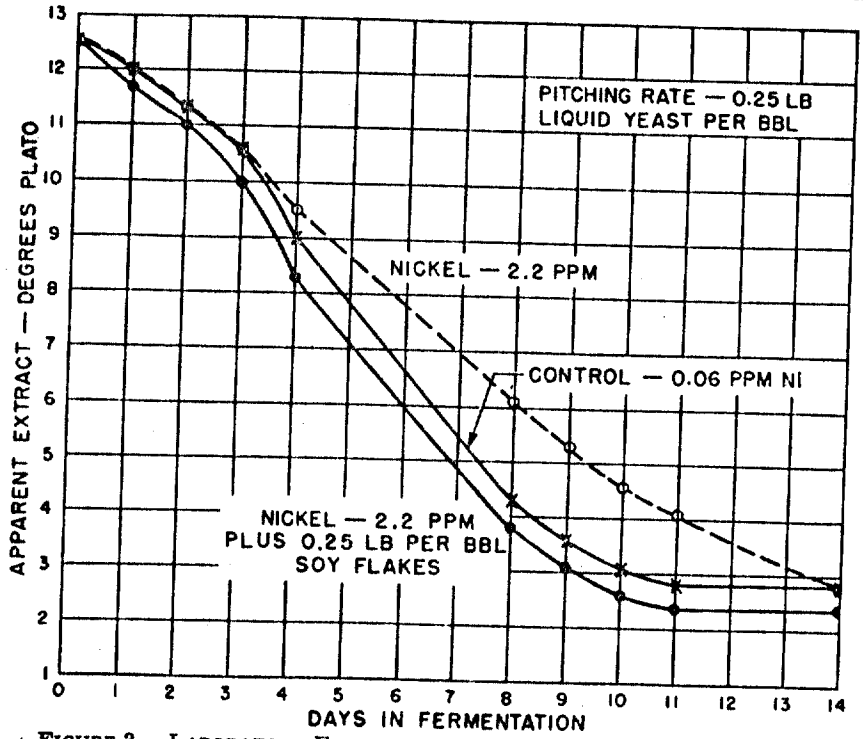

FIGURE 3. LABORATORY FERMENTATION AT 50°F OF BREWERY WORTS

Experimental Brewing Laboratory Tests

Further data were obtained on larger scale (4-gallon) fermentations as carried out on worts brewed in our experimental brewing laboratory and to which several levels of nickel salts were added at mashing. In each run four brews (70% malt-30% corn grits and 0.33 pound hops per barrel) were made comprising a control and three brews to which varying levels of nickel were added. Amounts of nickel based on the expected volume of 12°P wort of 0.5, 2.0, and 5.0 parts per million were employed, added at the beginning of mashing. The mashes appeared to proceed normally, with normal run-off times.

The distribution of nickel during processing was determined by sampling (a) the wort after boiling, (b) the beer after fermentation, (c) the yeast, and (d) the finished beer. To determine possible cumulative effects of nickel on fermentations, these runs were repeated in four successive fermentations, the respective yeast crops being used to pitch succeeding corresponding brews.

Table V shows the distribution of the nickel at the different processing stages. In the control brews, it will be seen that the nickel content of the worts is, as expected, very low, generally in the neighborhood of 0.05 part per million. In the brews containing nickel added in the mash tub, most of the added nickel appears in the wort. Only a relatively small proportion of nickel remains in the spent grains. The nickel remaining in the wort evidently is mostly in the soluble form and hence also only a minor amount is precipitated with the trub.

However, during fermentation, (in nickel-free equipment), a substantial proportion of the added nickel is removed along with the yeast. As a result, the beer shows considerably lower nickel content than the wort from which it is prepared. The proportion remaining in the beer constitutes about 30 to 50% of the amount in the filtered wort depending on initial nickel level; the higher percentages remaining in the brews having the higher nickel levels originally.

The data concerning nickel content of the yeast crops contained in Table V show a considerable increase in nickel content for the separated yeast from the nickel-containing brews. The original pitching yeast contained 5 parts per million of nickel on the dry basis. For brews containing 0.5 part per million of nickel in the wort, the separated yeast crop is shown to have in the neighborhood of 100 parts per million of nickel calculated on dry basis. For 2 parts per million in the wort, the yeast crop contains between 300 and 450 parts per million nickel, dry basis, while for the 5 parts per million brews about 600 parts per million of nickel were found in the yeast crops. In a previous paper from our laboratories[9], dealing with iron and copper, it was shown that there is a similar concentration of these metals in the yeast when they occur in the wort at higher-than-normal levels.

The course of the fermentation of the consecutive brews represented by the data of Table V is shown graphically in Figures 4 and 5. It is seen that for this particular wort a slight retarding effect in the first generation (Figure 4) is indicated for the brews containing 2 and 5 parts per million of nickel while the fermentation curve for the 0.5 part per million nickel is very close to the control.

However, in succeeding brews pitched with the yeasts, the retarding effect due to nickel apparently becomes more pronounced. This is evident in the curves for the third generation brews shown in Figure 5 by the greater spread between the curves representing fermentations at the different nickel levels.

Comparison of fermentation curves in Figure 4 with the curves in Figure 2 illustrates variations that may be expected in the fermentation response of different worts. In the case of Figure 4 the wort as prepared in our experimental brewing laboratory was fermented in 4-gallon pilot-scale fermenters while Figure 2 represents the course of fermentation in the long glass-tube fermenters of the worts as received from the brewery concerned with this problem. Aside from this difference in fermenter size, the two fermentations were identical. In both cases pitching rates were the same and the yeast used represented the first generation exposed to the nickel-containing wort. Yet, the differences in response to traces of nickel as between the two worts are evident and can be attributed chiefly to differences in their composition.

Considering the numerically high concentrations of nickel found in the yeast crops, the effect shown is not as great as might be expected from a highly toxic material if it were actually assimilated by the yeast cell. Possibly therefore the apparent increase in the nickel content of the yeast represents merely a precipitation of an insoluble nickel complex along with yeast, rather than actual assimilation by the yeast cell.

TABLE V

Distribution of Nickel in Pilot Brewery Tests

Samples	Controls no nickel added				0.5 ppm nickel added				2.0 ppm nickel added				5.0 ppm nickel added			
	Generation				Generation				Generation				Generation			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
a) Nickel added to mash—ppm*	nil	nil	nil	nil	0.50	0.50	0.50	0.50	2.0	2.0	2.0	2.0	5.0	5.0	5.0	5.0
	Nickel Found—Parts Per Million															
b) Wort	0.06	0.04	0.01	0.06	0.50	0.52	0.50	0.48	1.9	1.7	1.5	1.6	4.2	4.0	3.8	3.9
c) Filtered wort	0.05	0.04	0.01	0.04	0.50	0.45	0.50	0.46	1.4	1.6	1.6	1.5	4.1	4.0	3.5	3.2
d) Nickel dropout with spent grains (by difference, a minus b)	—	—	—	—	0.00	0.00	0.00	0.02	0.1	0.3	0.5	0.4	0.8	1.0	1.2	1.1
e) Nickel dropout with trub (by difference, b minus c)	0.01	0.00	0.00	0.02	0.00	0.07	0.00	0.02	0.5	0.1	—	0.1	0.1	0.0	0.3	0.7
f) Storage beer	0.01	0.01	0.01	0.01	0.16	0.15	0.13	0.11	0.56	0.49	0.49	0.50	1.7	1.8	2.0	1.5
g) Finished beer	0.02	0.02	0.02	0.01	0.19	0.15	0.17	0.10	0.60	0.50	0.49	0.49	1.7	1.8	1.9	1.4
h) Nickel dropout in fermenter (by difference, c minus f)	0.04	0.03	0.00	0.03	0.34	0.30	0.37	0.39	0.8	1.2	1.1	1.0	2.4	2.2	1.5	1.7
Yeast crops (dry basis)	2.0	1.0	9.4	16.2	90	116	133	90	290	450	456	335	560	630	600	500
Soluble nickel in beer as % of Ni in filtered wort	—	—	—	—	38	33	34	22	43	31	31	33	42	45	54	44

*Based on ppm in expected volume of 12°P wort.

724

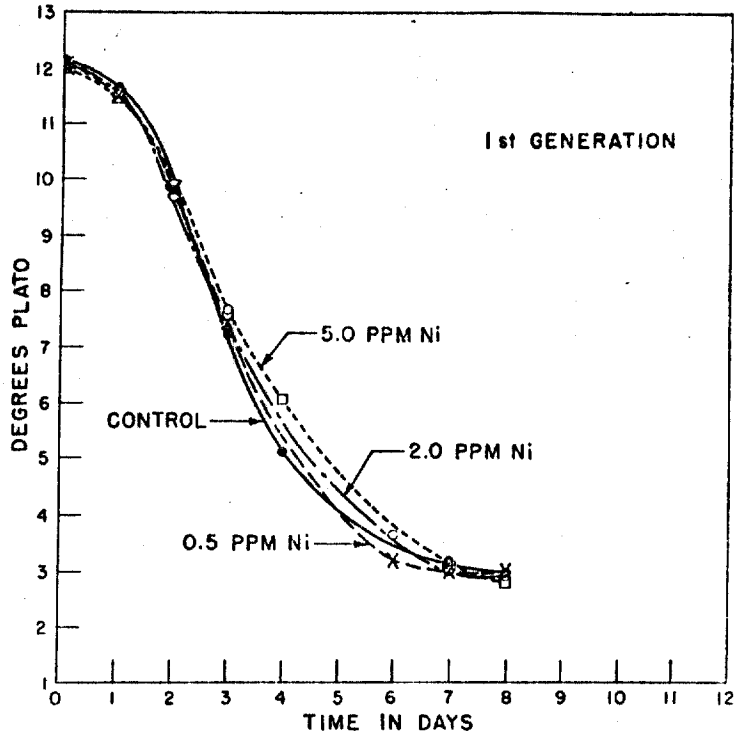

FIGURE 4. FERMENTATION CURVES OF WORTS PREPARED IN EXPERIMENTAL BREWING LABORATORY CONTAINING NICKEL ADDED DURING MASHING

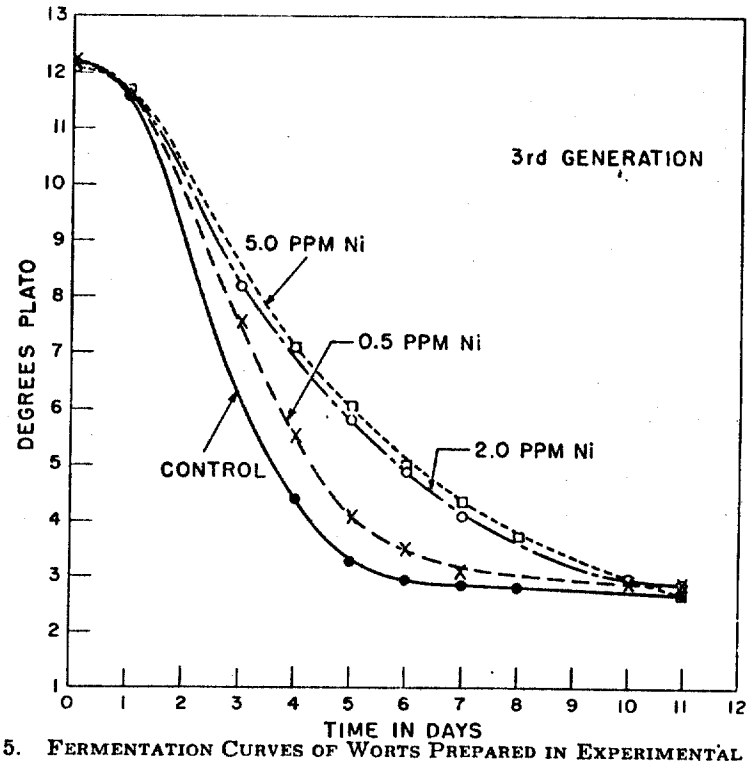

FIGURE 5. FERMENTATION CURVES OF WORTS PREPARED IN EXPERIMENTAL BREWING LABORATORY CONTAINING NICKEL ADDED DURING MASHING

The finishing operations performed on the beer in nickel-free equipment did not appreciably affect the nickel level; the finished beer has essentially the same nickel content as the storage beer.

Nickel as a Cause of Wildness

In a paper published from our laboratories in 1949, we described the behavior of gushing beer as being due to the formation of nuclei having surface characteristics of such a nature as to permit them to trigger the explosive evolution of CO_2 when the container is opened. A number of factors which could readily contribute to the formation of such nuclei in beer and thus serve to act as causes for wildness in beer were mentioned in that paper. These included trace metals, oxidation, maintenance of the beer at extremely low temperatures, etc. At that time, besides iron, nickel was recognized as one of the metals capable of producing this condition.

In our experiments it was found that nickel under certain conditions was not only capable of producing wildness, but actually appeared more potent than equivalent amounts of iron in this respect.

Table VI shows the results of tests of wildness on a series of beers containing respectively added iron and nickel. Following the addition of the metals these beers were stored at a temperature of −2°C (28°F) for the periods shown. After storage, the beers were removed from the cold box, allowed to warm up to room temperature (25°C — 77°F) — held at this temperature for one hour and then opened. Figures in the table reflect the relative amount of beer lost by gushing when the caps were removed.

TABLE VI

Wildness At 25° C After Storage At −2° C for Varying Periods

Additions	3 weeks	6 weeks	9 weeks	12 weeks	15 weeks
none-control	Q	Q	C-1	Q	C-¼
2 ppm nickel	40	65	136	100	175
2 ppm iron	C-¼	5	2	5	30

	2 weeks	3 weeks	5 weeks	7 weeks
none-control	C-¼	Q	C-¼	Q
0.5 ppm nickel	2	1	C-1-½	5
2.0 ppm nickel	45	40	20	85

Figures in table denote volume of beer overflowing when bottle was opened. "Q" denotes quiet when opened; "C" means beer formed a collar of foam of the height indicated in inches.

Summary

A precise method for the colorimetric determination of traces of nickel in malt beverages has been developed. By means of this method, the nickel content of brewing raw materials, commercial beers, and the distribution of nickel in the various steps of processing beer has been determined. The nickel content of brewing raw materials is generally low and commercial beers usually have a nickel content in the range of less than 0.05 part per million. If the nickel content is higher, pickup of the metal is indicated. Nickel in trace amounts, may under certain conditions, slow fermentation. The extent of this effect depends upon many factors besides the nickel level. When nickel is present in the wort a considerable proportion is separated with the yeast. In pilot brewery tests, high nickel levels in the yeast caused only moderate slowing of the fermentation. The finishing operations, performed in nickel-free equipment, did not affect the nickel levels in the beer and the finished beer contained 30-50% of the nickel originally in the wort. The presence of high levels of nickel in the finished beer seems to predispose it to the development of wildness.

Acknowledgement

The writers wish to thank Miss Rachelle Rothenberg and Mr. John Wylie for their help in obtaining much of the experimental and pilot brewery data.

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18(63) 291-304 (1955)

Traces of Nickel in Beers and Brewing†

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Introduction

THE influence of trace metals on the properties of beer has long occupied the attention of our laboratories. This is evidenced by papers which we have published at different times on the development of simple analytical procedures for determining trace metals in beer. Other papers similarly have discussed the effects of certain of these metals in brewing.

The present paper concerns nickel and will describe a simplified procedure for determining traces of this element in beer and brewing materials, and its effects. Our laboratories' concern with the effects of nickel goes back many years to a study of the influence of a series of metals, which included nickel, in catalyzing oxidation reactions in beer (1). This interest was, however, heightened when, in our studies of the problem of wild beer some years ago (2), we pointed out that traces of nickel as well as iron and other metals greatly accelerated tendencies of beers to develop gushing characteristics.

At that time a survey of the literature indicated the need for a practical, but at the same time sensitive and precise, method for the determination of nickel. One which would be sufficiently precise to determine amounts of the order of hundredths of a part per million seemed necessary. Such a method was accordingly developed and has recently been published (3).

Our interest in nickel recently was revived when a brewery consulted us about a fermentation problem which seemed, in their case, to arise as a result of the use of nickel equipment in the brewhouse. They had noticed a somewhat more sluggish fermentation in worts produced in nickel equipment than in companion brews from conventional equipment.

Our general interest in trace metals in the past has centered primarily on effects on shelf-life, gushing, foam, and similar properties of the finished product. There are, however, other effects which various trace metals can exert. In particular, effects on yeast and fermentation have long been a subject for consideration by fermentation chemists.

With the aid of the analytical method for nickel, already available, we were able accurately to follow variations in nickel content during processing at the above brewery and at the same time to confirm experimentally the possibility of effects on fermentations under certain conditions. The details of this study form the basis for the present paper.

In presenting this data we do not at all imply that nickel-containing equipment is unsuitable for use in the brewery. Much depends, of course, on the particular nature of the equipment. There is no perfect metal

†Paper presented at the Annual Meeting of the American Society of Brewing Chemists, in Philadelphia, and published in the *Proceedings* of the Society, 1955.

which can serve as a material of construction for all brewing equipment. For each use there are some advantages to each metal and also some disadvantages. Other metals such as copper and iron, which may be shown to have inhibitory effects on yeast growth or fermentation, or which are detrimental to beer stability, have a long and time-honored usage in the brewing industry. Certain nickel alloy equipment no doubt also has a definite place. On the other hand, pure nickel is indicated by the literature, and by the experience and tests to be reported here, as not as well suited for use, at least in the brewhouse. It is of importance in the use of any metal that precautions of proper design and maintenance be taken to prevent undue pickup of the metal.

Review of Literature

There are but few references to nickel in the brewing literature. Hagues (4) in 1931 discussed metallic contamination of beer in English breweries. He conducted an experiment in which a fermentation was carried out in a glass beaker in the presence of a piece of brightly polished nickel tubing. Fermentations were also conducted in small-scale fermenting vessels made of nickel and Monel metal. The surface-volume relationships of these small vessels were compared with regular brewery vessels and as a result, it was assumed that, based on their measurements, the analytical values for nickel pickup for the beer and yeast in these laboratory fermentations could be divided by a factor of 8.7 to obtain the expected results in a brewery-size vessel. Such data indicated pickup of nickel from pure nickel fermentors in the range of 1.7 to 2.4 parts per million in beer and from Monel vessels, much less, *i.e.*, 0.3 to 0.6 part per million. He therefore concluded that pure nickel would be undesirable for the construction of fermentors whereas Monel would be satisfactory. Small quantities of nickel gave the beer a "rough" flavor and produced a haze in a short time. He also mentioned that "the prolonged effect of this metal on yeast is unknown."

Mason (5) in 1940 reported on loss of weight due to corrosion of metal test assemblies in wort and beer at various stages of brewing under operating conditions. Considerable differences are apparent in his data as between loss in weight of pure nickel and Monel on the one hand, and the nickel alloy, Inconel, on the other. Moreover, of special interest, in view of the case history to be reported in the present paper, is the fact that corrosion for all three metals was appreciably greater at brewhouse stages than in fermentation or storage. This appears to be due largely to the temperature differences, in view especially of his results for different sections of the Baudelot cooler, although other factors may also be involved in the apparent differences between the effect of wort and fermented beer on the test pieces.

Although his tests regarding tolerance of fermented beer or ale to nickel salts present in solution were limited to observations only of haze, color, and flavor, Mason concluded from his data that Inconel is practically completely resistant to beer and that even nickel and Monel are sufficiently resistant to represent suitable materials for various pieces of brewing equipment, Monel being superior to nickel.

The paper by Essery (6) in 1945 on the estimation of traces of metals in wort included a method for nickel. Being essentially an analytical paper, it contained no data relating to the effects of nickel.

Doran (7) in 1946 reported on seven years' experience with the use of nickel-containing equipment under practical brewery conditions. He stated that after seven years' use, the inside nickel surface of the brew kettle was in excellent condition. This appears to have been the only large piece of equipment in which nickel surfaces were in contact with the product.

A paper by Andrews and Harrison (8) appeared in 1954 which proposed a colorimetric method employing *alpha*-furildioxime for the determination of nickel. This paper, which is devoted mostly to the analytical method, gives a few values for nickel content of beers and brewing ma-

terials. These values are in the range of nickel results found by us.

Method for Colorimetric Determination of Traces of Nickel

The general procedure as finally worked out (3) may be outlined as follows:† The sample is dry-ashed and the ash is taken up in dilute hydrochloric acid and extracted with a carbon tetrachloride solution of dithizone to remove the interference of copper and iron. The aqueous layer is treated with dimethylglyoxime and bromine and then made alkaline, developing the brown-to-red nickel color. The intensity of the color, which is directly related to the concentration in the range 0.0 to 0.5 part per million, is then read in a photometer. After the ashing step the reactions are conducted in a stoppered 15-ml graduated centrifuge tube and then transferred to a 10-ml volumetric flask for color development.

Nickel Content of Brewing Raw Materials

Of the raw materials entering into a brew, water is in the largest proportion, with the malt and adjuncts next. Table I gives the results on a series of scattered samples of American brewing waters varying in total solids content from 66 to 570 parts per million. Nickel was found to be absent in all samples within the sensitivity of the method, which is about 0.01 part per million. The water, in general, probably contributes negligible amounts of nickel, if any, to the finished product.

Table I: Nickel in brewing waters

Source	Total Solids	Nickel-ppm
New Jersey	66	0.00
Maryland	101	0.00
Ohio	201	0.00
Wisconsin	131	0.00
Texas	570	0.00
California	—	0.00

†*This procedure is described in detail on p. 285 of this issue.*

Table II gives the results on analysis of a typical series of malts, adjuncts, and hops. In the case of the malts, the nickel content varied from 0.09 to 0.90 part per million. In all the malts we have examined to date, we have never encountered any having nickel in excess of 1 part per million; generally, the nickel content is much below this level. A malt with even 0.9 part per million of nickel would contribute little more than about 0.1 part per million to the mash.

Table II: Nickel in brewing materials

Sample	Nickel-ppm
Malt A	0.50
Malt B	0.24
Malt C	0.13
Malt D	0.64
Malt E	0.90
Malt F	0.20
Malt G	0.09
Corn Grits A	0.10
Corn Grits B	0.31
Refined Grits A	0.05
Refined Grits B	0.09
Corn Flakes A	0.16
Corn Flakes B	1.77
Corn Flakes C	0.05
Corn Flakes D	0.10
Rice A	0.66
Rice B	0.35
Brewers' Soy Flakes	0.02
Hops A	3.00
Hops B	1.11
Hops C	1.75
Hops D	0.87

The adjuncts usually have been found to have very low nickel contents, except in the case of an occasional sample of corn flakes. This slightly higher level encountered in some corn flakes possibly represents a slight pickup from nickel equipment during processing of the material.

In the few samples of rices examined, the level of nickel has been similar to that found for malt.

The nickel content of hops has been found to be appreciably higher than some

of the other raw materials, values being up to 3 parts per million in the hop samples examined. However, because the total amount of hops used per barrel is so small compared with malt and adjuncts, it is obvious that the actual contribution of nickel to the brew from the hops is negligible. Hops containing 3 parts per million of nickel when used at a rate of 0.5 pound per barrel would add to the nickel content of the wort about 0.006 part per million assuming that it was all soluble.

Based on the above values, the nickel content of worts should generally be no more than about 0.1 part per million, and it is usually below this figure.

Nickel Content of Commercial Beers

A series of beer samples representing commercial products of breweries in the United States and Canada and some foreign countries, was examined. The nickel content of these samples is shown in Tables III and IV.

Table III: Typical nickel contents of beers

Brewery	Location	Nickel-ppm
1	U.S. — east	0.04
2	U.S. — east	0.01
3	U.S. — east	0.03
4	U.S. — midwest	0.04
5	U.S. — midwest	0.03
6	U.S. — midwest	0.02
7	U.S. — south	0.02
8	U.S. — west	0.03
9	U.S. — west	0.03
10	U.S. — southwest	0.04
11	Canada	0.04
Beers from breweries stated to use nickel or nickel-alloy equipment		
12	U.S. — east	0.17
13	U.S. — east	0.10
14	U.S. — east	0.04
15	U.S. — east	0.02
16	U.S. — midwest	0.01
17	U.S. — midwest	0.07
18	U.S. — midwest	0.04
19	Canada	1.80
20	Canada	0.44

Table IV: Nickel contents of some foreign beers

Brewery	Location	Nickel-ppm
1	Cuba	0.01
2	Denmark	0.01
3	England	0.01
4	Malta	0.02
5	Mexico	0.03
6	Norway	0.01
7	Venezuela	0.06

The first group of samples in Table III represents beers having nickel contents from about 0.01 to 0.04 part per million and is representative of the bulk of beer samples examined. The second group of results in Table III represents beers from breweries reported to contain some nickel equipment. The results in the latter group indicate that while nickel content of many of the samples is low, they are not all in the consistently low range noted for the first group. Those samples showing nickel levels in the higher range appear to represent pickup of the metal in the brewery. In this cursory survey no attempt was made to correlate the level of nickel found with the extent or kind of equipment involved.

The results in Table IV for beers brewed in foreign countries show that here again, for the samples investigated, the nickel contents are quite low.

Effect of Nickel on Fermentation

As stated, we were recently called upon to investigate a problem at a brewery which had installed nickel equipment in the brewhouse a few years earlier. This gave us an opportunity to study the effects of nickel on fermentation.

Following installation of this equipment, brews had been made both in the nickel equipment and in the older, conventional equipment. It had been found that the fermentations of the worts made in the nickel equipment were appreciably more sluggish than for the regular brews. The

temperature rise was also stated to be lower. To obtain a normal fermentation it was found necessary, as a regular practice, to use the two sets of equipment alternately and to add to nickel equipment brews some actively fermenting wort from conventional equipment on the third day of fermentation.

The nickel equipment at this brewery comprised a cereal cooker, mash tub, lauter tub, and kettle. All were clad with pure nickel on the surfaces exposed to the brews. The conventional equipment on the other hand consisted of iron, except for the kettle which was, as usual, copper.

After a suitably precise analytical procedure for the determination of nickel had been developed, the first step in our study of the problem was the determination of the nickel content of a series of wort samples from this brewery representative of the two types of equipment. We were able to confirm a marked difference in nickel content reflecting pickup from the equipment used. Nickel contents of 0.03 and 0.06 part per million were found for two kettle worts made in the conventional equipment, as compared with 0.96 and 2.2 parts per million for two worts prepared in the nickel equipment.

Since the beers were customarily mixed during fermentation, samples of finished beer representative of the two types of equipment could not be obtained separately. However, examination of samples of finished, blended beer showed 0.25 part per million of nickel to be present. This, of course, represents an appreciable level of nickel, and the sample would definitely be grouped with the higher nickel beers in Table III.

Laboratory fermentations

In all studies of effects of so-called yeast poisons in trace concentrations, it is necessary to take into consideration a wide variety of factors which by influencing cell metabolism may obscure the effects under investigation. Thus, the composition of the wort, the initial yeast cell concentration (pitching rate), fermentation temperature, aeration, and the surface-volume relationships of the fermentation vessels are all of the utmost importance in this connection. Retardation effects on fermentation therefore may or may not result from trace quantities of certain metals depending upon the extent to which cell-protective factors in the media, cell concentration, or stimulation of reproduction, may offset the particular injurious effect.

Thus, as a matter of practical experience, it is well known that rousing or aeration, or use of higher pitching rates or temperatures, are all sometimes resorted to when, for any reason, brewery fermentations become sluggish. In the same way, the addition of relatively small amounts of soy flakes is also known to stimulate yeast fermentations; this is now fairly common practice in certain conditions.

The possible influence of such factors was apparent in the preliminary stages of our experiments on this problem. We found, for example, that laboratory fermentations of worts from several local breweries to which varied amounts of nickel had been added did not behave uniformly. The wort from one brewery exhibited the fermentation-retarding effect of small amounts of nickel whereas wort from another brewery containing the same amount of added nickel fermented normally. In this case, as our subsequent experiments will indicate, the differences could no doubt be ascribed to the use of a small amount of soy in the case of the latter brewery.

Similarly, differences were observed along the same lines in small-scale laboratory fermentations, depending upon the degree of cell respiration permitted by aeration and stirring during the fermentation period. Thus, in laboratory flasks or bottles a definite retarding effect on fermentation due to 5 parts per million of nickel was observed if the flasks were undisturbed, whereas fermentation was normal if the flasks were shaken.

Based on these preliminary studies, further experimentation was carried out in such a way as to permit the control of

factors like aeration, air exposure, and initial yeast concentration. Two types of fermentation experiments were carried out.

a) On the relatively small quantities of wort samples received from the brewery in question, small-scale laboratory fermentations were conducted in long glass-tube fermentors to be described.

b) Additional fermentation tests in 4-gallon pilot-scale fermentors were carried out on worts brewed for the purpose in our experimental brewing laboratory.

Laboratory fermentations of brewery worts

Worts were obtained from the brewery as prepared respectively in the nickel and in the conventional equipment. These worts, containing respectively 0.96 and 0.06 part per million of nickel, were set up in the glass-tube fermentors and pitched at several pitching levels, using a lager yeast as obtained from a local brewery. Fermentations were conducted at 10°C (50°F).

The laboratory fermentors consist of 4-foot long glass tubes of 1⅞-inch diameter with a capacity of about two liters. The yeast levels employed for pitching were equivalent to 1.0, 0.5, and 0.25 pound of liquid yeast per barrel. Figures 1 and 2 show the course of the laboratory fermentation for the 0.25 and 1.0 pound per barrel levels. It will be seen that the fermentation of the wort prepared in the nickel equipment starts slowing up after about the third day. After about seven days for the higher yeast levels and ten days for the lower, the fermentations are nearly completed for the wort from the conventional equipment. On the other hand, the corresponding nickel worts still have three or four days to go before fermentation is complete. As would be expected, this effect of nickel is more pronounced in the fermentation conducted at the low pitching rate. The fermentation course for the run pitched at 0.5 pound per barrel was intermediate. The results obtained on these small fermentations thus seem to follow the pattern of the brewery fermentations as reported to us.

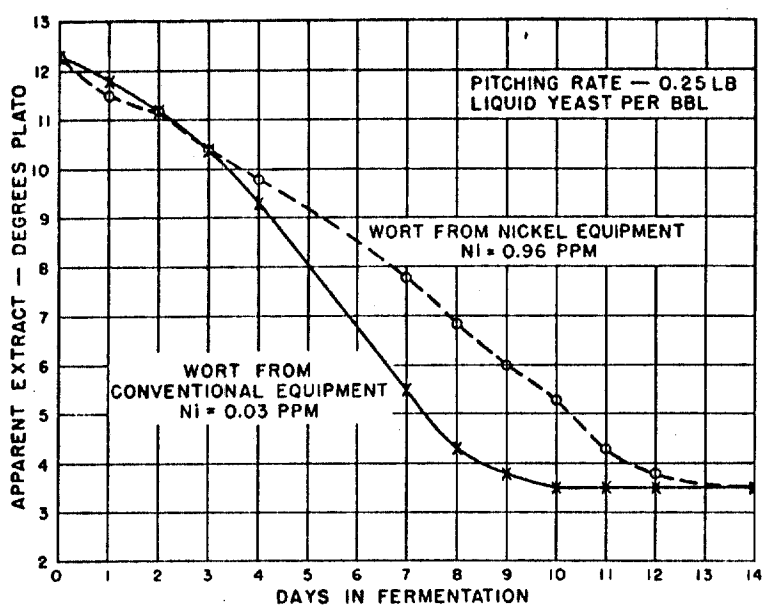

Figure 1: Laboratory fermentation at 50°F of brewery worts.

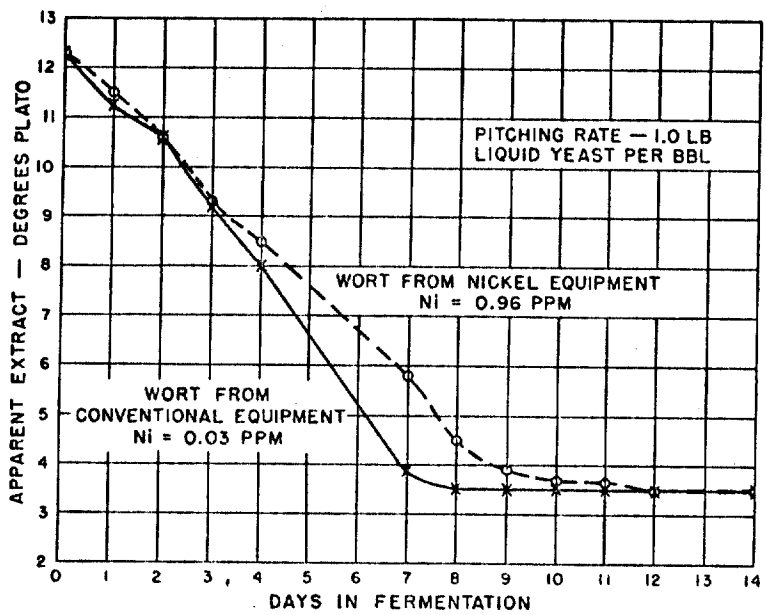

Figure 2: Laboratory fermentation at 50°F of brewery worts.

A similar laboratory fermentation experiment was conducted on a sample of this brewery's wort made in the nickel equipment, again in comparison with the control wort. In this experiment, one portion of the nickel wort, which contained 2.2 parts per million of nickel, was boiled with 0.25 pound of soy flakes per barrel of wort. After boiling, this wort was filtered before pitching, the comparison worts being similarly treated but without soy. All fermentations were pitched at the low level of 0.25 pound liquid yeast per barrel. The results of this test are shown in Figure 3. It is seen that the treatment with soy completely overcomes the inhibitory influence of the nickel on the fermentation.

Experimental brewing laboratory tests

Further data were obtained on larger scale (4-gallon) fermentations as carried out on worts brewed in our experimental brewing laboratory and to which several levels of nickel salts were added at mashing. In each run four brews (70% malt, 30% corn grits and 0.33 lb of hops per barrel) were made, comprising a control and three brews to which varying levels of nickel were added. Amounts of nickel, based on the expected volume of 12°P wort, of 0.5, 2.0, and 5.0 parts per million were employed, added at the beginning of mashing. The mashes appeared to proceed normally with normal run-off times.

The distribution of nickel during processing was determined by sampling (a) the wort after boiling, (b) the beer after fermentation, (c) the yeast, and (d) the finished beer. To determine possible cumulative effects of nickel on fermentations, these runs were repeated in four successive fermentations, the respective yeast crops being used to pitch succeeding corresponding brews.

Table V shows the distribution of the nickel at the different processing stages. In the control brews, it will be seen that the nickel content of the wort is, as expected, very low, generally in the neighborhood of 0.05 part per million. In the brews containing nickel added in the mash tub, most of the added nickel appears in

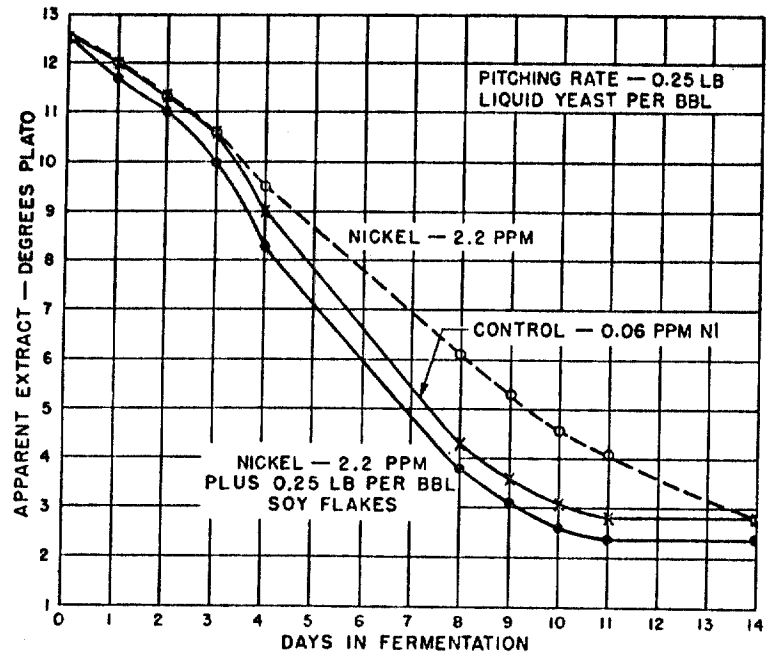

Figure 3: Laboratory fermentation at 50°F of brewery worts.

the wort. Only a relatively small proportion of nickel remains in the spent grains. The nickel remaining in the wort evidently is mostly in the soluble form and hence also only a minor amount is precipitated with the trub.

However, during fermentation (in nickel-free equipment), a substantial proportion of the added nickel is removed along with the yeast. As a result, the beer shows a considerably lower nickel content than the wort from which it is prepared. The proportion remaining in the beer constitutes about 30 to 50% of the amount in the filtered wort depending on initial nickel level, the higher percentages remaining in the brews having the higher nickel levels originally.

The data in Table V concerning nickel content of the yeast crops show a considerable increase in nickel content for the separated yeast from the nickel-containing brews. The original pitching yeast contained 5 parts per million of nickel on the dry basis. For brews in which the wort contained 0.5 parts per million of nickel, the separated yeast crop is shown to have in the neighborhood of 100 parts per million of nickel calculated on dry basis. The yeast crop from brews with 2 parts per million of nickel contained between 300 and 450 parts per million of nickel, dry basis; the yeast separated from the 5 parts per million brews contained about 600 parts per million of nickel (dry basis). In a previous paper from our laboratories (9), dealing with iron and copper, it was shown that there is a similar concentration of these metals in the yeast when they occur in the wort at higher than normal levels.

Table V: Distribution of nickel in pilot brewery tests

Samples	Controls no nickel added				0.5 ppm nickel added				2.0 ppm nickel added				5.0 ppm nickel added			
	Generation				Generation				Generation				Generation			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
a) Nickel added to mash—ppm*	nil	nil	nil	nil	0.50	0.50	0.50	0.50	2.0	2.0	2.0	2.0	5.0	5.0	5.0	5.0
	NICKEL FOUND — PARTS PER MILLION															
b) Wort	0.06	0.04	0.01	0.06	0.50	0.52	0.50	0.48	1.9	1.7	1.5	1.6	4.2	4.0	3.8	3.9
c) Filtered wort	0.05	0.04	0.01	0.04	0.50	0.45	0.50	0.46	1.4	1.6	1.6	1.5	4.1	4.0	3.5	3.2
d) Nickel dropout with spent grains (by difference, a minus b)	—	—	—	—	0.00	0.00	0.00	0.02	0.1	0.3	0.5	0.4	0.8	1.0	1.2	1.1
e) Nickel dropout with trub (by difference, b minus c)	0.01	0.00	0.00	0.02	0.00	0.07	0.00	0.02	0.5	0.1	—	0.1	0.1	0.0	0.3	0.7
f) Storage beer	0.01	0.01	0.01	0.01	0.16	0.15	0.13	0.11	0.56	0.49	0.49	0.50	1.7	1.8	2.0	1.5
g) Finished beer	0.02	0.02	0.02	0.01	0.19	0.15	0.17	0.10	0.60	0.50	0.49	0.49	1.7	1.8	1.9	1.4
h) Nickel dropout in fermentor (by difference, c minus f)	0.04	0.03	0.00	0.03	0.34	0.30	0.37	0.39	0.8	1.2	1.1	1.0	2.4	2.2	1.5	1.7
Yeast crops (dry basis)	2.0	1.0	9.4	16.2	90	116	133	90	290	450	456	335	560	630	600	500
Soluble nickel in beer as % of Ni in filtered wort	—	—	—	—	38	33	34	22	43	31	31	33	42	45	54	44

*Based on ppm in expected volume of 12°P wort.

725

The course of the fermentation of the consecutive brews represented by the data of Table V is shown graphically in Figures 4 and 5. It is seen that for this particular wort a slight retarding effect in the first generation (Figure 4) is indicated for the brews containing 2 and 5 parts per million of nickel whereas the fermentation curve for the 0.5 part per million nickel brew is very close to the control.

However, in succeeding brews pitched with the yeasts, the retarding effect due to nickel apparently becomes more pronounced. This is evident in the curves for the third generation brews shown in Figure 5 from the greater spread between the curves representing fermentations at the different nickel levels.

Comparison of fermentation curves in Figure 4 with the curves in Figure 2 illustrates variations that may be expected in the fermentation response of different worts. In the case of Figure 4, the wort as prepared in our experimental brewing laboratory was fermented in 4-gallon pilot-scale fermentors while Figure 2 represents the course of fermentation in the long glass-tube fermentors of the worts as received from the brewery concerned with this problem. Aside from this difference in fermentor size, the two fermentations were identical. In both cases pitching rates were the same, and the yeast used represented the first generation exposed to the nickel-containing wort. Yet, the differences between the two worts in response to traces of nickel are evident and can be attributed chiefly to differences in their composition.

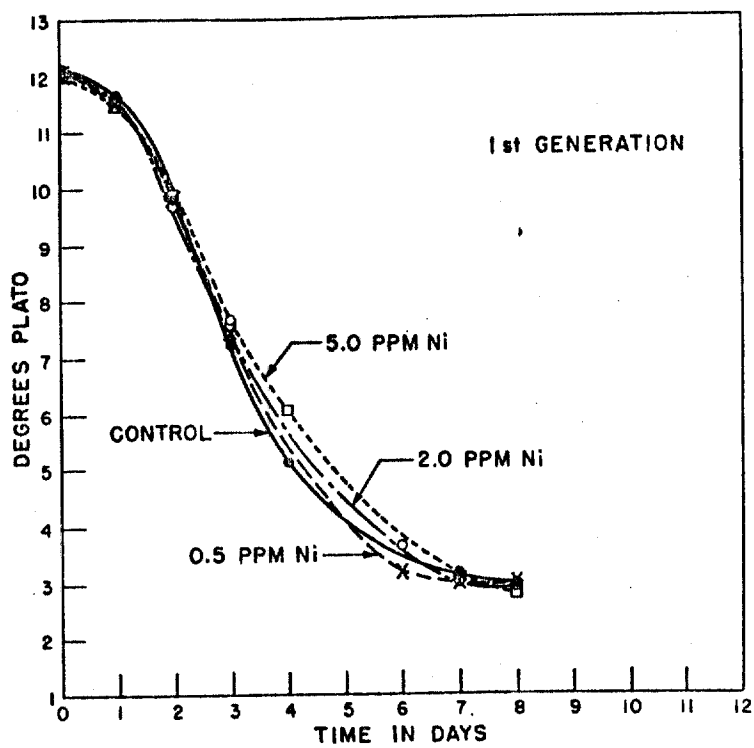

Figure 4: Fermentation curves of worts prepared in experimental brewing laboratory containing nickel added during mashing.

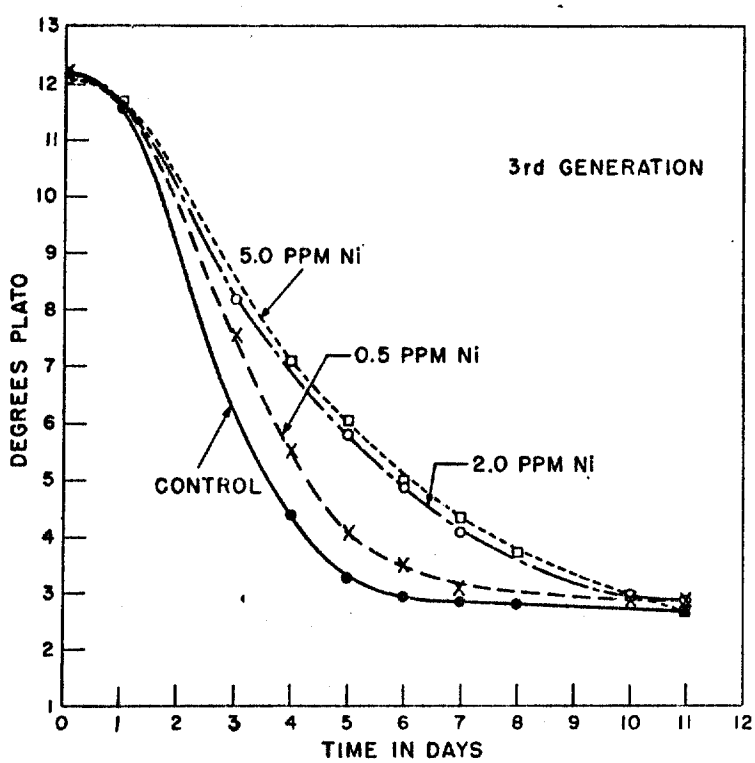

Figure 5. Fermentation curves of worts prepared in experimental brewing laboratory containing nickel added during mashing.

Considering the numerically high concentrations of nickel found in the yeast crops, the effect shown is not as great as might be expected from a highly toxic material if it were actually assimilated by the yeast cell. Possibly, therefore, the apparent increase in the nickel content of the yeast represents merely a precipitation of an insoluble nickel complex along with yeast, rather than actual assimilation by the yeast cell.

The finishing operations performed on the beer in nickel-free equipment did not appreciably affect the nickel level; the finished beer has essentially the same nickel content as the storage beer.

Nickel As a Cause of Wildness

In the paper published from our laboratories in 1949 (2), we described the behavior of gushing beer as being due to the formation of nuclei having surface characteristics of such a nature as to permit them to trigger the explosive evolution of carbon dioxide when the container is opened. A number of factors which could readily contribute to the formation of such nuclei in beer and thus serve to act as causes for wildness in beer were mentioned in that paper. These included trace metals, oxidation, maintenance of the beer at extremely low temperatures, etc. At that time, besides iron, nickel was recognized

as one of the metals capable of producing this condition.

In our experiments it was found that nickel under certain conditions was not only capable of producing wildness, but actually appeared more potent than equivalent amounts of iron in this respect.

Table VI shows the results of tests of wildness on a series of beers containing respectively added iron and nickel. Following the addition of the metals these beers were stored at a temperature of –2°C (28°F) for the periods shown. After storage, the beers were removed from the cold box, allowed to warm up to room temperature (25°C [77°F]), held at this temperature for one hour, and then opened. Figures in the table reflect the amount of beer lost by gushing when the caps were removed.

Table VI: Wildness at 25°C after storage at –2°C for varying periods

Additions	3 weeks	6 weeks	9 weeks	12 weeks	15 weeks
none (control)	Q†	Q	C-1	Q	C-¼
2 ppm nickel	40†	65	136	100	175
2 ppm iron	C-¼†	5	2	5	30
	2 weeks	**3 weeks**	**5 weeks**	**7 weeks**	
none (control)	C-¼	Q	C-¼	Q	
0.5 ppm nickel	2	1	C-1½	5	
2.0 ppm nickel	45	40	20	85	

†Figures in table denote volume (in ml) of beer overflowing when bottle was opened. "Q" denotes quiet when opened; "C" indicates beer formed a collar of foam of the height indicated in inches.

Summary

A precise method for the colorimetric determination of traces of nickel in malt beverages has been developed. By means of this method, the nickel content of brewing raw materials and commercial beers and the distribution of nickel in the various steps of processing beer have been determined.

The nickel content of brewing raw materials is generally low; the nickel content of commercial beers is usually in the range of less than 0.05 part per million. If the nickel content is higher than this, pickup of the metal in the brewery is indicated.

Nickel in trace amounts may, under certain conditions, slow fermentation. The extent of this effect depends upon many factors besides the nickel level. When nickel is present in the wort, a considerable proportion of it is separated with the yeast. In pilot brewery tests, high nickel levels in the yeast caused only moderate slowing of the fermentation. The finishing operations, performed in nickel-free equipment, did not affect the nickel levels in the beer. The finished beer contained 30 to 50 percent of the nickel originally present in the wort.

The presence of high levels of nickel in the finished beer seems to predispose it to the development of wildness.

ACKNOWLEDGMENT

The writers wish to thank Miss Rachelle Rothenberg and Mr. John Wylie for their help in obtaining much of the experimental and pilot brewery data.

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[*Résumé*]

Traces de nickel dans les bières et le brassage

Une méthode précise pour le dosage colorimétrique de traces de nickel, dans les boissons à base de malt, a été développée. A l'aide de cette méthode, on a dosé la teneur en nickel de matières premières pour la brasserie et de bières commerciales, ainsi que la distribution du nickel aux divers stades de la fabrication de la bière.

La teneur en nickel des matières premières de brasserie est généralement faible; la teneur en nickel des bières commerciales se situe généralement dans les environs de moins de 0.05 parties par million. Lorsque la teneur en nickel dépasse ce niveau, c'est que le métal trouve son origine dans la brasserie.

Le nickel sous forme de traces peut, dans certaines conditions, ralentir la fermentation. L'étendue de cette influence dépend de nombreux facteurs, en plus du taux de nickel. Quand il y a du nickel dans le moût, une proportion considérable est éliminée avec la levure. Dans des essais de brassins-pilotes, des taux élevés de nickel dans la levure ne provoquaient qu'un ralentissement modéré de la fermentation. Les opérations de finissage, faites dans des appareils sans nickel, n'exercèrent aucune influence sur la teneur de la bière en nickel. La bière finie contenait de 30 à 50 pour cent du nickel présent dans le moût au début.

De fortes teneurs de nickel dans la bière finie, semblent la prédisposer à devenir "sauvage" (wild).

[*Resumen*]

Huellas de níquel en la cerveza y durante su elaboración

Se ha desarrollado un método preciso para la determinación colorimétrica de huellas de níquel en bebidas a base de malta. Por este método se ha determinado el contenido de níquel en las materias primas de cervecería y en varias cervezas del

comercio, así como la distribución de este metal en las diferentes etapas de la elaboración de cerveza.

Por lo general hay muy poco níquel en las materias primas. La concentración de níquel en las cervezas del comercio es casi siempre menor de 0.05 partes por millón; una concentración mayor generalmente indica que este metal entró en la cerveza durante el proceso de elaboración.

El níquel, aún en cantidades tan pequeñas como huellas, bajo ciertas condiciones puede retardar la fermentación, el grado de retraso dependiendo de muchos otros factores. Cuando hay níquel en el mosto, una parte considerable de éste permanece en la levadura. Sin embargo, en ensayos de planta piloto se notó que aún niveles elevados de níquel en la levadura retrasaron la fermentación sólo moderadamente. Las operaciones finales de elaboración, al ser llevadas a cabo en equipos que no contienen niquel, no afectaron el nivel de este metal en la cerveza terminada, la cual contenía de 30 a 50 per ciento del níquel que originalmente había en el mosto.

La presencia de niveles elevados de níquel en la cerveza terminada parece predisponerla al desarrollo de la tendencia a derramarse súbitamente.

Arch. Ind. Health
16: 480-485 (1957)

Nickel Poisoning

IV. Chronic Exposure of Rats to Nickel Carbonyl: A Report After One Year Observation

F. WILLIAM SUNDERMAN, M.D., Ph.D.; JOHN F. KINCAID, Ph.D.; ANDREW J. DONNELLY, M.D., and BOB WEST, Ph.D., Philadelphia

Introduction

Over the past score of years several scattered reports have appeared in the literature attributing carcinogenic properties to nickel carbonyl.[1] Most of the evidence was obtained from studies on workers in the nickel industry who developed cancer of the respiratory passages after exposure to vapors of nickel carbonyl over a period of ten or more years. This subject has been reviewed in a previous publication.[1]

The present studies were initiated to study the effects produced by long, continued exposure of experimental animals to repeated sublethal doses of nickel carbonyl. Attention was also directed toward the possible carcinogenicity of the material. Surviving animals are still being studied from this point of view.

Methods

For our studies rats were exposed to sublethal concentrations of nickel carbonyl for a period of one year. The high resistance of the rat to pulmonary carcinoma is well known. The use of rats for these initial studies, therefore, seemed advisable, since any occurrence of pulmonary carcinoma from factors other than nickel carbonyl was unlikely.

In planning our experiments consideration was given to the use of both continuous and intermittent exposure. The latter was chosen. Intermittent exposure approaches more closely the variable concentrations that might occur in plant operations. Moreover, continuous exposure with nickel carbonyl would present formidable experimental difficulties not encountered with the intermittent type. For our purposes the animals were placed on a schedule of three 30-minute exposures per week. This schedule of repeated short exposures seemed reasonable, since the major effects from acute exposure are generally delayed 24 hours or longer.

Male albino rats of the Wistar strain were used. The rats were divided into four groups (C_1, X_1, X_2, Z) of 32 animals each and one group of 9 (C_2). They were kept under observation for a period of three weeks before the beginning of our studies. Throughout, they were fed a diet of Purina Dog Chow and given water as desired. At the time the exposures began the mean weight of the rats in Groups C_1, X_1, and Z was 273 gm. and the mean weight of the rats in Groups C_2 and X_2 was 145 gm. Groups X_1 and X_2 were exposed to nickel carbonyl at a concentration of 0.03 mg. per liter for 30 minutes three times weekly for a period of 52 weeks. Group Z was exposed to nickel carbonyl at a concentration of 0.03 mg. per liter for 30 minutes three times weekly for three weeks and later at a concentration of 0.06 mg. per liter for a total period of 52 weeks. Group C_1 served as control for Groups X_1 and Z. Group C_2 served as control for Group X_2.

The nickel carbonyl was administered by inhalation in exposure equipment recently described.[2] It was vaporized from a solution of nickel carbonyl dissolved in a mixture of 50% ethyl alcohol and ethyl ether. Control groups were administered inhalations of an alcohol-ether mixture without nickel carbonyl. Chemical analyses for nickel carbonyl present in the chamber were obtained throughout the 30-minute periods of exposure.[2]

Measurement of body weight of each animal was obtained at weekly intervals. Animals selected at random from each group were killed every two to three months during the year of exposure. Weights of vital organs of autopsied animals were noted. Sections of tissues were examined both histologically and chemically. Initially the animals were killed by the use of illuminating gas; later, by decapitation.

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This study was supported in part by contract of Jefferson Medical College with the Atomic Energy Commission.

From the Division of Metabolic Research, Jefferson Medical College, Rohm and Haas Company, Research Division, and The Institute for Cancer Research, Philadelphia.

TABLE 1.—*Groups of Rats Used in Nickel Carbonyl Studies*

Group	Conc., Mg./L.	Initial No.*	Animals Killed	Animals That Died †
C_1	0	32	10	11
C_2	0	9	1	5
X_1	0.03	32	4	23
X_2	0.03	32	1	20
Z	0.06	32	9	19

* Surviving animals are being retained and observed for tumor development.

† Several animals listed in this column were killed in a moribund condition.

Results

Mortality Changes.—The fate of the rats used in these studies is summarized in Table 1. It may be noted that the mortality in the control groups is relatively high. This may be due to the alcohol-ether vapor to which these animals were subjected. During most of the year's exposures the control animals, as well as the other animals, were subjected to an atmosphere containing alcohol-ether at a concentration of approximately 3 mg. per liter for 90 minutes each week. When these studies were about 75% complete, the amount of alcohol-ether used as the solvent was reduced, and so the concentration of the solvent vapors during exposure dropped to approximately 0.5 mg. per liter.

Weight Changes.—The weight curves comparing control and exposed animals are presented in Figure 1. The mean weight of the control rats (Group C_1) at the end of the 52-week experimental period was 465 gm. (SEM±16.7), as compared with a mean of 394 gm. (SEM±23.7) and 346 gm. (SEM±13.7) for the rats in Groups X_1 and Z, respectively. When Groups X_1 and Z are compared with the control group by the conventional Fisher *t*-test,[3] this observed failure to grow at the same rate as the controls is significant. (For Groups X_1 and C_1, $P=0.05$; for Groups Z and C_1, $P<0.01$.)

The amount of nickel in the liver and kidney of the animals subjected to chronic exposure to nickel carbonyl was determined according to our modification of the method of Alexander, Godar, and Linde.[1,4] The data in Table 2 show that the concentrations of nickel in these organs are increased after both acute and chronic exposures. Since there is little change in the mean weights of liver and kidney, respectively, as shown in Table 3, the total contents of nickel in these organs are obviously increased.

A summary of the changes in organ weights of the rats subjected to chronic exposure is presented in Table 3. Significant increases were observed in the weights of the lungs, heart, and adrenals of the exposed animals (Groups X and Z).

Pathologic Changes

All of the chronically exposed test animals had more extensive inflammatory lesions in lungs than those encountered in the controls. Only a few of the rats in the control groups had pneumonitis, whereas all of the others had severe inflammatory disease, including pneumonia, purulent bronchitis, bronchiectasis, abscesses, granulomata,

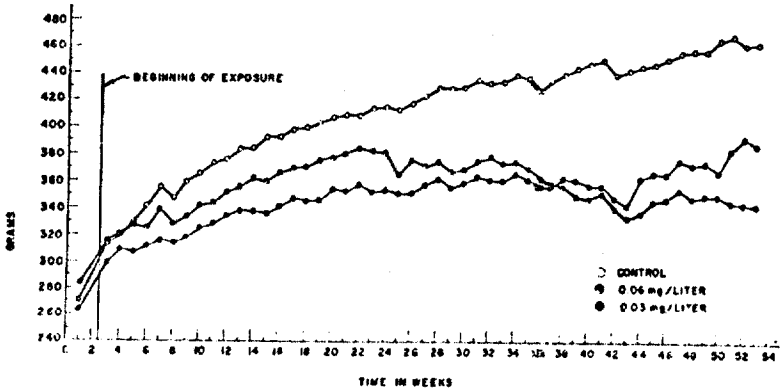

Fig. 1.—Weight curves of rats exposed to nickel carbonyl.

TABLE 2.—*Nickel in Tissues of Animals After Exposure to $Ni(CO)_4$*

Group	Ni, μg., Gm. of Wet Tissue	
	Mean Liver Value	Mean Kidney Value
Control	0.12 (SE±0.02)	0.40 (SE±0.13)
Chronic*	0.20 (SE±0.05)	1.21 (SE±0.05)
Acute $L.D._{50}$ exposure †	0.48 (SE±0.29)	0.95 (SE±0.13)

* These analyses were made on tissues from animals killed 6 to 12 months after the start of the program.
† These analyses were made on tissues obtained 48 to 168 hours after exposure.

and empyema. Many of the exposed rats had a contiguous pericarditis, and a number had suppurative lesions involving the thoracic walls. In approximately one-third of the chronically exposed animals the pulmonary exudate contained large numbers of histiocytic foam cells (Fig. 2). Squamous metaplasia was present in bronchiectatic walls of several rats (Fig. 3). This metaplasia was accompanied by distortions of the epithelium resembling neoplasia. In at least one animal this change was so striking that invasive carcinoma was simulated to a remarkable degree. This change was characterized by an apparent invasion of the surrounding fibrotic tissue by cords and islands of epithelial cells with moderately hyperchromatic nuclei and practically no organized pattern (Fig. 4).

No significant differences in the pulmonary lesions were apparent as a result of the different dosage levels, 0.03 mg. per

TABLE 3.—*Per Cent of Total Body Weight of Various Organs of Rats Subjected to Chronic Exposure of Nickel Carbonyl*

Group	Organ Weight as Per Cent of Total Body Weight, Per Cent ± S.E.	Per Cent Increase
C Liver	2.97±0.11	
X	3.07±0.15	
Z	2.95±0.19	
C Lung	0.69±0.17	
X	2.20±0.18	219
Z	1.69±0.22	145
C Heart	0.33±0.04	
X	0.53±0.03	61
Z	0.52±0.13	60
C Kidney	0.36±0.04	
X	0.40±0.02	
Z	0.43±0.03	
C Adrenal	0.0057±0.0006	
X	0.0119±0.0012	109
Z	0.0124±0.0009	118

C = control.
X = 0.03 mg. per liter for 30 minutes, three times weekly for one year.
Z = 0.06 mg. per liter for 30 minutes, three times weekly for one year.

Fig. 2.—Alveolar spaces in periphery of lu[illegible] crowded with vacuolated macrophages (foam cell[illegible]

lter (Groups X_1 and X_2) and 0.06 m[illegible] per liter (Group Z).

Of the extrathoracic pathologic lesio[illegible] the ones most frequently encountered in [illegible] groups were colitis and cecitis. These we[illegible] often ulcerative and were accompanie[illegible]

Fig. 3.—Section of bronchiectatic lung w[illegible] squamous metaplasia of bronchial epithelium ar[illegible] the purulem exudate.

Fig. 4.—Islands and cords of epithelial cells in a brous stroma from a peribronchial area. The pat-ern is practically indistinguishable from carcinoma individual fields are considered as isolated enti-es.

Fig. 5.—Edge of mesenteric lymph node with residual lymphoid tissue enclosing cystic spaces. This cystic node was adjacent to an intestinal ulcer.

y regional mesenteric lymphadenitis and lema, with cyst-like dilatations in the larger odes (Fig. 5). Some of the lymph-filled yst-like distortions were up to 3 cm. in ameter. A few nodes contained large umbers of polymorphonuclear leucocytes, articularly around the cavity walls. Some f these cystic nodes were immediately ad-acent to the ulcers in the colon wall.

The hearts, livers, kidneys, and adrenal lands of the test animals did not exhibit ny constant histopathologic distortions con-dered significant to this study.

In the control animals killed in good con-tion (11 rats) the majority had lungs hich were essentially normal. A severe hronic meningitis was discovered in one ntrol animal, and another had a large in-ammatory and fibroblastic mass in one high. Because of the apparently unrelated mplications and the pulmonary lesions in e rats discovered dead or dying, only e killed animals warrant comparisons with e test animals. A number of isolated thologic lesions were found incidentally and were not considered related to the study.

Comment

There is little doubt that chronic exposure of rats to nickel carbonyl is attended by impairment of health. This is evidenced by the decreased rates of growth, the higher mortality rates, and the higher incidence of pathologic lesions in the exposed animals as compared with the controls. The exposed animals were less active and appeared apathetic; their coats lacked luster, and their noses were frequently congested and covered with blood. The substantial enlargements of the heart, lungs, and adrenals noted in the exposed groups are further indications of damage to vital organs.

The use of rats as test animals in determining the carcinogenicity of inhalants is recognized as a severe challenge. According to Ratcliffe [5] and Willis [6] the incidence of spontaneous pulmonary neoplasms in rats is negligible. Stewart [7] indicates that induction of lung tumors in rats is diffi-

cult. However, the lungs of rats cannot be considered completely refractory to specific carcinogens, since pulmonary tumors have been induced by urethan[8] and aerosols of radioactive cerium[9] and beryllium sulfate.[10]

Nickel carbonyl, an agent known to produce fibroblastic lesions in lungs,[11,12,13] has been considered as a possible pulmonary carcinogen in human beings. As yet the carcinogenicity has not been proven experimentally. Despite this, nickel carbonyl cannot be declared noncarcinogenic and it must remain suspect. Our present studies may actually heighten the suspicion, since squamous metaplasia is evoked or abetted in the bronchial epithelium of rats chronically exposed to the vapors.

The amount and degree of metaplasia produced in the exposed animals is considered important in relation to carcinogenicity. Smith has indicated that "undifferentiated, anaplastic, or squamous cell cancers compose the majority of lung cancers seen in man. Attention should therefore be directed toward materials or conditions leading to dedifferentiation or metaplasia of pulmonary tissue."[14] Squamous metaplasia of the bronchial epithelium is commonly found in bronchiectatic lungs with suppurative bronchitis in the rat, and such pulmonary lesions were often found in our exposed animals. The evidence, therefore, that chronic exposure to nickel carbonyl produces cancer of the respiratory passages in the rat remains suspicious but inconclusive.

Summary

Studies are reported on the effects of chronic exposure of rats to nickel carbonyl. Several groups of animals were exposed to sublethal concentrations of nickel carbonyl for a period of one year.

Chronic exposure of rats to nickel carbonyl is attended by impairment of health, decreased rates of growth, and high mortality rates. The weights of the hearts, lungs, and adrenals of the exposed rats were significantly higher than those of the controls.

Rats chronically exposed to nickel carbonyl developed extensive pulmonary lesions, including a remarkable degree of squamous metaplasia of the bronchial epithelium. This metaplasia has been considered in relation to carcinogenesis. Although the presence of inflammatory lesions complicates the interpretation of the metaplasia, nevertheless the amount and degree of the metaplasia that was observed warrants continued investigation of nickel carbonyl as a possible carcinogenic agent. Surviving animals are being retained and observed for tumor development.

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Correction

In the article "Control of Heat Casualties at Military Training Centers," by Constantin P. Yaglou and Comdr. David Minard (MC), U. S. N., in the October issue of the Archives, the formula near the top of the left column on page 303 is incorrect. The correct formula is as follows:

$$\text{Globe temp.} = 0.74 \times 0.95 \times (\text{black globe temp.} - \text{shade air temp}) + \text{shade air temp.}$$

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STUDIES OF NICKEL CARCINOGENESIS:

ALTERATIONS OF RIBONUCLEIC ACID FOLLOWING INHALATION OF NICKEL CARBONYL

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The prevalence of pulmonary cancer among nickel workers was first reported by Baader in 1937[1] and has been confirmed by numerous subsequent investigations.[2, 11, 12, 32, 36, 61] Most noteworthy is the statistical study by Doll in 1958,[11, 12] which indicated that, between the years 1938 and 1956, 35.5 per cent of nickel workers in Glamorganshire, Wales, died of cancer of the lung and nose. These observations have led the Ministry of Pensions and National Insurance of Great Britain officially to recognize pulmonary cancer as an occupational disease among industrial workers exposed to "nickel produced by decomposition of a gaseous nickel compound."[12, 19]

The relation of the inhalation of nickel to pulmonary carcinogenesis has been the subject of a series of investigations in our laboratory.[46, 48, 51] Our attention has been focused primarily upon nickel carbonyl, because of the extreme toxicity, high volatility, and catalytic activity of this compound.[26, 27, 47, 50, 52] Previous papers have reported the experimental induction of pulmonary cancers in rats exposed to low concentrations of the vapor of nickel carbonyl[46, 48] and have demonstrated that nickel is present in tobacco smoke.[51] It has been estimated that the amount of nickel inhaled annually by a heavy cigarette smoker is approximately 3 times that which is carcinogenic for the rat.[51]

Our interest in the binding of nickel by ribonucleic acid (RNA) was stimulated by the report by Wacker and Vallee in 1959[58, 59] that ribonucleic acids isolated from diverse biologic sources contained large concentrations of trace metals, including nickel, chromium, manganese, iron, lead, copper, and zinc. A series of investigations has indicated that these metals form an integral part of ribonucleic acid molecules.[17, 56-59] Intramolecular metal bonds between the nitrogenous bases are believed to constitute a tertiary structure of ribonucleic acid that stabilizes the hydrogen-bonded secondary structure.[17, 57] Trace metals have been demonstrated to influence the structure and biologic activity of ribonucleic acids from viruses,[8, 13, 21, 34] tumor cells,[5] and lens cells,[9] and also to influence the synthesis of ribonucleic acids by a variety of microorganisms.[4, 55, 62] Wacker, Vallee, and their associates have suggested that metal bonds, by maintaining the molecular configuration of ribonucleic acid, may bear a functional relation to protein synthesis and to the transmission of genetic characteristics.[17, 59]

Recognition of the presence of metals in nucleic acids is a relatively recent development, for Neuberg and Roberts, in 1949, stated that "the nucleic acids as such are free from metals."[37] It may be noted that Pirie in 1945[39] had mentioned that traces of 12 metals, in addition to calcium and sodium, remained in preparations of tomato bushy stunt virus after prolonged dialysis, and Zittle in 1946[64] had noted that copper was present in commercial preparations of ribonucleic acid. Jungner in 1951[24] demonstrated by means of spectrographic analyses that magnesium was present in purified nucleic acids. This observation has been followed by numerous studies of the affinity

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Dr. Sunderman is Instructor in Medicine, and member of the Division of Metabolic Research.

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This is the thirteenth paper in a series of studies of nickel poisoning.

Presented at the meeting of the American Society of Clinical Pathologists, Chicago, Illinois, September 5, 1962.

of nucleic acids for magnesium and related cations.[6, 7, 14, 16, 20, 38, 41, 44, 54, 60, 65] These investigations have indicated that magnesium is bound primarily to the phosphate groups of nucleic acids and that purified nucleoproteins undergo association-dissociation reactions in response to changes in magnesium concentration.[14, 20, 38, 54] *In vitro* studies have demonstrated that nucleic acids also have affinity for mercury,[25, 42, 53] silver,[42] beryllium,[15] indium,[42] and the alkaline earths.[40, 45] The demonstration by Loring and associates in 1957[33, 35] that iron and copper are present as stable metal complexes within the structure of tobacco mosaic virus RNA was followed by the studies of Wacker and Vallee, which have already been mentioned.

The present investigation has been directed, *first*, to a comparison of the content of trace metals in ribonucleic acids isolated from a variety of rat tissues, and, *second*, to a study of the influence of inhalation of nickel carbonyl upon the nickel content of ribonucleic acids.

EXPERIMENTAL PROCEDURE

Precautions

Metal-free water, prepared by distillation and ion exchange, was used throughout the study. All glassware was cleaned with nitric acid immediately before use. Spectrographic analyses were made of phenol and all other reagents used in the preparation of RNA in order to ensure that these reagents were not contaminated with metals. Cellophane dialysis tubing was washed for two 4-day periods, first with disodium ethylenediaminetetraacetic acid (EDTA) (0.01 M) and then with metal-free water.

Preparation and Characterization of RNA

Preparation of RNA from rat tissues was performed by a modification of the method of Kirby[28-30] based upon the method of Gierer and Schramm.[18] The rats were white males of the Wistar strain, weighing from 200 to 250 Gm. For each preparation of RNA, a group of 24 or 32 rats was killed by cervical fracture. The lungs, liver, kidneys, and leg muscles were immediately excised, drained of blood, freed of fibrous tissue by dissection, and frozen by dropping onto solid carbon dioxide. The respective organs were pooled, weighed, and minced. For each preparation of RNA, 30 to 120 Gm. of tissue were used. A cold solution of phosphate buffer (pH 7.3, 0.05 M) containing sodium chloride (0.14 M) and naphthalene disulfonic acid, disodium salt (0.5 Gm. per 100 ml.), was added to the tissues in a volume of 10 ml. per Gm. The tissues were homogenized in an ice-cooled, motor-driven, all glass tissue grinder. The homogenates were then filtered through a Saran screen.

To each homogenate was added an equal volume of aqueous phenol (90 per cent weight per volume). The mixture was shaken mechanically for 1 hr. at 20 C. and centrifuged for 1 hr. This and subsequent centrifugations were performed at 2000 r.p.m. at 4 C. The aqueous supernatant fluid was added to an equal volume of aqueous phenol. The mixture was shaken for 15 min. and centrifuged for 20 min. Subsequent steps were performed at 4 C. The aqueous supernatant fluid was washed 4 times with 0.7 volume of ethyl ether to remove phenol. After the last washing, residual ether was volatilized by bubbling nitrogen through the solution. Potassium acetate was added to achieve a concentration of 2 Gm. per 100 ml., and RNA was precipitated by addition of 2 volumes of cold ethanol.

The precipitated RNA was separated by centrifugation, washed with cold ethanol-water (3:1), and dissolved in 50 to 100 ml. of water. Residual ethanol was removed with a rotary evaporator. Polysaccharides were precipitated by addition of 1 volume of dipotassium hydrogen phosphate solution (2.5 M), 0.05 volume of phosphoric acid (33 per cent volume in volume), and 1 volume of 2-methoxyethanol. The mixture was centrifuged for 15 min., and the aqueous supernatant fluid was removed. After addition of a few drops of toluene, the supernatant fluid was transferred to cellophane dialysis sacs. The sacs were placed in Oxford dialysis apparatuses, and the contents were dialyzed for 48 hr. at 4 C. against a volume of 5 l. of water, which was changed 6 times. The contents of the sacs were removed, and sufficient potassium acetate was added to

achieve a concentration of 2 Gm. per 100 ml. RNA was precipitated by addition of 2 volumes of cold ethanol. The precipitate was collected by centrifugation, washed 3 times with cold ethanol-water (3:1), and dried in a vacuum desiccator over phosphorus pentoxide for 48 hr. The RNA was ground to a fine powder with a glass mortar and pestle and once again was dried *in vacuo*.

In certain experiments, sodium chloride precipitation was used for fractionation of RNA as described by Laskov and coworkers.[31] Immediately following phenol extraction, high molecular weight RNA was precipitated with 3 M sodium chloride during 48 to 72 hr. at 4 C. The NaCl-precipitable and NaCl-soluble fractions of RNA were then treated as described previously.

Measurements of nitrogen were made by the method of Johnson,[23] measurements of phosphorus were made by the method of Youngburg and Youngburg,[63] and paper chromatographic identification of purine and pyrimidine bases and of pentoses was performed by the methods of Smith.[43] Ultraviolet absorption measurements of RNA dissolved in water (25 μg. per ml.), were obtained with the Beckman model DU spectrophotometer, or with the Bausch and Lomb model 505 ratio-recording spectrophotometer.

For studies of the phase transition of RNA during heating, a solution containing 25 μg. of RNA per ml. of buffer (0.025 M ammonium acetate in 0.025 M NaCl, pH 6.8) was prepared immediately before use. The cuvet holder of the Bausch and Lomb spectrophotometer was fitted with an electric heating element, which was regulated to increase the temperature of the RNA solution within the cuvet at a constant rate of 1 C. per min.

Spectrographic Analyses

Emission spectrography in the range of 2500 to 3500 Å was performed with a large Bausch and Lomb Littrow spectrograph. The DC arc technic was used with an excitation current of 10 amp. and 250 v. Samples of RNA (15 to 25 mg.) were weighed and transferred directly to high purity graphite electrode cups that contained 2 mg. of sodium chloride as a carrier. A series of 15-sec. "roll-down" exposures was performed with each sample, in order to ascertain the time required for complete volatilization of the metals. All spectrographic analyses were performed in triplicate or quadruplicate. A series of standard samples was analyzed with each set of determinations, and measurements of the recovery of metals added to RNA were made. Densitometry of the spectral emission lines was performed with an Applied Research Laboratory densitometer-comparator. The spectral lines of copper and iron were found to be consistently too intense for quantitative determination. Satisfactory recovery and reproducibility were achieved with measurements of manganese, tin, lead, chromium, and zinc. The spectral lines of nickel were usually present but were not sufficiently intense or reproducible to permit quantitative measurements. It was noted that maximal emission from nickel did not occur until the emission from sodium, potassium, and most of the other metals had been completed. Because of these difficulties, spectrographic analyses of nickel were abandoned in favor of chemical measurements.

Ultraviolet Spectrophotometric Measurements of Nickel

The colorimetric method of Kincaid and associates[26] was modified to permit measurements of nickel in samples of RNA weighing 15 to 30 mg. In order to minimize contamination and loss, the solvent extraction and recovery procedures were all performed in a single centrifuge tube. Spectrophotometric measurements were made in the ultraviolet spectrum at 320 mμ, rather than 385 mμ. The specificity of this modified procedure remained unchanged from that of the original method,[26] but the sensitivity of the modification was increased by more than 10-fold. All analyses were performed in triplicate.

Principle

After destruction of organic material by wet digestion, nickel was separated from interfering elements by chloroform extrac-

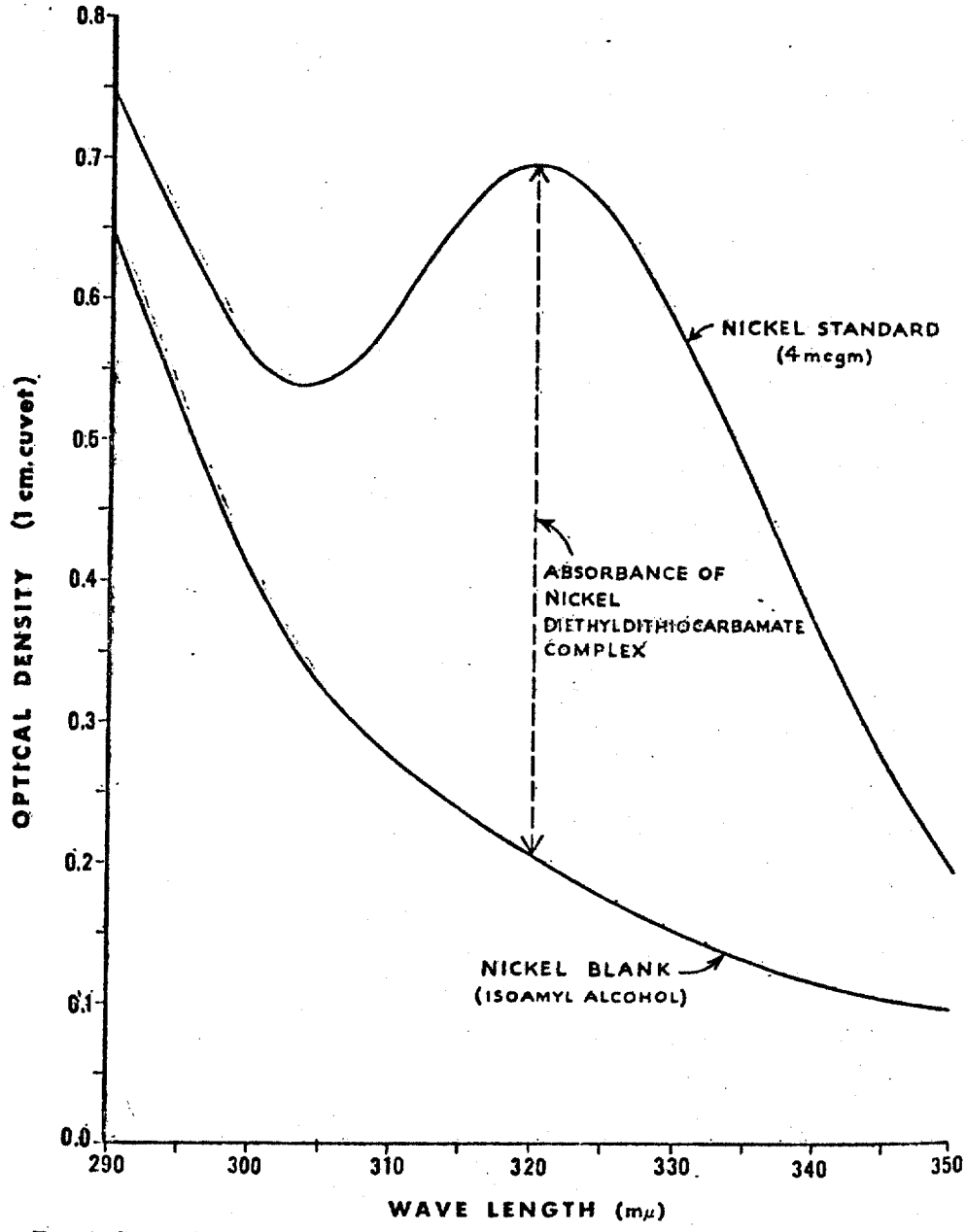

FIG. 1. Spectral absorption curve pertaining to ultraviolet spectrophotometric method for nickel.

tion of nickel dimethylglyoxime at alkaline pH. Nickel dimethylglyoxime was recovered from chloroform by extraction with dilute hydrochloric acid. Nickel was converted to the diethyldithiocarbamate complex and extracted with isoamyl alcohol. Spectral absorption curves of the nickel diethyldithiocarbamate complex in isoamyl alcohol are illustrated in Figure 1. A calibration curve obtained by the modified procedure is illustrated in Figure 2. It may be seen that for practical purposes, the lower limit of sensitivity of the procedure is approximately 0.1 μg. of nickel.

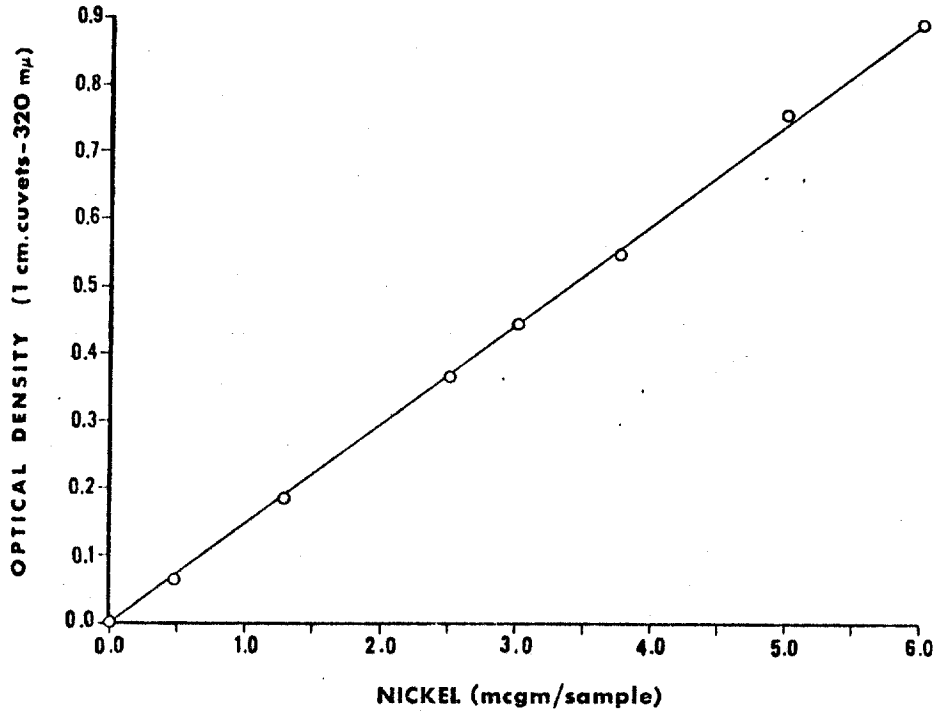

FIG. 2. Calibration curve pertaining to spectrophotometric method for nickel

Reagents

1. *Mixed acid solution.* One part of concentrated sulfuric acid (sp. gr., 1.84) mixed with 1 part of perchloric acid (70 to 72 per cent) and 3 parts of nitric acid (sp. gr., 1.42).

2. *Dimethylglyoxime solution.* Dimethylglyoxime, 0.25 Gm., dissolved in 50 ml. of 95 per cent ethanol and diluted with water to 250 ml.

3. *Sodium diethyldithiocarbamate solution,* 0.2 Gm. per 100 ml.

4. *Citrate buffer "A."* Diammonium citrate, 200 Gm., was dissolved in 600 ml. of water, adjusted to pH 9.0 to 9.5 with ammonium hydroxide, and transferred to a 1-l. separatory funnel. Ten milliliters of dimethylglyoxime solution were added. The mixture was extracted 3 times with 30-ml. portions of chloroform. The solution was filtered and diluted to 1 l.

5. *Citrate buffer "B."* Diammonium citrate, 200 Gm., was dissolved in 600 ml. of water, adjusted to pH 9.0 to 9.5 with ammonium hydroxide, and transferred to a 1-l. separatory funnel. Sodium diethyldithiocarbamate solution, 10 ml., was added. The mixture was extracted with 20-ml. portions of carbon tetrachloride until the solvent layer was colorless. The solution was filtered and diluted to 1 l.

6. *Hydrochloric acid,* 0.5 N.

7. *Dilute ammonium hydroxide solution,* 2 per cent (volume per volume).

8. *Nickel stock standard.* Metallic nickel, 500 mg., was dissolved in 20 ml. of nitric acid (1:1) and diluted with water to 1 l.

9. *Nickel working standard* (2.5 μg. Ni per ml.). Nickel stock standard, 5 ml., was diluted to 1 l. with 0.5 N hydrochloric acid.

Procedure

A sample of RNA weighing 15 to 30 mg. was transferred to a 30-ml. Kjeldahl flask. Concentrated nitric acid, 5 ml., was added, and the contents of the Kjeldahl flask were digested almost to dryness. The flask was cooled, and 5 ml. of mixed acid solution were added. The contents of the flask were digested until the volume was approximately 2 ml. The flask was cooled, and the contents

were transferred with several small washes of water to a 50-ml., glass-stoppered centrifuge tube. Citrate buffer "A," 10 ml., was added, and the tube was placed in a cold water bath in a hood. The contents of the tube were adjusted to pH 8.50 by addition of concentrated ammonium hydroxide. (Measurements of pH were facilitated by use of a probe type pH electrode.) Dimethylglyoxime solution, 5 ml., was added. The tube was shaken for 1 min. and allowed to stand for 5 min. Chloroform, 15 ml., was added, and the tube was shaken for 1 min. The tube was centrifuged for 5 min. at 2000 r.p.m., and the aqueous supernatant fluid was aspirated and discarded. Twenty milliliters of 0.5 N hydrochloric acid were added. The tube was shaken for 1 min. and centrifuged for 5 min. The infranatant chloroform was aspirated and discarded. Carbon tetrachloride, 2.5 ml., was added, the tube was shaken for 1 min. and centrifuged for 5 min., and the infranatant carbon tetrachloride was aspirated and discarded. Citrate buffer "B," 5 ml., was added, and the tube was placed in a cold water bath in a hood. The contents of the tube were adjusted to pH 8.50 by addition of concentrated ammonium hydroxide. Sodium diethyldithiocarbamate solution, 5 ml., was added, followed by 4 ml. of isoamyl alcohol. The tube was shaken vigorously for 2 min., allowed to stand for 15 min., and shaken again for 2 min. The tube was centrifuged for 5 min., and the infranatant aqueous phase was aspirated and discarded. The isoamyl alcohol phase was transferred to a 1-cm. quartz cuvet, and measurement of optical density was made at 320 mμ. The optical density of the unknown sample was compared with that of blank samples and of standards containing 2.5 μg. of nickel.

TABLE 1

CONCENTRATION OF NICKEL IN FIVE PREPARATIONS OF RAT LIVER RIBONUCLEIC ACID (RNA)*

Preparation	εP (260 mμ)	Nickel Concentration
		μg./Gm. RNA
I	9,600	60
II	10,200	56
III	10,000	59
IV	8,900	46
V	9,100	51
Mean	9,560	54†

* RNA precipitable with 3 M NaCl.

† *Cf.* Wacker and Vallee's value of 61.[59]

Exposures of Rats to Nickel Carbonyl

Nickel carbonyl was administered to rats by inhalation in an exposure chamber by means of a procedure that has been previously described.[49] Groups of 32 rats were exposed for periods of 30 min. to nickel carbonyl in a concentration of 0.60 mg. per l. of air. Continuous measurement of the concentration of nickel carbonyl present in the exposure chamber was performed by a method previously described.[26] Control groups of 32 rats were placed in the exposure chamber and treated similarly, but they were not exposed to nickel carbonyl. After 24 hr., the rats were killed by cervical fracture, and tissues were immediately excised and frozen for isolation of RNA.

RESULTS

The preparations of RNA were hard, white powders that were completely soluble in water. The yield of RNA from liver and kidney was 400 to 500 mg. per 100 Gm., and the yield from lung and muscle was 100 to 150 mg. per 100 Gm. The nitrogen content of rat liver RNA was 11.8 per cent (weight per weight), and the phosphorus content was 6.7 per cent (weight per weight). These values yield an N:P ratio of 1.76, and they are in agreement with Kirby's values for liver RNA from Wistar strain rats of 11.65 per cent nitrogen, 6.75 per cent phosphorus, and an N:P ratio of 1.72.[28] Rat lung RNA was found to contain 11.6 per cent nitrogen (weight per weight) and 6.1 per cent phosphorus (weight per weight) and thus to yield an N:P ratio of 1.90. The extinction per mole of phosphorus, $(\epsilon P)_{260}$, for rat liver RNA was 10,600, and for rat lung RNA, 10,100. Paper chromatographic fractionation of the products of perchloric acid hydrolysis of liver and lung RNA demonstrated guanine, adenine, cystosine, and uracil. Ribose was the single sugar that was detected by paper chromatography.

TABLE 2

DIALYSIS OF RAT LIVER RIBONUCLEIC ACID (RNA)

Duration of Dialysis	Concentration of Nickel in RNA
days	*μg./Gm. RNA*
0	660
¼	160
1	66
2	56
3	58
4	54
5	50

To illustrate the reproducibility of measurements of nickel present in RNA, the results of analyses of 5 different preparations of high molecular weight liver RNA are listed in Table 1. The mean concentration of nickel in liver RNA was 54 μg. per Gm. This mean value correlates substantially with the value of 64 μg. of nickel per Gm. reported by Wacker and Vallee for a preparation of high molecular weight liver RNA from Hisaw strain rats.[59] The 5 preparations of liver RNA listed in Table 1 were each obtained from a different shipment of Wistar strain rats and were processed at intervals of 6 weeks to 3 months. The consistency of these measurements does not support the view that the association of metals with RNA may be artifactitious.[22]

All the RNA preparations were dialyzed against multiple changes of metal-free water for a period of 2 days. As demonstrated in Table 2, dialysis of rat liver RNA for shorter periods was not adequate to remove unbound nickel. The variations that were observed with prolongation of dialysis up to 5 days were not significant, inasmuch as they were within the range observed with replicate preparations.

The concentrations of nickel in RNA from rat liver, lung, kidney, and muscle are summarized in Table 3. Approximately 90 per cent of RNA from rat liver was precipitable with 3 M sodium chloride. The concentration of nickel in this NaCl-precipitable fraction agreed closely with that in the total liver RNA. The remaining fraction, which was soluble in 3 M sodium chloride, constituted approximately 10 per cent of the total liver RNA. The concentration of nickel in this NaCl-soluble fraction averaged 180 μg. per Gm.

The proportions of NaCl-soluble and NaCl-precipitable fractions of lung RNA were the inverse of those of liver RNA. Less than 10 per cent of lung RNA was precipitable with 3 M sodium chloride. Accordingly, the concentration of nickel in total lung RNA agreed closely with that in the NaCl-soluble fraction. With lung as with liver, the concentration of nickel in the NaCl-soluble RNA was approximately 3 times that in the NaCl-precipitable fraction. The mean value of 350 μg. of nickel per Gm. of NaCl-soluble lung RNA is equivalent to 3.0 mM of nickel per mole of RNA phosphorus.

TABLE 3

CONCENTRATION OF NICKEL IN RIBONUCLEIC ACID (RNA) FROM VARIOUS TISSUES

Tissue	Preparation	Number of Preparations	Mean εP (260 mμ)	Nickel Concentration	
				Mean	Range
				μg./Gm. RNA	
Rat liver	Total	5	10,600	57	50-66
	NaCl-precipitable	5	9,560	54	46-60
	NaCl-soluble	3	10,200	180	100-225
Rat lung	Total	3	10,100	350	320-390
	NaCl-precipitable	2	9,700	110	100-110
	NaCl-soluble	2	7,900	350	340-360
Rat kidney	Total	3	8,800	190	140-240
Rat muscle	Total	3	8,300	310	200-380
Human lung	Total	3	8,800	160	130-180

TABLE 4

REPLICATE SPECTROGRAPHIC ANALYSES OF A SINGLE PREPARATION OF RAT KIDNEY RIBONUCLEIC ACID (RNA)

Analysis	Concentration of Trace Metals				
	Cr	Mn	Pb	Sn	Zn
	μg./Gm. RNA				
I	67	37	200	9	1400
II	44	47	180	7	1300
III	39	55	130	6	1600
IV	44	57	90	4	900
Mean	48	49	150	6.5	1300

Alterations in the nickel concentration in lung and liver RNA following the inhalation of nickel carbonyl are listed in Table 6. In rats sacrificed 24 hr. after 30-min. exposure to nickel carbonyl, the concentration of nickel in the NaCl-precipitable fraction of lung RNA increased from 110 to 270 μg. per Gm., whereas the concentration of nickel in the NaCl-soluble fraction decreased from 340 to 230 μg. per Gm. The concentration of nickel in NaCl-precipitable liver RNA increased from 59 to 160 μg. per Gm., whereas that in NaCl-soluble liver RNA was not significantly altered.

In order to detect alterations in the stric-

TABLE 5

SPECTROGRAPHIC ANALYSES OF RIBONUCLEIC ACID (RNA) FROM VARIOUS TISSUES

Tissue	Preparation	Concentration of Trace Metals*				
		Cr	Mn	Pb	Sn	Zn
		μg./Gm. RNA				
Rat liver	NaCl-precipitable	9 (8-11)	5 (4-5)	66 (57-79)	34 (22-41)	220 (170-270)
Rat liver	NaCl-soluble	28 (11-44)	10 (7-18)	140 (100-180)	35 (24-52)	1500 (1400-1600)
Rat lung	NaCl-precipitable	230 (180-280)	46 (44-50)	820 (760-870)	560 (260-760)	1800 (900-2600)
Rat lung	NaCl-soluble	310 (200-480)	61 (48-78)	580 (530-610)	64 (26-100)	7500 (6300-8200)
Rat kidney	Total	65 (42-81)	44 (37-54)	170 (90-220)	8 (5-9)	1200 (1000-1300)
Rat muscle	Total	220 (90-390)	45 (41-49)	290 (180-400)	12 (10-15)	720 (350-1010)
Human lung	Total	35 (21-49)	23 (13-30)	87 (65-110)	5 (3-7)	460 (270-610)

* Mean and range of 3 preparations.

As listed in Table 3, the concentrations of nickel in rat kidney and muscle RNA averaged 190 and 310 μg. per Gm., respectively. Also included in Table 3 are the concentrations of nickel in RNA from normal human lung, obtained at surgery, which were found to average 160 μg. per Gm.

Replicate spectrographic analyses of a single preparation of rat kidney RNA are listed in Table 4 to illustrate the variability of these analyses. The results of spectrographic analyses of RNA from various tissues are tabulated in Table 5. Each of the values represents the mean of triplicate analyses of 3 different preparations of RNA. It may be noted that the greatest mean concentration of each of the metals was found in the RNA preparations from rat lung.

TABLE 6

CONCENTRATION OF NICKEL IN LUNG AND LIVER RIBONUCLEIC ACID (RNA) FOLLOWING INHALATION OF NICKEL CARBONYL*

Tissue	Preparation	Nickel Concentration†	
		Control group	Exposed group ($Ni(CO)_4$)
		μg./Gm. RNA	
Lung	NaCl-precipitable	110	270
	NaCl-soluble	340	230
Liver	NaCl-precipitable	59	160
	NaCl-soluble	120	110

* Exposure to 0.60 mg. of $Ni(CO)_4$ per L. of air for 30 min.

† Mean of triplicate analyses.

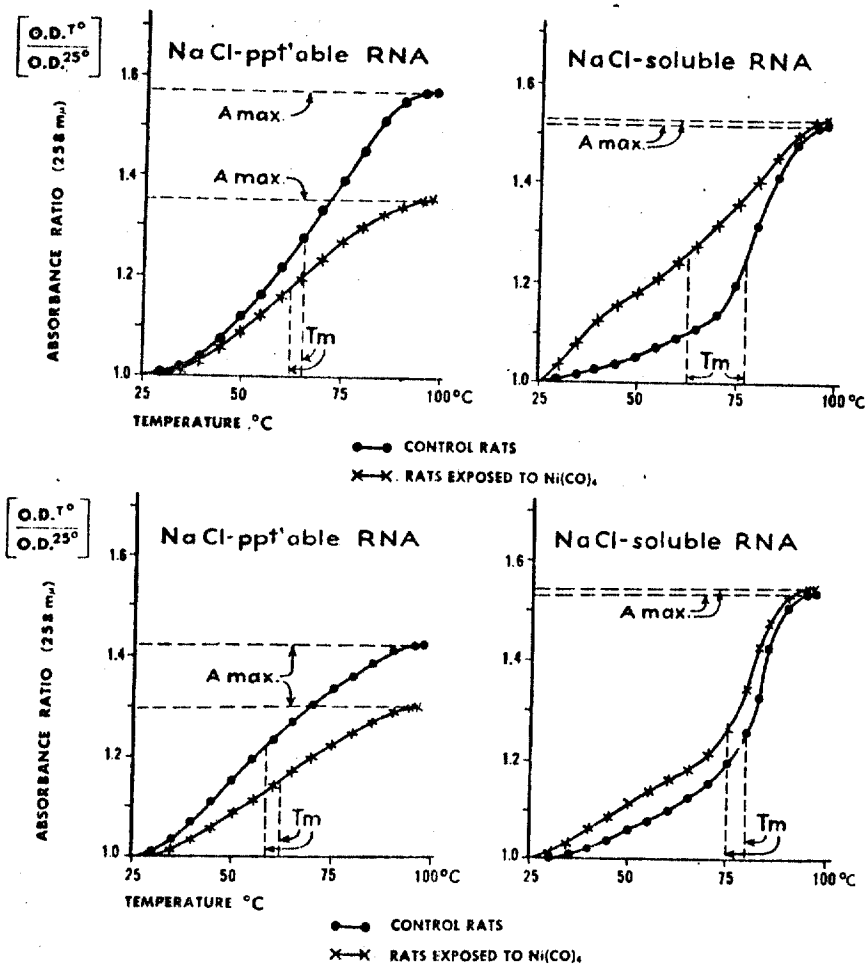

FIG. 3 (upper). Influence of inhalation of nickel carbonyl upon phase-transition curves of pulmonary ribonucleic acids.
FIG. 4 (lower). Influence of inhalation of nickel carbonyl upon phase-transition curves of hepatic ribonucleic acids.

ture of RNA following inhalation of nickel carbonyl, attention was directed to the increase in ultraviolet absorption that occurs when solutions of RNA are heated.[10, 17, 21] Phase-transition curves for solutions of RNA are illustrated in Figures 3 and 4. In these graphs, the ordinate is the ratio of the absorbance of an RNA solution at temperature T to its absorbance at 25 C., and the abscissa is the temperature range from 25 C. to 100 C. In accordance with customary nomenclature,[17] the Tm represents the temperature at the midpoint of the phase-transition, and the A_{max} represents the maximal absorbance ratio.

In Figure 3 are phase-transition curves for NaCl-precipitable and NaCl-soluble lung RNA from control rats and from rats exposed to nickel carbonyl. Following exposure, NaCl-precipitable lung RNA underwent a significant diminution in the maximal absorbance ratio with relatively little change in the midpoint temperature. On the other hand, NaCl-soluble lung RNA underwent a significant diminution in midpoint temperature with relatively little change in maximal absorbance ratio.

As previously noted (Table 6), the concentration of nickel in the NaCl-precipitable lung RNA increased following exposure of

the rats to nickel carbonyl, whereas that in the NaCl-soluble fraction decreased. These alterations in nickel concentration and in phase-transition curves support the view that diminutions in maximal absorbance ratio are primarily associated with an increase in metal-bonding of RNA, whereas diminutions in midpoint temperature are primarily associated with disruption of hydrogen bonds.[17]

In Figure 4 are illustrated the phase-transition curves for NaCl-precipitable and NaCl-soluble liver RNA from control rats and from rats exposed to nickel carbonyl. Again, the NaCl-precipitable fraction (which increased in its concentration of nickel) underwent a significant diminution in maximal absorbance ratio with little attendant change in midpoint temperature. NaCl-soluble liver RNA from exposed rats demonstrated little change in nickel concentration, maximal absorbance ratio, or midpoint temperature.

DISCUSSION

Histopathologic study of the lungs of rats sacrificed 24 hr. after exposure to nickel carbonyl did not produce evidence of edema, atelectasis, or inflammation. The weights of lungs from the exposed group did not differ from those of the control group, and the yields of RNA from the 2 groups were equivalent. These considerations furnish evidence that the RNA isolated from the lungs of the exposed animals was of intrinsic origin.

The observation that inhalation of nickel carbonyl is attended by alterations in the concentration of nickel and in certain physical-chemical properties of lung RNA may point to a mechanism of pulmonary carcinogenesis. A review of the literature has failed to reveal previous reports of a chemical carcinogen that produced similar effects, *in vivo*, upon the chemical or physical properties of ribonucleic acid; however, Belman and co-workers[3] have recently demonstrated similar changes in the phase-transition curve of salmon sperm DNA following *in vitro* exposure of the DNA to the bladder carcinogens, 2-amino-1-naphthol and 1-amino-2-naphthol.

SUMMARY

In previous studies, pulmonary carcinomas have been induced in rats by exposure to the inhalation of nickel carbonyl. As a continuation of these investigations, measurements were undertaken of nickel and other trace metals in ribonucleic acid (RNA) from tissues of normal rats and of rats exposed to nickel carbonyl.

An ultraviolet spectrophotometric method was devised for the determination of nickel in samples of RNA containing as little as 0.4 μg. of nickel. Measurements of chromium, manganese, lead, tin, and zinc in RNA were made by means of emission spectrography.

Rat lung RNA was found to be rich in trace metals and to contain nickel, chromium, manganese, lead, tin, and zinc in quantities that exceed those present in RNA from rat liver, kidney, and skeletal muscle.

Acute exposure of rats to nickel carbonyl resulted in an increase in the concentration of nickel in NaCl-precipitable lung RNA and a decrease in the concentration of nickel in NaCl-soluble lung RNA. The concentration of nickel in NaCl-precipitable liver RNA increased following exposure of rats to nickel carbonyl, whereas that in NaCl-soluble liver RNA was not significantly altered.

Following inhalation of nickel carbonyl, changes that were consistent with an increase in metal-bonding were observed in the phase-transition curves of NaCl-precipitable RNA from liver and lung. The change that was observed in the phase-transition curve of NaCl-soluble lung RNA was consistent with disruption of hydrogen bonds.

SUMMARIO IN INTERLINGUA

In previe studios, carcinoma pulmonari esseva inducite in rattos per exponer le animales al inhalation de carbonyl de nickel. In continuation de iste investigationes, mesurationes esseva effectuate del nickel e altere oligometallos in acido ribonucleic ab tissu de rattos normal e ab tissu de rattos exponite a carbonyl de nickel.

Esseva elaborate un methodo de spectrophotometria ultraviolette pro le determination de nickel in specimens de acido ribo-

nucleic continente solmente 0.4 μg. de nickel. Mesurationes de chromo, manganese, plumbo, stanno, e zinc in acido ribonucleic esseva effectuate per medio de spectrographia emissionic.

Esseva trovate que acido ribonucleic ab le pulmon del ratto es ric in oligometallos. Illo contine nickel, chromo, manganese, plumbo, stanno, e zinc in quantitates excedente illos presente in acido ribonucleic ab le hepate, le ren, e le musculo skeletic del mesme animal.

Le acute exposition de rattos a carbonyl de nickel resultava in un augmento del concentration de nickel in acido ribonucleic pulmonari precipitabile per NaCl e in un reduction del concentration de nickel in acido ribonucleic pulmonari solubile in NaCl. Le concentration de nickel in acido ribonucleic hepatic precipitabile per NaCl cresceva post le exposition de rattos a carbonyl de nickel, durante que le concentration de nickel in acido ribonucleic hepatic solubile in NaCl non esseva alterate a grados significative.

Post le inhalation de carbonyl de nickel, alterationes compatibile con un augmento del ligaminage de metallo esseva observate in le curvas de transition phasic de acido ribonucleic precipitabile per NaCl in le caso de material obtenite ab hepate e pulmon. Le correspondente alteration observate in le caso de acido ribonucleic solubile in NaCl in le caso de material obtenite ab le pulmon esseva compatibile con disruption de ligamines de hydrogeno.

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MEASUREMENTS OF NICKEL IN BIOLOGICAL MATERIALS BY ATOMIC ABSORPTION SPECTROMETRY

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Quantitative determinations of trace metals by atomic absorption spectrometry were first described by Walsh in 1955.[21] The theory and analytical applications of atomic absorption spectrometry have been summarized by Allan,[3] David,[4] Elwell and Gidley,[5] Malmstadt and Chambers,[10] Robinson,[14] Russell and co-workers,[15] Willis,[22] and Zettner.[23] The limits of sensitivity for the detection of nickel by atomic absorption spectrometry have been estimated by Allan,[1,2] Fuwa and Vallee,[6] Kinson and Belcher,[9] Menzies,[11] Robinson,[14] and Willis.[22] In the present paper, an atomic absorption procedure is described which facilitates quantitative measurements of nickel in biological materials. The method has been employed for determinations of nickel in urine, ribonucleic acids, and serum proteins.

METHOD

Principle

After organic material has been destroyed by wet digestion, nickel ion is converted to nickel dimethylglyoxime, and extracted into chloroform at alkaline pH. Nickel is recovered from chloroform by extraction with dilute hydrochloric acid. Aspiration of the extract into the burners of the atomic absorption spectrometer produces thermal molecular dissociation and dispersion of nickel atoms throughout the flames. A small proportion of the nickel atoms becomes excited to emit light, but most of the atoms remain in the ground state and are capable of absorbing specific wave lengths of incident light. These discrete wave lengths are provided by a lamp with a hollow cathode constructed of metallic nickel. The beam of light from the hollow cathode lamp is passed several times through the flames and then is focused upon the entrance slit of a diffraction grating monochromator. The absorption of light is proportional to the concentration of nickel in the sample.

Precautions

Metal-free water, which is employed throughout the procedure, is prepared by ion-exchange and by distillation in an all-glass still. Glassware is cleaned with nitric acid immediately before use. Analyses are performed in duplicate or triplicate. A calibration curve is prepared with each group of analyses.

Reagents

1. *Nitric acid*, reagent grade, sp. gr. 1.5.
2. *Sulfuric acid*, reagent grade, sp. gr. 1.84.
3. *Hydrochloric acid*, 0.5 N.
4. *Ammonium hydroxide*, reagent grade, 29 per cent (w/v).
5. *Chloroform*, reagent grade.
6. *Dimethylglyoxime solution*. Dimethylglyoxime, 0.25 Gm., is dissolved in 50 ml. of absolute ethanol and diluted to 250 ml. with water.
7. *Citrate buffer*. Diammonium citrate, 200 Gm., is dissolved in 600 ml. of water and adjusted to pH 9.0 to 9.5 with concentrated ammonium hydroxide. The solution is transferred to a 1-liter, separatory funnel and 10 ml. of dimethylglyoxime solution are added. The mixture is extracted 3 times with 30 ml. of chloroform. The solution is passed through a retentive filter paper, and diluted to 1 liter with water.
8. *Phenolphthalein indicator solution*. Phe-

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nolphthalein, 1 per cent (w/v) is dissolved in ethanol, 70 per cent (v/v).

9. *Nickel stock standard solution.* Metallic nickel, 500 mg., is transferred to a 1-liter volumetric flask, dissolved in 20 ml. of nitric acid, and diluted to volume with water.

10. *Nickel working standard solutions.* Nickel stock standard solution, 1, 2, 3, 4, and 5 ml., is transferred to 500 ml. volumetric flasks and diluted to volume with water. These working standard solutions contain 1, 2, 3, 4, and 5 μg. of nickel per ml.

Atomic Absorption Apparatus

The principles and operation of the atomic absorption spectrometer* have previously been described by Sunderman and Carroll.[18] For measurements of nickel, 3 Beckman oxygen-acetylene burners are mounted in series upon the burner manifold. The cannulas of the burners are attached to 6-cm. lengths of polyethylene tubing,† in order that the sample may be aspirated from a test tube. The quartz mirrors of the optical system are focused so that the beam of light from the nickel hollow cathode lamp passes 5 times through the flames, providing an absorption path length of approximately 30 cm. The output of the photomultiplier tube amplifier circuit is recorded graphically by means of a strip-chart recorder.‡ The amplification of the recorder is increased to provide full-scale deflection with a 5-mv. signal.

Procedure

Digestion of urine. Fifty-milliliter samples of urine are transferred to 100-ml. Kjeldahl flasks. Twenty milliliters of nitric acid and a few Pyrex beads are added to each flask and the contents of the flask are digested to a volume of approximately 10 ml. The flask is cooled and 3 ml. of sulfuric acid are added. Digestion is continued until the appearance of white fumes of sulfur trioxide. If charring occurs, nitric acid is added as needed to clarify the digestion mixture. Three-milliliter quantities of water are added to each flask 3 times. Following each addition of water, digestion is continued until the appearance of white fumes.

Digestion of ribonucleic acid (RNA) or protein. Samples of RNA or protein weighing 15 to 50 mg. are transferred to 100-ml. Kjeldahl flasks. Five milliliters of nitric acid and a few Pyrex beads are added to each flask, and the contents of the flask are digested to a volume of 1 to 2 ml. The flask is cooled and 1 ml. of sulfuric acid is added. The digestion is continued as described for samples of urine.

Blank and standard samples containing 0, 1, 2, 3, 4, and 5 μg. of nickel are digested by the same procedure that is used for the unknown samples.

Extraction of nickel. Each digestion flask is cooled to room temperature in a water bath. Five milliliters of citrate buffer are added and the contents of the flasks are again cooled to room temperature. One drop of phenolphthalein indicator is added, and the contents of each flask are titrated with ammonium hydroxide until the appearance of a pink color. After the solution has cooled, 3 ml. of dimethylglyoxime solution are added and the contents of the flask are mixed. Three milliliters of chloroform are added; the flask is stoppered with a Teflon plug, and the contents of the flask are shaken for 1 min. The flask is allowed to stand for approximately 10 min., in order to complete the separation of the aqueous and chloroform phases. Using a long Pasteur pipet, the chloroform phase is removed quantitatively and transferred to a 12-ml. centrifuge tube. Extraction of the aqueous phase is repeated with a second 3-ml. volume of chloroform. In analyses of urine, a white precipitate of inorganic salts remains in the aqueous phase. One milliliter of 0.5 N hydrochloric acid is added to the centrifuge tube which contains the combined chloroform extracts. The tube is stoppered, shaken vigorously for 1 min., and centrifuged at 2500 r.p.m. for 3 min. The chloroform phase is removed by aspiration and discarded.

* Atomic absorption spectrometer, Model 82-362, Jarrell-Ash Company, Waltham, Massachusetts.

† Polyethylene "Intramedic" tubing, No. PE-160, Clay-Adams Company, New York, New York.

‡ Strip-Chart recorder, Model No. E15-1, Brown-Honeywell Regulator Company, Philadelphia, Pennsylvania.

Adjustment of the atomic absorption apparatus. The power supply to the nickel hollow cathode lamp is adjusted to 30 ma. The beam of light from the hollow cathode lamp is focused upon the monochromator aperture. Monochromator entrance and exit slits of 25 μ are placed in the slit-holders. The monochromator is adjusted to measure the absorption at 2320.0 Å. The oxygen regulator valve is opened first, followed by the acetylene regulator valve, and the burners are ignited. The gas regulator valves are adjusted to provide an oxygen pressure of 15 p.s.i. and an acetylene pressure of 9.5 p.s.i.

An opaque card is placed in front of the monochromator entrance slit and the recorder is adjusted to 100 per cent absorption. The card is removed and the recorder is adjusted to 0 per cent absorption while water is aspirated through the burners. Fluctuations in the baseline are minimized by use of the damping adjustment. The stability of the burners, optical system, and electronic system is verified by monitoring the base line while water is aspirated through the burners.

Measurements of atomic absorption. The polyethylene tubes connected to the burners are inserted into a test tube which contains 1 of the standards or samples. The absorption reading does not become stable until the sample has been aspirated into the flames for 2 to 3 sec. The atomic absorption of the sample is then recorded for 5 sec. Immediately following the aspiration of each sample, water is aspirated through the burners until the absorption reading has returned to the base line.

At the conclusion of the analyses, water is aspirated through the burners for 5 min. The acetylene regulator valve is closed and the burners are dried in a stream of oxygen for 1 to 2 min.

Calculations. Measurements of per cent absorption (% A) are converted into absorbance (O.D.) units by the equation:

$$\text{Absorbance (O.D.)} = 2 - \log_{10}(100 - \%\,A).$$

A calibration curve is used in computing the concentrations of nickel in the unknown samples.

EXPERIMENTAL STUDIES AND RESULTS

Instrumental Parameters

The sensitivity of detection of nickel with the atomic absorption spectrometer was determined for the nickel emission lines at 2310.9 Å, 2320.0 Å, 2345.5 Å, 3002.5 Å, 3050.8 Å, 3414.8 Å, 3461.7 Å, and 3524.5 Å. These measurements confirmed Allan's report[1] that greatest sensitivity was achieved with the emission line at 2320.0 Å. Under the operating conditions described for the procedure, the sensitivity (1% A) of detection of nickel in aqueous solution without preliminary extractions was 0.10 p.p.m.

Comparisons were made of the measurements of nickel with fuel mixtures of acetylene and oxygen, acetylene and air, propane and oxygen, and propane and air. The sensitivities which were obtained with acetylene-oxygen flames were greater than those obtained with the other fuel mixtures. A systematic evaluation was made of measurements of nickel with oxygen pressures ranging from 7 to 18 p.s.i., and with acetylene pressures ranging from 5 to 12 p.s.i. Optimal sensitivity was achieved with an oxygen pressure of 15 p.s.i. and an acetylene pressure of 9.5 p.s.i.

In order to prevent disturbance of the flames by drafts of air, as well as to reduce the auditory and visual irritation produced by the flames, a flame-shield was constructed of 3/16-in. asbestos sheeting. The flame-shield surrounded the burner and optical systems of the atomic absorption apparatus. Use of the flame-shield improved the constancy of the recorder baseline, and thereby resulted in a slight increase in the sensitivity of the apparatus for the detection of nickel. To avoid inhalation of nickel, the atomic absorption spectrometer was operated within a fume hood.

Additions of octyl alcohol, caprylic alcohol, and Sterox* to standards and urine samples did not improve the performance of the burners or increase the sensitivity of the method. Additions of a variety of organic solvents, including isopropyl alcohol,

* Sterox is a non-ionic detergent produced by the Monsanto Chemical Corporation, St. Louis, Missouri.

propyl alcohol, and acetone, were likewise without significant effect.

Digestion and Extraction Procedures

The sensitivity of the atomic absorption spectrometer was insufficient to permit direct measurements of nickel in normal urine; therefore, it was necessary to employ a chemical procedure to concentrate nickel, prior to atomic absorption spectrometry. Destruction of organic materials is an essential preliminary step in such procedures. The wet digestion technic which is described in the present method was found to be more rapid and convenient than the digestion procedure previously developed in our laboratory, which used a mixture of perchloric, sulfuric, and nitric acids.[8,16]

Several methods were attempted for extraction and concentration of nickel from the digestion mixture. In 1 approach, the digestion mixture was adjusted to pH 8.5, and sodium diethyldithiocarbamate was added. Nickel diethyldithiocarbamate was extracted into isoamyl alcohol, and the isoamyl alcohol extract was aspirated directly into the burners of the atomic absorption apparatus. In a second approach, the digestion mixture was adjusted to alkaline pH and dimethylglyoxime was added. Nickel dimethylglyoxime was extracted into butanol, and the butanol extract was aspirated directly into the burners. Dissatisfaction with the results obtained with both of these approaches led to the development of the extraction technic which is described here. Under the conditions outlined for the procedure, extraction of nickel dimethylglyoxime with chloroform was found to be preferable to the use of carbon tetrachloride, methylene chloride, ethylene dichloride, or a mixture of equal parts of chloroform and carbon tetrachloride.

Extraction of the chloroform phase with 1 ml. of 0.5 N hydrochloric acid, as described above, resulted in a mean recovery of 80.3 per cent (S.D. = ±3.4) of the nickel which was originally present in the digestion mixture. The recovery of nickel could be increased to 96 per cent by a second extraction of the chloroform phase with 1 ml. of hydrochloric acid. Since the concentration of nickel in the combined hydrochloric acid extracts was only 60 per cent of that in the first hydrochloric acid extract, greatest sensitivity of analysis was accomplished by a single extraction with hydrochloric acid.

Tests for Interference

To a series of standard samples containing 1 μg. of nickel were added soluble salts containing 100 μg. of metals which might possibly constitute sources of interference. The metals that were tested included sodium, magnesium, aluminum, potassium, calcium, vanadium, chromium, manganese, iron, cobalt, copper, zinc, arsenic, strontium, molybdenum, silver, cadmium, gold, mercury, lead, bismuth, and uranium. Cobalt and chromium salts were the only apparent sources of interference. Measurements with salts of cobalt and chromium from several commercial sources indicated that the apparent interferences were probably attributable to contamination of the reagents with traces of nickel.

To a series of standard samples containing 1 μg. of nickel were added sodium salts containing 100 μg. of anions which might possibly constitute sources of interference. The anions that were tested included borate, citrate, oxalate, nitrite, nitrate, fluoride, metasilicate, phosphate, sulfite, sulfate, thiosulfate, persulfate, chloride, and bromide. None of these anions interfered in the measurement of nickel.

Calibration Curve

As illustrated in Figure 1, the calibration curve obtained with the atomic absorption procedure adhered to the Beer-Lambert law with quantities of nickel in the range of 1 to 5 μg. With quantities of nickel greater than 5 μg., the calibration curve deviated from linearity. Since the slope of the calibration curve was influenced by small fluctuations in gas pressures and in monochromator adjustments, it was necessary to prepare a calibration curve with each set of analyses. It may be noted from Figure 1 that the atomic absorption technic was approximately 20 per cent less sensitive than the laborious ultraviolet spectrophoto-

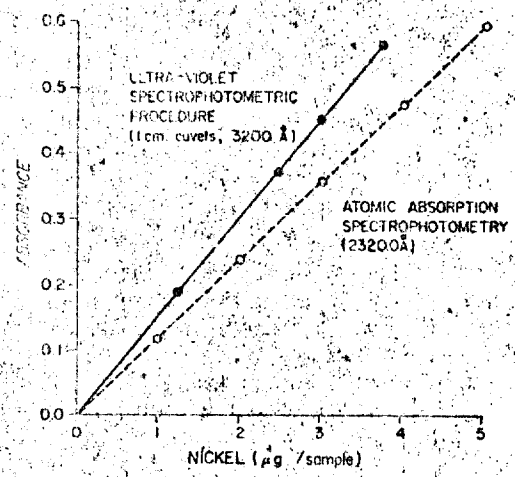

FIG. 1. Comparison of nickel calibration curves by spectrophotometry and atomic absorption.

metric method which was previously reported.[16]

Measurements of Precision and Recovery

Statistical analysis of differences between duplicate measurements of nickel in urine samples from 17 normal persons indicated that the S.D. of the method was ±0.12 μg. of Ni per 100 ml., (coefficient of variation = 6.3 per cent). Ten replicate determinations of nickel were performed upon a single specimen of human β-globulin.* The samples of β-globulin taken for analysis ranged in weight from 33 to 38 mg. and contained an average of 1.61 μg. of nickel. The mean concentration of nickel was 45.9 μg. of Ni per Gm. of protein (S.D. ±2.7), with a range of values from 41.3 to 50.2. The coefficient of variation of replicate analyses was 5. 9 per cent.

The recovery of 2.5 μg. of nickel added to 4 samples of urine averaged 96 per cent, with a range from 94 to 97. The recovery of 1 μg. of nickel added to 4 samples of human β-globulin averaged 101 per cent, with a range from 96 to 104.

Measurements of Nickel in Urine

Measurements of the urinary excretion of nickel by 17 normal persons are summarized in Table 1. The subjects were physicians and laboratory technologists who had no occupational exposure to nickel. The mean concentration of nickel in urine was 1.8 μg. per 100 ml. (S.D. = ± 0.8), with a range from 0.4 to 3.1 μg. per 100 ml. The mean excretion of nickel in urine was 19.8 μg. per 24 hr. (S.D. = ±10.0), with a range from 7.2 to 37.6 μg. per 24 hr. As indicated in Table 1, the observed concentrations of nickel in urine are comparable to the concentrations reported by previous investigators.[7, 8, 12, 13]

Measurements of Nickel in Ribonucleic Acids

Measurements of nickel were made upon specimens of ribonucleic acids isolated from the ultracentrifugal (109,000 × *g*) supernatants of homogenates of lungs and livers of normal rats, by methods previously developed in our laboratory.[16, 17, 19] The mean concentration of nickel in 5 preparations of supernatant RNA from rat lung was 48 μg. of Ni per Gm. of RNA with a range from 34 to 64. The mean concentration of nickel in 5 preparations of supernatant RNA from rat liver was 29 μg. of Ni per Gm. of RNA, with a range from 21 to 39. In studies which are currently in progress, the concentrations of nickel in preparations of RNA from normal rats are being contrasted with the concentrations of nickel in RNA from rats which have been chronically exposed to the inhalation of nickel carbonyl to carcinogenic levels.

Measurements of Nickel in Serum Proteins

The mean concentrations of nickel in purified preparations of human serum proteins are listed in Table 2. Electrophoretic fractions of serum proteins from a normal subject were prepared in our laboratory by continuous-flow electrophoresis, lyophilization, lipid extraction, and dialysis, as previously described.[20] Purified serum proteins, prepared by cold-ethanol fractionation, were obtained from 2 commercial sources.* As indicated in Table 2, the

* Pentex Biochemicals, Inc., Kankakee, Illinois.

* Nutritional Biochemicals Corporation, Cleveland, Ohio, and Pentex Biochemicals, Inc., Kankakee, Illinois.

TABLE 1

RESUMÉ OF NORMAL VALUES FOR URINE NICKEL

Author	Year	No. of Subjects	Urine Nickel		
			Mean	S.D.	Range
			μg./100 ml.		
Kinkaid and associates[8]	1956	69	1.1	±0.9	0–3.0
Perry and Perry[13]	1959	24	2.0	±2.6	1.0–7.0
Morgan[12]	1960		4.0	±2.0	0–8.0
Imbrus and associates[7]	1963	154	1.0	±0.7	0.1–8.1
Present study	1965	17	1.8	±0.8	0.4–3.1
Present study	1965	17	19.8*	±10.0*	7.2–37.6*

* These results are given in micrograms per 24 hr.

TABLE 2

CONCENTRATIONS OF NICKEL IN PURIFIED SERUM PROTEINS

Serum Protein Fractions	Continuous-Flow Electrophoresis	Cold-Ethanol Fractionation*	Cold-Ethanol Fractionation†
	μg. Ni/Gm. protein		
Albumin	9	5	7
α-Globulins	22		
Fraction IV-1		<1	<1
Fraction IV-4		7	10
β-Globulin	51	93	46
γ-Globulin	19	3	<1

* Nutritional Biochemicals Corp., Cleveland, Ohio.

† Pentex Biochemicals, Inc., Kankakee, Illinois.

highest concentrations of nickel were present in preparations of serum β-globulin. Attempts to isolate and to characterize a nickel-binding constituent of serum β-globulin are currently being undertaken in our laboratory.

DISCUSSION

The sensitivity of the atomic absorption procedure for the determination of nickel in biological materials is greater than the sensitivity of the colorimetric procedures[8, 12] and less than the sensitivities of the ultraviolet spectrophotometric[16] and emission spectrographic technics.[7, 13] The time required for measurements of nickel by the atomic absorption procedure is approximately one half of that required for the ultraviolet spectrophotometric procedure. The cost of an atomic absorption spectrometer is less than that of a suitable emission spectrograph, and the atomic absorption spectrometer is more adaptable to use in clinical laboratories. For these reasons, the atomic absorption procedure should be especially useful for routine determinations of urinary nickel, in the detection of industrial exposures to the inhalation of nickel carbonyl.[8, 12]

SUMMARY

An atomic absorption procedure has been developed which facilitates quantitative measurements of nickel in biological materials, including urine, ribonucleic acids (RNA), and serum proteins.

The sensitivity of detection of nickel by the atomic absorption spectrometer (0.10 p.p.m.) was insufficient to permit direct measurements of nickel in normal urine. Therefore, it was necessary to employ a dimethylglyoxime extraction procedure to concentrate the nickel, prior to atomic absorption spectrometry.

The coefficients of variation of measurements of nickel in urine and human β-globulins were 6.3 and 5.9 per cent, respectively. The recovery of nickel added to urine averaged 96 per cent, with a range from 94 to 97, and the recovery of nickel added to human β-globulin averaged 101 per cent, with a range from 96 to 104.

The mean concentration of nickel in 24-hr.

collections of urine from 17 normal subjects was 1.8 μg. per 100 ml. (S.D. = ±0.8), with a range from 0.4 to 3.1. The mean urinary excretion of nickel was 19.8 μg. per 24 hr. (S.D. = ±10.0), with a range from 7.2 to 37.6.

The mean concentration of nickel in 5 preparations of ribonucleic acids from ultracentrifugal supernatants of homogenates of rat lung was 48 μg. of Ni per Gm. of RNA, with a range from 34 to 64. The mean concentration of nickel in 5 preparations of RNA from ultracentrifugal supernatants of homogenates of rat liver was 29 μg. of Ni per Gm. of RNA, with a range from 21 to 39.

Measurements of nickel were performed upon fractions of human serum proteins, prepared by continuous-flow electrophoresis, and by cold-ethanol precipitation. The highest concentrations of nickel were found in preparations of serum β-globulins.

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Spectrophotometric Measurements of Serum Nickel

F. William Sunderman, Jr.

An ultraviolet spectrophotometric procedure has been developed for the measurement of serum nickel. Samples of serum are lyophilized and the dry residues are subjected to acid digestion. Nickel is separated from interfering elements by chloroform extraction of nickel dimethylglyoximate at alkaline pH. Nickel is converted to the diethyldithiocarbamate complex and is extracted with isoamyl alcohol. The absorbance of nickel bisdiethyldithiocarbamate is measured as the difference between the absorbance maximum (325 mμ) and the absorbances at 2 background wave lengths (295 and 355 mμ). The concentrations and volumes of the reagents have been adjusted in order to achieve a sensitivity of 0.002 ppm (2 μg./L.). The recovery of 2.5 μg. of nickel added to 8 samples of serum averaged 104% (S.D. ± 8), with a range from 91 to 115%. The mean concentration of nickel in serum from 23 normal subjects was 2.2 μg./100 ml. (S.D. ± 1.8), with a range from 0.1 to 7.7 μg./100 ml. The median concentration of serum nickel was 1.7 μg./100 ml.

CLINICAL INTEREST in measurements of serum nickel has been stimulated by the report of elevated concentrations of nickel in serums of patients with myocardial infarction. Using a spectrographic method for semiquantitative estimations of nickel, D'Alonzo and Pell (*1*) have observed that serum nickel concentrations were greater than 50 μg./100 ml. in 19 of 20 patients with acute myocardial infarction. The differences between the concentrations of serum nickel in patients with myocardial infarction and in control subjects were highly significant ($p = 0.000002$). Based upon these findings, D'Alonzo and Pell (*1*) have

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speculated that nickel may either be involved in the etiology of myocardial infarction, or that an enzyme containing nickel may be released into the serum following myocardial infarction.

As a preliminary step in investigation of nickel metabolism in myocardial infarction, a spectrophotometric procedure for measurement of serum nickel has been developed in our laboratory. Previously, the technic of Kinkaid *et al.* (*2*) for the determination of nickel in biological materials was modified in order to increase its sensitivity by approximately 10-fold (*3*). Further refinements in this method for nickel analysis are described in the present paper which have resulted in an additional twofold increase in sensitivity, and have enabled measurements to be made of the concentrations of nickel in serums from normal subjects.

Materials and Methods

Principle

Samples of serum are lyophilized and the dry residues are subjected to acid digestion. Nickel is separated from interfering elements by chloroform extraction of nickel dimethylglyoximate at alkaline pH. Nickel is converted to the diethyldithiocarbamate complex and is extracted with isoamyl alcohol. The absorbance of nickel bisdiethyldithiocarbamate is measured as the difference between the absorbance at the adsorption maximum (325 mμ) and the mean of the adsorbances at 2 background wavelengths (295 and 355 mμ).

Precautions

Each batch of reagents is analyzed to ensure that it is not contaminated with nickel. Metal-free water, obtained by distillation and ion exchange, is used for the preparation of solutions. Glassware is cleaned with warm nitric acid and rinsed with metal-free water immediately before use. Analyses are performed in duplicate, and measurements of the recovery of nickel added to serum are included in each group of determinations.

Reagents

Nitric acid Reagent grade, s.g. 1.5.
Sulfuric acid Reagent grade, s.g. 1.84.
Hydrogen peroxide solution Reagent grade, 30% (w/v).
Ammonium hydroxide solution Reagent grade, 29% (w/v).
Dilute ammonium hydroxide solution 1% (w/v).
Hydrochloric acid 0.5N.
Chloroform
Carbon tetrachloride

Isoamyl alcohol
Octyl alcohol

Dimethylglyoxime solution Dimethylglyoxime, 0.25 gm., is dissolved in 50 ml. of absolute ethanol and diluted with water to 250 ml. This solution is stored in the refrigerator and is stable for at least 1 month.

Sodium diethyldithiocarbamate stock solution Soduim diethyldithiocarbamate trihydrate, 0.2 gm., is dissolved in 100 ml. of water. This solution is prepared each week and stored in the refrigerator.

Sodium diethyldithiocarbamate working solution A 1:10 dilution (with water) of the stock solution is prepared immediately prior to use.

Citrate buffer A Diammonium citrate, 200 gm., is dissolved in 600 ml. of water, adjusted to pH 9.0-9.5 with concentrated NH_4OH, and transferred to a 1-L. separatory funnel. Ten ml. of dimethylglyoxime solution are added and the mixture is extracted 4 times with 30 ml. of chloroform. The aqueous solution is filtered through Whatman No. 4 filter paper and diluted to 1 L. with water.

Citrate buffer B Diammonium citrate, 200 gm., is dissolved in 600 ml. of water, adjusted to pH 9.0-9.5 with concentrated NH_4OH, and transferred to a 1-L. separatory funnel. Stock sodium diethyldithiocarbamate solution, 5 ml., is added and the mixture is extracted with 20 ml. of CCl_4 until the solvent layer is colorless (at least 4 extractions). The aqueous solution is filtered through Whatman No. 4 filter paper and diluted to 1 L. with water.

Nickel stock standard Metallic nickel, 500 mg., is dissolved, with heating, in 20 ml. of HNO_3 (1:1) and diluted with water to 1 L.

Nickel working standard, (0.5 μg./ml.) Nickel stock standard, 1 ml., is diluted to 1 L. with 0.5N HCl.

Procedure

Collection of Blood and Separation of Serum

Venepuncture is performed with a No. 19-gauge platinum-ruthenium needle.* Samples of blood (100 ml.) are withdrawn using all-glass 50-ml. hypodermic syringes. The blood is dispensed into 50-ml. centrifuge tubes, and is allowed to clot at room temperature for 30 min. before the serum is removed. Special care should be taken to minimize hemolysis.

Lyophilization and Digestion

Transfer 20-ml. samples of serum to 50-ml. Kjeldahl digestion flasks. The serum is frozen by immersing the flasks in a mixture of dry ice

*Supplied by the Hamilton Co., Whittier, Calif.

and acetone, and the flasks are connected to the manifold of a lyophilization apparatus. When lyophilization is complete, the flasks are removed from the manifold. Add 20 ml. of HNO_3 to the dried serum samples, as well as to 2 empty digestion flasks ("blank" samples) and to 2 digestion flasks containing 1.0 μg. of nickel ("standard" samples). Pyrex beads are dropped into each flask to prevent bumping, and 0.5 ml. of octyl alcohol is added to prevent foaming. The flasks are heated on a Kjeldahl digestion apparatus, and are boiled down to a volume of 1–2 ml. Additional octyl alcohol is introduced dropwise, as necessary, to prevent foaming. The flasks are allowed to cool. Add 3 ml. of concentrated H_2SO_4 and continue the digestion. Whenever charring occurs, 4 drops of concentrated HNO_3 are added immediately so that the color of the digestion mixture returns from black to yellow. When the contents of each flask reach a volume of 1–2 ml., 4 drops of 30% H_2O_2 solution are added in order to bleach the yellow color. If charring occurs after the addition of H_2O_2, 4 drops of HNO_3 are added as before. With repeated additions of H_2O_2 and HNO_3 the digestion mixture eventually becomes colorless. Boiling is continued for at least 15 min. after the last addition of H_2O_2.

Nickel Extraction

Each digestion flask is cooled to room temperature. Add 5 ml. of citrate buffer A and again cool the flask to room temperature. The contents of the flask are adjusted to pH 8.5 by addition of concentrated NH_4OH. The measurement of pH in the Kjeldahl flask is facilitated by the use of a probe-type pH electrode. Add 3 ml. of dimethylglyoxime solution. The contents of the flask are mixed with a "Vortex" rotatory mixer and then are allowed to stand for 5 min. Add 3 ml. of chloroform and shake the contents of the flask for 1 min. The flask is allowed to stand for approximately 10 min. in order to separate the aqueous and chloroform phases. Using a Pasteur pipet, the chloroform phase is transferred to a 50-ml. Teflon-stoppered centrifuge tube. Extraction of the aqueous phase is repeated with 2 additional 3 ml. volumes of chloroform. Add 20 ml. of dilute NH_4OH solution to the combined chloroform extracts. The contents of the centrifuge tube are mixed with the Vortex mixer. After centrifugation at 1500 rpm for 10 min., the upper (NH_4OH) phase is aspirated and discarded. The washing of the chloroform extracts is repeated with 2 additional 20-ml. volumes of dilute NH_4OH solution. Add 20 ml. of 0.5N HCl to the combined chloroform extracts. The contents of the centrifuge tube are mixed with the Vortex mixer. After centrifugation at 1500 rpm for 10 min., the lower (chloroform) phase is aspirated and discarded. Add 2 ml.

of carbon tetrachloride (CCl_4) to the HCl extract. The mixture is centrifuged and the lower (CCl_4) phase is aspirated and discarded. Add 5 ml. of citrate buffer B to the centrifuge tube and adjust the contents to pH 8.5 by addition of concentrated NH_4OH. Add 2 ml. of sodium diethyldithiocarbamate working solution. The contents of the tube are mixed with the Vortex mixer and allowed to stand for 15 min. Add 3 ml. of isoamyl alcohol; the tube is stoppered, and the contents are mixed for 1 min. After centrifugation at 1500 rpm for 15 min., the lower (HCl) phase is aspirated and discarded, and the isoamyl alcohol phase is transferred to a quartz spectrophotometer cuvet of 1-cm. pathlength and 2-ml. capacity.

Spectrophotometry and Computations

Spectrophotometry is performed in a Beckman Model DK-2A ratio-recording spectrophotometer using isoamyl alcohol as the reference solution. The absorbance curve is recorded from 290 to 360 mμ. The corrected absorbance of nickel bisdiethyldithiocarbamate is computed graphically as illustrated in Fig. 1, or is calculated by the following equation:

$$A_{corr.} = A_{325\ m\mu} - \frac{A_{295\ m\mu} + A_{355\ m\mu}}{2}$$

Experimental Studies and Results

Collection of Blood

No differences were observed between the concentrations of serum nickel in duplicate samples of blood which were obtained via platinum-ruthenium needles and via Teflon needles. Likewise, no differences were found between the concentrations of serum nickel in duplicate samples of blood which were collected in all-glass syringes and in plastic transfusion bags. In blood samples which were grossly hemolyzed, the concentrations of serum nickel were as much as 1.5 μg./100 ml. higher than in control samples. The concentration of nickel in a sample of serum which was allowed to remain in contact with the blood clot for 8 hr. was 0.8 μg./100 ml. higher than in a control sample of serum which was separated from the blood slot 30 min. after collection. On the basis of these findings, it would appear to be important to avoid hemolysis and prolonged contact of serum with the blood clot.

Digestion of Serum

The acid-digestion procedure was facilitated by prior lyophilization of the serum samples. Constant attention was necessary during the acid digestion in order to avoid charring and to prevent loss of the

sample through bumping or foaming. It usually required 12–14 hr. to complete the digestion. Although the digestion was more rapid when perchloric acid ($HClO_4$) was employed instead of H_2O_2, $HClO_4$ was not selected for routine use owing to the possible hazard of explosion.

Extraction of Nickel Dimethylglyoximate

According to Kinkaid (*2*), Stary (*4*), and Sandell (*5*), separation of nickel from the other metals which are present in biological materials may be accomplished by chloroform extraction of nickel dimethylglyoximate. Although iron and copper are potential sources of interference, inclusion of the citrate ion in the buffer keeps ferric phosphate in aqueous solution, and traces of copper dimethylglyoximate which are soluble in chloroform may be removed by repeated extractions with dilute NH_4OH (*5*). In the present investigation, no interference in the determination of nickel was observed when ferric or cupric ions were added to serum samples in quantities equivalent to 200 μg./100 ml. of serum. Maximum recovery of nickel was accomplished by 3 extractions

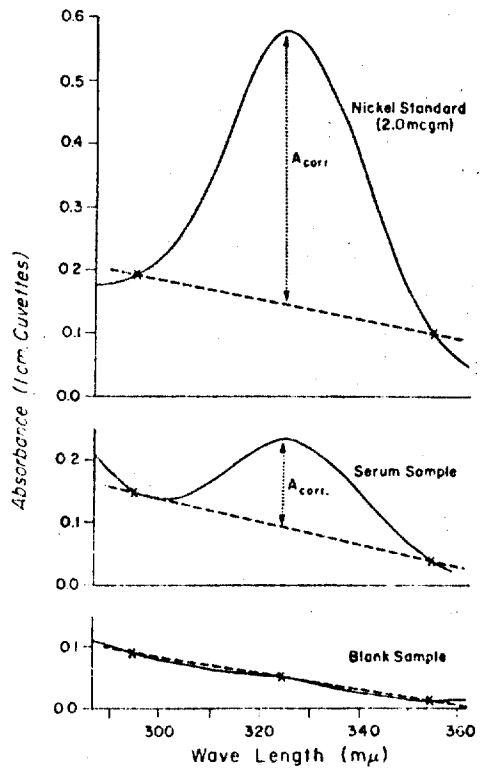

Fig. 1. Spectral absorption curves of nickel bisdiethyldithiocarbamate. (See text).

of the nickel dimethylglyoximate with 3 ml. of chloroform followed by a single re-extraction of the chloroform with 20 ml. of 0.5N HCl.

Extraction of Nickel Bisdiethyldithiocarbamate

The optimum concentration of diethyldithiocarbamate ion was studied by including sodium diethyldithiocarbamate trihydrate in the final reaction mixture in quantities ranging from 0.1 to 10 mg. Greatest sensitivity was achieved by use of 0.4 mg. of sodium diethyldithiocarbamate, rather than 10 mg., as previously described (*3*). Our findings indicate that the sensitivity of measurements of nickel by the ultraviolet spectrophotometric technic, which was previously employed in our laboratory, was limited by the presence of too great an excess of diethyldithiocarbamate ion, resulting in unnecessarily high "blank" absorbance at 325 mμ. The optimum volume of isoamyl alcohol for extraction of the nickel bisdiethyldithiocarbamate was found to be 3 ml. Increasing the volume of the isoamyl alcohol extract to 4 ml. diminished the sensitivity of analysis, owing to dilution. Additional extractions with isoamyl alcohol did not improve the sensitivity or precision of the method.

Spectrophotometric Measurements

The precision of replicate analyses was improved by use of the Allen technic of correcting for variability in "base line" absorbance (*6*). The most satisfactory results were obtained when absorbance meas-

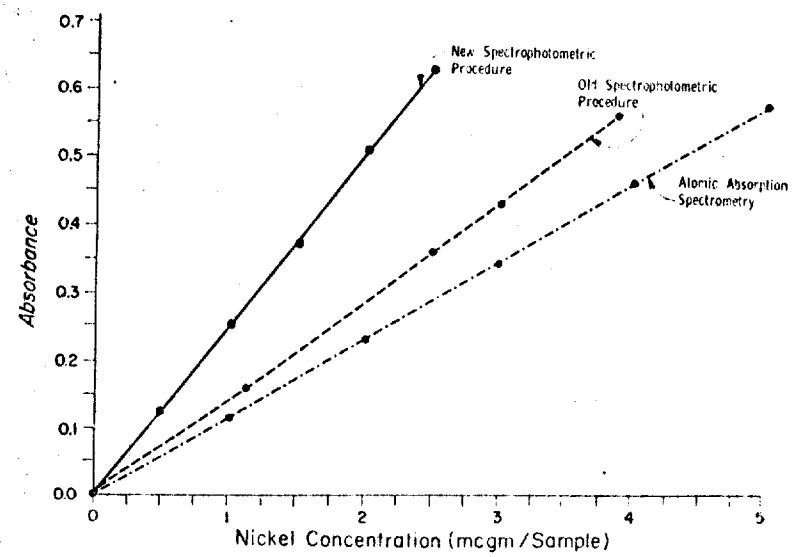

Fig. 2. Comparison of nickel calibration curves. (See text.)

urements were made at the absorption peak of nickel bisdiethyldithiocarbamate (325 mμ) and at 2 background wavelengths which were equidistant on either side of the peak (295 and 355 mμ, respectively). As shown in Fig. 1, a straight line could be drawn through the absorbance readings of "blank" samples at 295, 325, and 355 mμ.

As illustrated in Fig. 2, the calibration chart of absorbance versus nickel concentration was linear with quantities of nickel ranging from 0.5 to 2.5 μg. per sample. It may be noted from the relative slopes of the calibration curves that the determination of nickel by the present procedure is more sensitive than by the previous spectrophotometric procedure (*3*), or by the atomic absorption procedure (*7*) which is used in our laboratory for measurements of nickel in urine.

Sensitivity, Precision, and Recovery

Under the described conditions, the gram atom absorptivity at 325 mμ was 37,400. The limit of detection, expressed on the basis of a corrected absorbance of 0.01 absorbance unit, was 0.04 μg. of nickel per sample. With 20-ml. samples, an absorbance reading of 0.01 was approximately equivalent to 0.2 μg. of Ni per 100 ml. of serum (i.e., 2 parts per billion). The range of variation of duplicate analyses of 23 normal serums was 0.0–0.4 μg. Ni per 100 ml., and the S.D. of replicate measurements was ± 0.20 μg. Ni per 100 ml. The coefficient of variation of replicate measurements of nickel in normal serum was 9.1%. The mean recovery of 2.5 μg. of Ni added to eight 20-ml. samples of serum was 104% with a S.D. of ± 8% and a range of recovery from 91 to 115%.

Measurements of Serum Nickel

A frequency-distribution curve for the concentration of nickel in serums from 23 normal persons is portrayed in Fig. 3. The subjects were 9 male and 14 female technologists, age 20–35 years, who had no occupational exposure to nickel. The mean concentration of serum nickel was 2.2 μg./100 ml. (S.D. = ± 1.8), with a range of 0.1–7.7 μg./

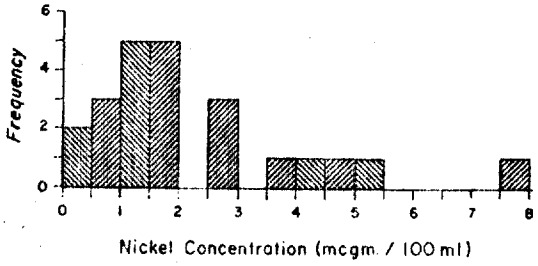

Fig. 3. Frequency distribution graph of concentrations of nickel (Spectrophotometric measurements) in serums from 23 normal adults. Mean, 2.2; median, 1.7; range, 0.1–7.7; S.D., ± 1.8.

100 ml. The median concentration of serum nickel was 1.7 μg./100 ml. The quantity of nickel in 1 sample was barely detectable, with a corrected absorbance value of 0.005. The concentration of nickel in this serum was estimated to be 0.1 μg./100 ml.

Discussion

A résumé of published values for the normal concentration of nickel in plasma and serum is given in Table 1. The mean concentration of serum nickel which was observed in the present investigation is lower than the mean concentrations which have been obtained by spectrographic technics. All of the investigators have found that the concentrations of nickel in normal serum or plasma were near the limits of detection of their analytical methods. In contrast to the limit of detection of 2 parts per billion by the present procedure, the limit of detection by the spectrographic method of Paixao and Yoe was 20 parts per billion (*11*), and that of Herring *et al.* was 7 parts per billion (*12*). Butt *et al.* (*13*) did not indicate the limit of detection of nickel by their spectrographic technic; however, they stated that nickel was not detected in 18 of their 48 serums.

Published values for the normal concentration of nickel in whole blood and erythrocytes are summarized in Table 2. Cluett (*8*), Paixao and Yoe (*11*), and Butt *et al.* (*13*) have reported that the mean con-

Table 1. Résumé of Normal Concentrations of Nickel in Plasma and Serum Using Various Methods*

Author	Date	Subjects (No.)	Nickel (μg./100 ml.) Range	Mean
		PLASMA		
Cluett (*8*)	1956	1	—	1.2
Koch *et al.* (*9*)	1956	—	1.0– 8.5	3.0†
Monacelli *et al.* (*10*)	1956	12	1.0– 6.0	4.0
Paixao & Yoe (*11*)	1959	39	0.0–18.0	2.3
Herring *et al.* (*12*)	1960	109	0.0–27.0	6.0
		SERUM		
Butt *et al.* (*13*)	1964	48	—	5.3–6.2‡
Gofman *et al.* (*14*)	1964	39	0.0–18.0	—
Present study	1966	23	0.1– 7.3	2.2§

*All authors used a spectrographic method, with these exceptions: spectrophotometric method used by Cluett and in the present study; X-ray spectroscopic method used by Gofman *et al.*

†S.D., ± 1.9.

‡Range of mean (Ni not detected in 18 serums).

§S.D., ± 1.8.

Table 2. Résumé of Normal Concentrations of Nickel in Whole Blood and Erythrocytes Using Various Methods*

Author	Date	Subjects (No.)	Nickel (μg./100 ml.) Range	Mean
		WHOLE BLOOD		
Cluett (*8*)	1956	8	2.0– 9.0	4.1
Paixao & Yoe (*11*)	1959	—	—	3.6†
Imbus *et al.* (*15*)	1963	153	0.9– 9.8§	4.2‡
Butt *et al.* (*13*)	1964	47	—	32.7
		ERYTHROCYTES		
Paixao & Yoe (*11*)	1959	40	0.0–16.0	5.1
Herring *et al.* (*12*)	1960	106	0.0–31.0	5.3

*All authors used a spectrographic method except Cluett who used a spectrophotometric method.
†Calculated from values for plasma and erythrocytes.
‡S.D., ± 2.8.
§Ninety-fifth percentile.

centrations of nickel in whole blood or erythrocytes were higher than in serum or plasma. Although the normal concentrations of nickel in whole blood or erythrocytes have not been established in our laboratory, our observation of increased concentrations of nickel in hemolyzed serum supports the view that the concentration of nickel is higher in erythrocytes than in serum. Contrary findings have been reported by Herring *et al.* (*12*), who noted a slightly lower mean concentration of nickel in erythrocytes than in plasma. Herring and associates did not detect any significant differences between the nickel concentrations in blood from males and females or from Negroes and Caucasians.

In a previous investigation (*7*), measurements were made of the concentrations of nickel in purified preparations of human serum proteins obtained by continuous-flow electrophoresis and by cold-ethanol fractionation. The highest concentrations of nickel were consistently found in the beta globulin fractions. Our conclusion that a nickel-binding globulin is present in human serum has recently been confirmed by Himmelhoch *et al.* (*17*). Fractions of dialyzed human serum obtained by gradient chromatography on DEAE-cellulose columns were analyzed for nickel and other metals by emission spectrography. Himmelhoch *et al.* identified a nickel-binding protein which was distinctly separated from the proteins which bound iron, zinc, and manganese. Based upon the presence of nickel in a single protein fraction, Himmelhoch and his associates speculated that serum nickel may be a constituent of a specific metalloprotein.

Although the spectrophotometric method for the measurement of serum nickel is intended primarily for use in investigations of nickel metabolism in myocardial infarction, the technic should also be valuable in the diagnosis of poisoning from nickel carbonyl and other toxic nickel compounds. It is anticipated that measurements of serum nickel may furnish an index of the severity of acute nickel poisoning, and serve as a guide to the efficacy of chelation therapy. In addition, the procedure may provide a means of detecting chronic exposure to inhalation of nickel, a condition which is associated with a high incidence of cancer of the respiratory tract (*16*). Measurements of serum nickel in patients with myocardial infarction and in experimental animals exposed to nickel carbonyl are currently being undertaken in our laboratory and will be described in subsequent publications.

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The Journal of Nutrition
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Nickel Deprivation in Chicks [1]

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ABSTRACT To investigate the effects of nickel deprivation, 12 chicks (group A) were grown in isolation from environmental nickel, and were fed a diet which contained 44 ppb of nickel. Twelve control chicks (group B) were treated similarly and were fed the same diet with added nickel (3.4 ppm Ni as $NiCl_2$). Nickel deprivation was produced in the chicks of group A, based upon the presence of diminished mean concentrations of nickel in serum and liver on day 30 (serum Ni = 1.6 ± 0.3 μg/liter in group A, versus 4.2 ± 0.4 μg/liter in group B, $P < 0.001$; hepatic Ni = 64 ± 6 μg/kg dry weight in group A, versus 82 ± 9 μg/kg dry weight in group B; $P < 0.01$). After 30 days, there were no significant differences between groups A and B in (a) body weights, (b) appearance of legs on physical and X-ray examinations, (c) hematological parameters, (d) concentrations of serum cholesterol, or (e) histological appearance of organs and tissues by light microscopy. Electron microscopic examination demonstrated dilatation of perimitochondrial rough endoplasmic reticulum in hepatocytes of nickel-deprived chicks. J. Nutr. *102:* 259–268, 1972.

INDEXING KEY WORDS nickel · endoplasmic reticulum

Nielsen and associates (1, 2) have reported that the feeding to chicks of diets which contain 40 to 80 ppb of nickel results in the development of an apparent nickel deficiency syndrome. They observed that nickel-deficient chicks had (a) swollen hock joints, (b) reduced length-to-width ratios of the tibias, (c) orange-yellow discoloration of the legs, (d) scaly dermatitis of the legs, (e) fat-depleted livers, and (f) enhanced accumulation of a tracer dose of ^{63}Ni in liver, spleen and aorta. In one experiment (2), the nickel-deficient chicks had depressed rates of growth. These abnormalities did not occur in control chicks which were fed the identical diet with 3 to 5 ppm of nickel added as nickel chloride. The present investigation has been undertaken in an endeavor to confirm these observations by Nielsen and collaborators (1, 2).

MATERIALS AND METHODS

Twenty-four male chicks of the White Rock strain [4] were brought to the laboratory within 3 hours after hatching. Twelve of the chicks (group A) were fed a low nickel diet (see below), and were kept in plastic isolation cages (fig. 1) in a small, controlled-environment room. The remaining 12 chicks (group B) were fed the same diet with added nickel chloride (see below), and were kept in identical isolation cages in a similar, adjacent room. Except for the investigators, no one was permitted to enter either room for the 30-day experiment. The rooms were brightly illuminated throughout the experiment; the room temperatures were maintained at 29 to 32°, and the relative humidity was maintained at 40 to 60%. The rooms were continually ventilated with fresh, filtered air, which was not permitted to recirculate. The chicks were housed in groups of five or six per

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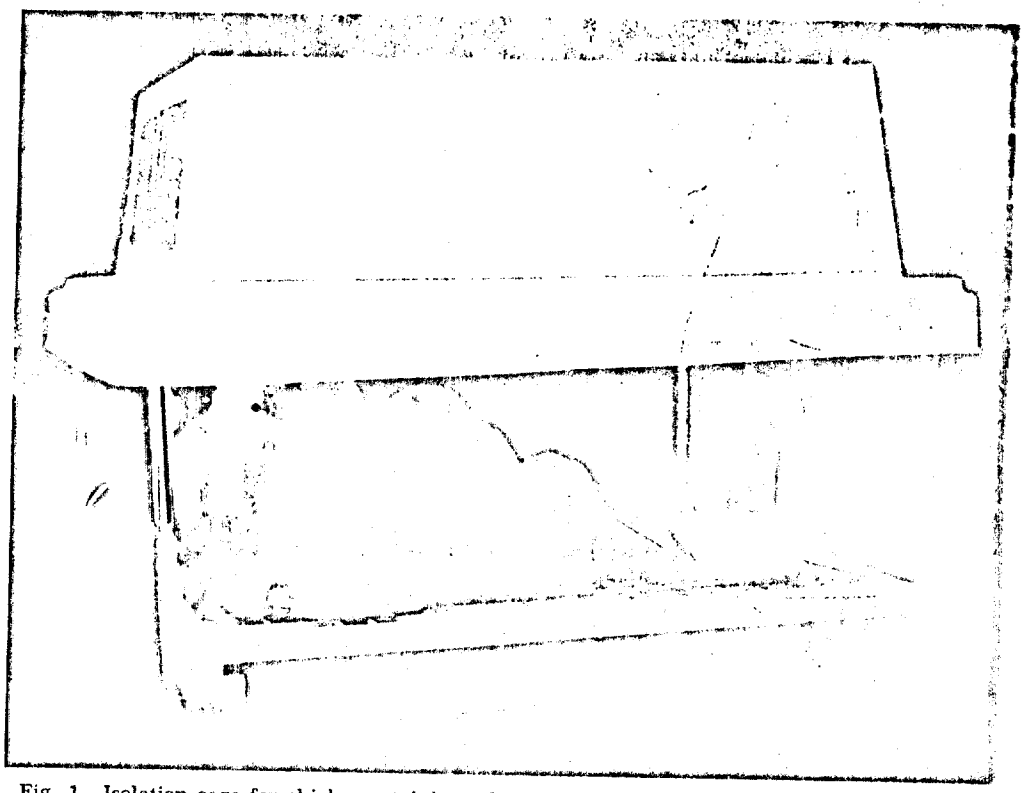

Fig. 1 Isolation cage for chicks containing: plastic grid (of a type commonly used in fluoresc lighting fixtures); plastic Petri dish for foods; and plastic water dispenser (as commonly sold in shops for use in canary cages). (The plastic cages and filter-paper covers were purchased from C worth Division, Becton, Dickinson and Co., Rockland County, N. Y.).

isolation cage during the first 15 days and in groups of three or four per cage during the second 15 days. The isolation cages were replaced with clean cages every 2 days. Plastic Petri dishes were used as food containers. Three circular holes (2.5 cm diameter) were cut in the cover of each Petri dish, in order to prevent the chicks from scratching the diet out of the dish, and in order to minimize contamination of the diet with excreta. The cages and their contents (plastic grids and plastic containers for food and water) were first scrubbed with soap, then immersed for 1 hour in 2 N nitric acid, and finally rinsed with copious water which had been demineralized by ion-exchange [5] and freshly distilled in an all-glass still. All equipment and materials used for the chicks which were fed the low nickel diet (group A) were strictly separated from those used for the chicks fed the nickel-supplement diet (group B).

The chicks were allowed to consu their diets and demineralized, distill water ad libitum. The low nickel di (tables 1, 2 and 3) was similar to the di which was employed in experiment no. of Nielsen and Higgs (2), with min modifications.[6] Nonnutritive fiber [7] w purified by soaking 0.5 kg of fiber in 3 lit of 0.8 N hydrochloric acid for 1 hour. T nonnutritive fiber was collected on Wh man no. 1 filter paper by means of sucti

[5] Continental Water Systems, Inc., Ridgefield, N.J

[6] Based upon a personal communication from Forest H. Nielsen, U. S. Department of Agricult Beltsville, Md., the following modifications were ma a) L-arginine was increased from 5 to 10 mg/ b) retinyl palmitate was increased from 4 to 8 n kg; c) DL-α-tocopheryl acetate was increased from to 40 mg/kg; d) pyridoxine hydrochloride was creased from 1 to 2 mg/kg, and e) neomycin sulf was added in a concentration of 25 mg/kg.

[7] Solka-Floc BW-40, Brown Co., Berlin, N.H.

TABLE 1

Low nickel diet for chicks

Ingredient	g Ingredient per kg diet	μg Ni per g ingredient	μg Ni per kg diet
Skimmed milk powder [1]	625	0.033	20.63
Corn meal, yellow degerminated enriched [1]	275	0.074	20.35
Corn oil [1]	50	0.019	0.95
Nonnutritive fiber [2] (purified before use)	30	0.005	0.15
L-Arginine [3]	10.0	0.039	0.39
L-Glycine [1]	8.0	0.007	0.056
Choline chloride [1]	0.30	0.009	0.003
Vitamin–antibiotic mixture (see table 2)	0.70	0.049	0.034
Mineral mixture (see table 3)	1.00	2.06	2.06
	1000.00		44.62 [4]

[1] Nutritional Biochemicals Corp., Cleveland, Ohio.
[2] Solka-Floc BW-40, Brown Co., Berlin, N.H.
[3] Calbiochem Corp., Los Angeles, Calif.
[4] This computed concentration of nickel in the diet agrees closely with the analyzed concentration of 44 μg Ni/kg of mixed diet (see Results).

TABLE 2

Vitamin–antibiotic mixture for low nickel diet

Ingredient	g Ingredient per kg diet	μg Ni per g ingredient	μg Ni per kg diet
Niacin [1]	0.0150	<0.01	0.000
Menadione [1]	0.0006	<0.01	0.000
DL-α-Tocopheryl acetate (250 IU/g) [2]	0.0100	0.10	0.004
Vitamin D_3 (200,000 IU/g) [2]	0.0040	0.18	0.001
Retinyl palmitate (250,000 IU/g) [2]	0.0080	0.22	0.002
Biotin [1]	0.0001	0.45	0.000
Pyridoxine hydrochloride [1]	0.0020	<0.01	0.000
Folic acid [1]	0.0010	<0.01	0.000
Vitamin B_{12} (0.1% triturate) [1]	0.0050	0.46	0.002
Thiamin hydrochloride [1]	0.0020	0.23	0.000
Neomycin sulfate [1]	0.0250	0.50	0.013
D-Glucose [3]	0.5973	0.020	0.012
	0.7000		0.034

[1] General Biochemicals Co., Chagrin Falls, Ohio.
[2] Nutritional Biochemicals Corp., Cleveland, Ohio.
[3] Calbiochem Corp., Los Angeles, Calif.

TABLE 3

Mineral mixture for low nickel diet

Ingredient	g Ingredient per kg diet	μg Ni per g ingredient	μg Ni per kg diet
Zinc oxide (ZnO) [1]	0.0500	<0.01	0.000
Potassium iodide (KI) [2]	0.0005	3.3	0.002
Cupric sulfate ($CuSO_4 \cdot 5H_2O$) [3]	0.0075	5.8	0.044
Iron powder (Fe, dissolved in HCl before use) [2]	0.0350	12.0	0.420
Manganous sulfate ($MnSO_4 \cdot 5H_2O$) [2]	0.2400	6.6	1.584
D-Glucose [4]	0.6670	0.020	0.013
	1.0000		2.063

[1] Alpha Inorganics Co., Beverly, Mass.
[2] Johnson, Matthey Chemicals, Ltd., London, England.
[3] Fisher Scientific Co., Pittsburgh, Pa.
[4] Calbiochem Co., Los Angeles, Calif.

ıltration with a Büchner funnel. The resultant fibrous cake was washed four times with 200 ml of 0.4 N hydrochloric acid, and 10 times with 200 ml of demineralized, distilled water. The fibrous cake was dried over anhydrous calcium carbonate in a vacuum desiccator. All other ingredients of the low nickel diet were obtained in

highest available purity from three commercial sources, and the nickel concentration of each sample was analyzed in triplicate by atomic absorption spectrometry (see below). The sample of each ingredient which contained the lowest concentration of nickel was used for preparation of the diet. The control chicks (group B) were fed the same diet as group A, except for supplementation with 3.4 ppm of nickel as nickel chloride. The nickel chloride ($NiCl_2 \cdot 6H_2O$) was prepared from spectrographically pure nickel powder [8] and ultrapure hydrochloric acid.[9] The diets were freshly mixed each week in acid-washed plastic carboys, and were stored in a cold room at 4°. The nickel concentrations of the diets were measured each week, in order to monitor for nickel contamination.

Atomic absorption spectrometry of nickel was performed by the method of Nomoto and Sunderman (3) with minor modifications (4, 5), employing an atomic absorption spectrometer.[10] The limit of detection of nickel was 2 μg/kg of diet or tissue (2 ppb), expressed as the concentration of nickel in 5 g of diet or tissue that yielded a recorder signal equal to twice the baseline noise level. The recovery of nickel added to eight specimens of mixed diet in concentration of 25 ppb averaged 98% (range = 92 to 107%). The experiment which is described in this paper was preceded by three preliminary studies of 3, 10 and 20 days' duration, which established (a) the precision and sensitivity of the method for nickel analysis of diets and tissues, (b) the efficacy of the precautions to minimize nickel contamination, and (c) the nutritional adequacy of the nickel-supplemented diet.

The chicks were examined twice each week by an animal caretaker who is skilled at detecting abnormalities in the growth and development of White Rock chickens. On days 10 and 20, the chicks were weighed, and the circumferences of the hock joints were estimated by encircling each hock with a loop of surgical suture thread. Each suture was cut and its length was measured with an accuracy of ± 0.5 mm. On day 28, blood was collected from wing veins for measurements of serum total cholesterol by the method of Leffler et al. (6), and for hematological studies, including microhematocrit, erythrocyte count, leukocyte count, and blood film stained with Wright's stain. On day 29, physical examination of each chick was performed by two independent observers who did not know whether the chick had received the low-nickel or the nickel-supplemented diets. On day 30, each chick was narcotized with carbon dioxide; the thorax was opened and the chicken was exsanguinated by cardiac puncture, with precautions to avoid nickel contamination, as previously described (3). Specimens of serum (10 ml) and liver (5 g) were taken for nickel analyses. Specimens of liver (1 g) were diced and dried at 110° for 96 hours for measurements of water content, in order that the results of nickel analyses could be expressed on the basis of both wet weight and dry weight of tissue. Care was taken that the specimens of liver were representative of the entire liver, containing portions of each hepatic lobe. Probability computations based upon the null hypothesis for differences between mean values for measurements of groups A and B were performed by Student's *t* test (8).

Autopsies of the chicks were performed by a veterinary pathologist. The following tissues were taken for histological examinations by light microscopy: heart, lung, kidney, liver, pancreas, spleen, duodenum, adrenal, testis and brain. Electron microscopy was performed upon livers from four chicks which had been fed the low nickel diet, and from two of the control chicks. Portions of each lobe of the livers were rapidly diced in 3% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, washed in 0.1 M sodium phosphate buffer, and postfixed in 2% (w/v) osmium tetraoxide in 0.1 M sodium phosphate buffer. The liver samples were dehydrated with ethanol and propylene oxide, and were embedded in Epon-araldite.[11] Four blocks from each liver were taken for study. Thin sections were cut with an ultramicrotome, and 10 grids were prepared from each block. The sections were stained with lead citrate and uranyl

[8] Johnson, Matthey Chemicals, Ltd., London, England.
[9] E. Merck and Co., Darmstadt, West Germany.
[10] Model 403, Perkin-Elmer Corp., Norwalk, Conn.
[11] DuPont Co., Wilmington, Del.

acetate, and each grid was examined by electron microscopy. Random squares on each grid were selected for photography. Postmortem X-ray photographs were taken of the chicks' left legs, so that precise measurements could be made of the lengths and widths of the tibias.

RESULTS

Nickel concentrations in diets. The low nickel diet which was used in this investigation contained an average of 44 μg Ni/kg (44 ppb), based upon six separate analyses (range = 38 to 49 ppb). These nickel analyses of the mixed diet agree closely with the computed nickel concentration of 44.6 μg Ni/kg, based upon summation of the nickel contents of the ingredients of the diet (tables 1, 2 and 3). The nickel-supplemented diet which was used in this investigation contained an average of 3.4 mg Ni/kg (3.4 ppm), based upon six separate analyses (3.1 to 3.6 ppm).

Survival data. Three of the chicks which were fed the low nickel diet (group A) died on days 16, 19 and 23, respectively. One of the chicks which was fed the nickel-supplemented diet (group B) died on day 21. Clinical observations and postmortem examinations indicated that these chicks all died from acute respiratory disease. A cavernous hemangioma of the neck may have contributed to the death of one of the chicks in group A.

Nickel concentrations in serums and livers. As indicated in table 4, the mean nickel concentration in serums of the chicks which were fed the low nickel diet (group A) was 38% of that in the control chicks (group B) ($P < 0.001$). The concentrations of serum nickel in the control chicks (group B) did not differ significantly from those which were previously found in adult chickens grown on Connecticut farms (mean = 3.6 ± 0.4 μg/liter) (7). The mean nickel concentration in livers of chicks which were fed the low-nickel diet (group A) was 78% of that in the control chicks (group B), ($P < 0.01$).

TABLE 4

Studies of nickel deprivation in chicks [1]

	Low Ni diet [2] (A)	Ni-supplemented diet [3] (B)
Serum Ni conc., μg/liter	1.6 ± 0.3 (*9*)	4.2 ± 0.4 (*11*) [a,4]
Hepatic Ni conc.		
Wet weight, μg/kg	18 ± 2 (*9*)	23 ± 2 (*11*) [b,4]
Dry weight, μg/kg	64 ± 6 (*9*)	82 ± 9 (*11*) [b,4]
Body weights, g		
Day 10	120 ± 14 (*12*)	120 ± 10 (*12*)
Day 20	198 ± 31 (*10*)	207 ± 17 (*12*)
Day 29	262 ± 45 (*9*)	276 ± 36 (*11*)
Hock circumferences, mm		
Day 10	30.9 ± 1.3 (*24*)	30.4 ± 1.2 (*24*)
Day 20	41.2 ± 2.4 (*20*)	37.4 ± 2.0 (*24*)
Dimensions of left tibias, mm		
Length	55 ± 4 (*9*)	57 ± 2 (*11*)
Greatest diameter	7.6 ± 0.7 (*9*)	8.3 ± 0.8 (*11*)
Width at mid-shaft	5.0 ± 0.5 (*9*)	5.2 ± 0.6 (*11*)
Length-to-width ratio [5]	11.0 ± 1.0 (*9*)	11.0 ± 0.9 (*11*)
Conc. of serum cholesterol, mg/dl	127 ± 19 (*9*)	141 ± 28 (*11*)
Hematological parameters		
Hematocrit, %	22.4 ± 2.0 (*9*)	23.1 ± 2.7 (*11*)
Erythrocyte count, cells × 10^6/mm^3	1.72 ± 0.21 (*9*)	1.76 ± 0.30 (*11*)
Erythrocyte volume [6], μ^3/cell	132 ± 13 (*9*)	132 ± 11 (*11*)

[1] The values listed in the table indicate the means ± SD. The number of items in each group is printed in italics within parentheses.
[2] Diet containing 44 ppb of Ni.
[3] Diet containing 3.4 ppm of Ni.
[4] Probability based upon null hypothesis for differences between mean values for groups A and B, computed by Student's *t* test. The superscript "a" indicates that the *P* value is < 0.001; "b" indicates that the *P* value is < 0.01. Where no letter superscript is indicated, the *P* value is > 0.05.
[5] Tibial length divided by the width at mid-shaft.
[6] Hematocrit (percentage) × 10, divided by erythrocyte count (10^6/mm^3).

Physical examinations. On day 29 (the day before they were killed), the chicks all appeared to be healthy. No differences in appearance or behavior were discerned between the chicks fed the low nickel diet (group A) and those fed the nickel-supplemented diet (group B). Specifically, there were no deformities of the hocks, no discoloration of the legs, no dermatitis of the shank skin, and no abnormalities of gait, such as were observed by Nielsen and co-workers (1, 2). As shown in table 4, there were no significant differences between groups A and B in the mean body weights nor in the mean circumferences of the hocks at any time during the experiment.

X-ray examinations of the leg bones. No differences were noted between groups A and B in regard to the maturation, cortical density or major dimensions of the femoral, tibial, tarsal or metatarsal bones. Contrary to the findings of Nielsen and co-workers (1, 2), the mean lengths, widths and length-to-width ratios of the tibias of chicks which were fed the low nickel diet (group A) did not differ significantly from the corresponding values in the control chicks (group B), based upon measurements of bone dimensions on postmortem roentgenograms (table 4).

Serum cholesterol and hematological studies. Measurements of serum cholesterol and of blood hematocrit, erythrocyte count and mean corpuscular volume in chicks which were fed the low nickel diet (group A) did not differ significantly from those in the control chicks (group B) (table 4). The morphology of erythrocytes, leukocytes and platelets appeared to be identical in stained blood films from groups A and B. No megaloblastic changes were detected.

Autopsy findings. One of the chicks which was fed the low nickel diet (group A) was found to have a few lymphocytic foci in the myocardium and lungs, such as typically develop in response to infection by avian leukemia viruses. Gross examinations and light microscopy failed to disclose any pathological lesions in any of the other chicks. There were no significant differences between groups A and B in the histological appearance of any organs or tissues, based upon examinations by light microscopy.

Hepatic ultrastructure. In hepatocytes of three of the four chicks in group A which were examined, there was unequivocal dilatation of the perimitochondrial rough endoplasmic reticulum (fig. 2). This distinctive abnormality was present in random distribution in 15 to 20% of hepatocytes in all of the sections of these livers. Such dilatation of perimitochondrial endoplasmic reticulum was not detected in the control chicks (group B), nor has it been encountered previously in several years of experience of one of the authors (C.N.B.) in ultrastructural studies of avian and mammalian hepatocytes. The nuclei, nucleoli and mitochondria of hepatocytes from the chicks in group A appeared to be normal. The hepatocytes of the control chicks (group B) did not show any ultrastructural abnormalities.

DISCUSSION

The present investigation was successful in producing nickel deprivation in chicks, based upon the presence of mean concentrations of nickel which were significantly lower in serums and livers from the chicks fed the low nickel diet than in serums and livers from the control chicks. However, the present study did not provide definite evidence of a nickel deficiency syndrome. The most unusual finding of the present study was the occurrence of ultrastructural lesions in hepatocytes of some of the nickel-deprived chicks. The livers from the nickel-deprived and control chicks were all sectioned, fixed and processed simultaneously, so that it is unlikely that these changes could be artifactual. It may be speculated that these lesions of the perimitochondrial endoplasmic reticulum might be related to the diminution of hepatic fat in nickel-deprived chicks, as reported by Nielsen and Higgs (2). In the present study, the body weights of chicks in the nickel-deprived and control groups at 20 days of age averaged 198 ± 31 and 207 ± 17 g, respectively, compared with mean weights of 125 and 117 g in nickel-deprived and control groups of White Rock chicks at 21 days of age, as reported by Nielsen and Sauberlich (1). Similarly, in the present study, the body weights of

Fig. 2 Hepatic parenchymal cells showing perimitochondrial areas of distended endoplasmic reticulum. (Lead citrate-uranyl acetate stain, × 22,000). M = mitochondrion; ER = endoplasmic reticulum; N = nucleus; n = nucleolus.

chicks in the nickel-deprived and control groups at 29 days of age averaged 262 ± 45 and 276 ± 36 g, respectively, compared with mean weights of 212 and 214 g in corresponding groups of White Rock chicks at 28 days of age, as reported by Nielsen and Higgs (2). The greater rates of growth which were found in the present study may possibly be attributed to the selection of only male chicks, whereas the chicks which were studied by Nielsen and associates (1, 2) were randomly selected, without regard to sex. Differences between the responses of the chicks in the present investigation and in the studies of Nielsen et al. (1, 2) may also possibly be attributed to differences in the environmental systems which were employed. Nielsen and co-workers (1, 2) used a completely enclosed system, whereas in the present study the chicks were exposed to unfiltered room air for approximately 3 minutes each day while they were being fed and watered. In the studies of Nielsen et al. (1, 2) the ambient humidity was probably high, owing to the enclosed environmental system, whereas in the present study the relative humidity ranged from 40 to 60%.

The most important differences between the results of this study and the results previously reported by Nielsen and coworkers (1, 2), were the failure of the present investigators to observe any discoloration, dermatitis or deformity of the legs of the chicks which were deprived of nickel. Nielsen and associates (1, 2) indicated that these findings were somewhat inconsistent. Thus, deformities of the hocks were more evident in their first study (1) than in their second study (2); conversely, dermatitis of the shanks was present in their second study (2), but not in their first study (1). Nielsen and Higgs (2) stated that: ". . . the nickel deficiency described here is, at best, borderline, and further improvements in diets and methods are needed." An unsuccessful attempt to produce nickel deficiency in rats has been reported by Smith (9), who concluded that: ". . . if nickel is required by the rat, the dietary requirement is less than 80 ppb." An attempt to develop a diet containing less than 5 ppb of nickel is currently being undertaken in our laboratory, using skimmed milk which has been collected and processed without any contact with metal, and corn meal which has been extracted with hydrochloric acid. In future investigations, it is planned to increase the dietary content of iron, in order to avoid any possibility of iron deficiency. This possibility was suggested by the relatively low hematocrits and erythrocyte counts which were found in blood samples from both of the experimental groups.

In the present study, there was no significant difference between the mean concentrations of serum cholesterol in chicks which received 44 ppb or 3.4 ppm of nickel in their diets. Speculation that nickel-deprivation might influence the concentration of serum cholesterol was stimulated by Schroeder's report (10) that the mean concentration of serum cholesterol was significantly higher in rats fed a diet containing 200 ppb of nickel than in rats fed the same diet with 5 ppm of nickel added to the drinking water.

The criteria necessary for a trace element to be considered as an "essential" nutrient have recently been reviewed by Smith (9), Mertz (11), and Schroeder and Nason (12). Whereas nickel was formerly regarded as an "abnormal" trace element (13), Schroeder and Nason (12) have concluded that nickel is "possibly essential" for nutrition. Evidence in support of the view that nickel may possibly play an essential physiological role includes a) the report by Nomoto et al. (7) that serum nickel is maintained within relatively narrow ranges of concentrations in 16 different animal species; b) the identification by Himmelhoch et al. (14) and Nomoto et al. (7) of a nickel-containing protein ("nickeloplasmin") in human and rabbit serums; and c) the finding by D'Alonzo and Pell (15), Sunderman et al. (16) and McNeely et al. (17) that pathological alterations of serum nickel concentrations occur in several common diseases of man.

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ADDENDUM

Perimitochondrial dilatation of the endoplasmic reticulum of hepatocytes, such as was observed in the present study, has previously been described in the hepatocytes of rats which were fed a diet deficient in vitamin E and Factor 3 (Piccardo, M. G. and Schwarz, K. 1958 The electron microscopy of dietary necrotic liver regeneration. In: Liver Function, ed., R. W. Brauer. Publ. no. 4 of American Institute of Biological Sciences, Washington, D. C., p. 528). According to Piccardo and Schwarz, perimitochondrial dilatation of endoplasmic reticulum was the earliest ultrastructural evidence of dietary degeneration of the hepatocytes. The onset of this lesion occurred during the latent period before their rats developed any obvious signs of a dietary deficiency syndrome. Therefore, the occurrence of the same ultrastructural lesion in the hepatocytes of the nickel-deprived chicks in the present study may be consistent with the production of a subclinical nickel deficiency syndrome.

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Effect of Nickel on Cultured Rat Embryo Muscle Cells

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Cultured rat embryo muscle cells were exposed to nickel-containing medium in order to study the specific effect of nickel on mitotic cells. Nickel caused a marked decrease in mitotic index and induced abnormal mitotic figures such as multipolar spindles, C-metaphase-like shapes, disoriented bipolar spindles, lagging chromosomes, and unequal cytoplasmic divisions. Histochemical staining for protein-bound sulfhydryl groups showed that nickel caused the retention of stain in telophase and post-telophase stages, indicating that nickel may be causing mitotic abnormalities by interfering with the sulfhydryl groups involved in the spindle mechanism. Although the exact nature of the primary target is not known, the increased stainability at telophase and post-telophase stages indicates a delay or interference with the conversion of protein-bound sulfhydryls into free —SH groups, which is generally indicated by lack of stainability for —SH reagents after anaphase. The possibility exists that nickel may be interfering with —SH or —S—S groups of the spindle fibers or with a sulfhydryl enzyme, which might be responsible for the dissolution of the mitotic spindle.

Additional key words: Nickel carcinogenesis, Spindle abnormalities, Sulfhydryl-rich proteins, Muscle cultures.

The role of nickel in the induction of tumors of muscle origin in laboratory animals has been the subject of extensive study in this laboratory for the past several years.[16-18, 20] Cultured rat embryo muscle cells exposed to dilute solutions of nickel compounds have consistently shown responses resembling those found during *in vivo* nickel tumorigenesis.[6] Basrur and Gilman[6] have shown that exposure of young cultures to nickel-containing medium causes destruction of cells, especially those in mitosis, and that the uptake of tritiated thymidine is reduced drastically in young cultures following exposure to nickel. Older cultures of embryonic muscle in which differentiation is well advanced, however, were less severely affected, indicating that the mitotic cells are more susceptible to the action of nickel than the nonmitotic (differentiating) myoblasts. The purpose of the present study is to examine the effect of nickel on mitotic cells and on the spindle proteins and to test whether cell division is inhibited by the primary interaction of nickel with —SH groups assumed to be involved in the control of cell division[19] and in tumor induction.[11, 14, 15]

MATERIALS AND METHODS

Cultures were set up from the limb muscle of 16- to 17-day-old rat embryos in Connaught's H597 growth medium supplemented with 20 per cent heat-inactivated calf serum, 0.08 per cent sodium bicarbonate, and 100 IU per ml. of potassium penicillin G.[5] Each Leighton tube was inoculated with 2.0 ml. of cell suspension containing approximately 2×10^5 cells per ml. prior to incubating at 38° C. All tubes were supplied with fresh medium 1 day after initiating the cultures.

The nickel solution used for treatment of cultures was made from powdered nickel sulfide stirred in phosphate-buffered saline for 3 hours at room temperature.[6] The amount of nickel present in the solution was determined quantitatively in a Spectronic-20 colorimeter.[31]

Cultures were treated with 0.2 ml. of the prepared nickel solution at a final concentration of 1.0 μg. of nickel per ml. of culture medium. Mitotic indices of controls and nickel-treated cultures were determined from the total number of nondividing cells and cells in all stages of division in 20 fields selected at random from each slide. The incidence of abnormal mitoses, also scored from 20 fields in each cover slip, was recorded as the percentage of the total number of dividing cells. These experiments were repeated on three sets of cultures using the same stock of nickel solution for treatment. In all instances the cultures were 1-day-old at the time of exposure to nickel. The controls and the experimental series in this group were replaced with fresh medium 1 hour prior to treatment.

In another series of experiments cultures were exposed to nickel, starting from the first day after explantation in order to study the influence of the age of cultures at the time of treatment. In this series, the duration of exposure to nickel was kept constant (24 hours), and the mitotic indices in controls and treated cultures were estimated after colchicine administration for 6 hours prior to harvesting. Three cover slip cultures were

included for each treatment series and controls, and a minimum of 500 cells were scored from each cover slip culture.

For the demonstration of spindle apparatus 24-hour-old cultures were treated with nickel for 24 and 48 hours. The treated cultures and the corresponding controls were fixed in formol sublimate and stained with 1.0 per cent toluidine blue, according to the toluidine blue-molybdate method of Löve and Walsh for RNA proteins.[25]

Tests for sulfhydryl-rich spindle proteins were performed on controls and nickel-treated cultures following the mercury orange method of Bennett and Yphantis[8] and the dihydroxy dinaphthyl disulfide (DDD) method of Barrnett and Seligman.[3, 4] The cover slip cultures were fixed in 80 per cent ethanol containing 1.0 per cent trichloroacetic acid for 1 hour and then transferred to mercury orange for 24 hours or to DDD for 1 hour at 60° C., followed by staining with a diazonium salt for 10 minutes. The diazonium salt used for coloring the sulfhydryl complex[2] oxidized with DDD was 1 per cent aminodimethoxybenzanilide (fast blue RR).

To determine the specificity of staining reactions, control cultures were treated with sulfhydryl-blocking reagent, 0.001 M iodine, with a trace of potassium iodide, before staining with mercury orange and DDD.[2]

RESULTS

The normal sequence of growth in untreated rat embryo muscle cultures, from 24 hours to 6 days after explantation is shown in Figures 1 to 4. The predominant cell types in the young cultures were fibroblast-like (Fig. 1), although even in 24-hour-old cultures some myoblasts in the process of differentiation were generally noted. These multinucleate myoblasts steadily increased in number (Figs. 2 and 3) and, by the sixth day, well differentiated muscle elements with cross-striations were abundant (Fig. 4).

The growth pattern as evidenced by the total number of cells per field, and the mitotic index in controls and cultures treated with nickel are shown in Table 1. The mean number of cells per field in the cultures subjected to continuous exposure to nickel for 72 hours was less than half of that noted in the corresponding controls. The effect of nickel on the mitotic index when cultures of different ages (1 to 6 days old) were exposed to nickel for the same duration (24 hours) is presented in Figure 5. The peak of mitotic activity was noted in controls between the third and sixth day after the initiation of cultures. Mitotic index in cultures exposed to nickel showed a marked reduction at all stages of growth irrespective of the age of culture at the time of treatment (Fig. 5).

Cultures of all ages exposed to nickel-containing medium exhibited a large percentage of pyknotic nuclei. In all treated cultures a majority of the affected cells could be distinctly identified as cells at some stage of mitosis. Nickel-treated cultures consistently showed a higher percentage of abnormal mitotic cells than the corresponding controls. The most common types of abnormalities were multipolar spindles (Figs. 6, 8, and 9), laggards (Figs. 7, 8, and 19), C-metaphase-like figures (Fig. 6), and change of polarity in the mitotic apparatus in such a way that the long axis of the spindle was at right angles to the long axis of the cell (Figs. 7 and 18). The frequency of aberrant mitoses in controls and nickel-treated cultures was estimated from cultures which were not treated with colchicine (Table 2). A high incidence of abnormal spindles was noted among the mitotic cells in cultures treated with nickel for 48 hours. The spindle fibers of the bipolar mitotic cells noted in the treated cultures generally appeared less distinct than those of untreated cells.

In Figures 10 to 13 the mitotic cells in control cultures stained according to the mercury orange method are shown. With the exception of nucleoli, which appeared orange-red, the interphase cells in these cultures remained unstained. The mitotic cells, however, were stained a deep orange-red, the intensity reaching a maximum in metaphase (Fig. 10). In later stages, the stainability decreased gradually (Figs. 11 and 12) so that cells in post-telophase stages were indistinguishable from interphase cells, except for the fact that in the former the deeply stained spindle remnants were noticeable (Fig. 13). The mitotic cells of nickel-treated cultures stained with mercury orange are shown in Figures 14 to 17. All stages, especially telophase (Fig. 16) and post-telophase stages (Fig. 17), seemed to be more intensely stained than controls. The spindle fibers of abnormal mitotic apparatus exhibiting altered orientation (Fig. 18) and unequal segregation of nuclear and cytoplasmic substances (Fig. 19) showed an intense positive reaction with mercury orange as did the midbody[23] in cytokinesis (Figs. 20 and 21).

The chromosomes were stained a deep orange-red in all mitotic stages, whereas the post-telophase and interphase chromatin was unstained with mercury orange.

With the DDD staining procedure sulfhydryl sites stained red-violet, and as with mercury orange, the reaction was confined to the mitotic cells (Figs. 22 to 25). The abnormal mitoses of nickel-treated cultures were more intensely stained than the controls (Figs. 26 to 29).

The mitotic apparatus and the cytoplasm in its immediate vicinity were stained either moderately positve as in controls or strongly positive as in nickel-treated cultures, with both —SH reagents used in the present study. However, the two compounds exhibited a marked difference in their affinity for chromosomes. With the DDD method the chromosomes were unstained at all stages (Figs. 22 to 29), whereas they were stained a deep orange-red in all mitotic stages with the mercury orange (Figs. 14 to 21).

Cultures exposed to a sulfhydryl-blocking agent, 0.001 M iodine prior to staining with DDD or mercury orange, were completely negative in staining reaction.

DISCUSSION

The main effects of nickel on rat embryo muscle cultures in the present study appear to be a suppression of division in a majority of cells and the production of aberrant mitoses. The relatively low cell counts

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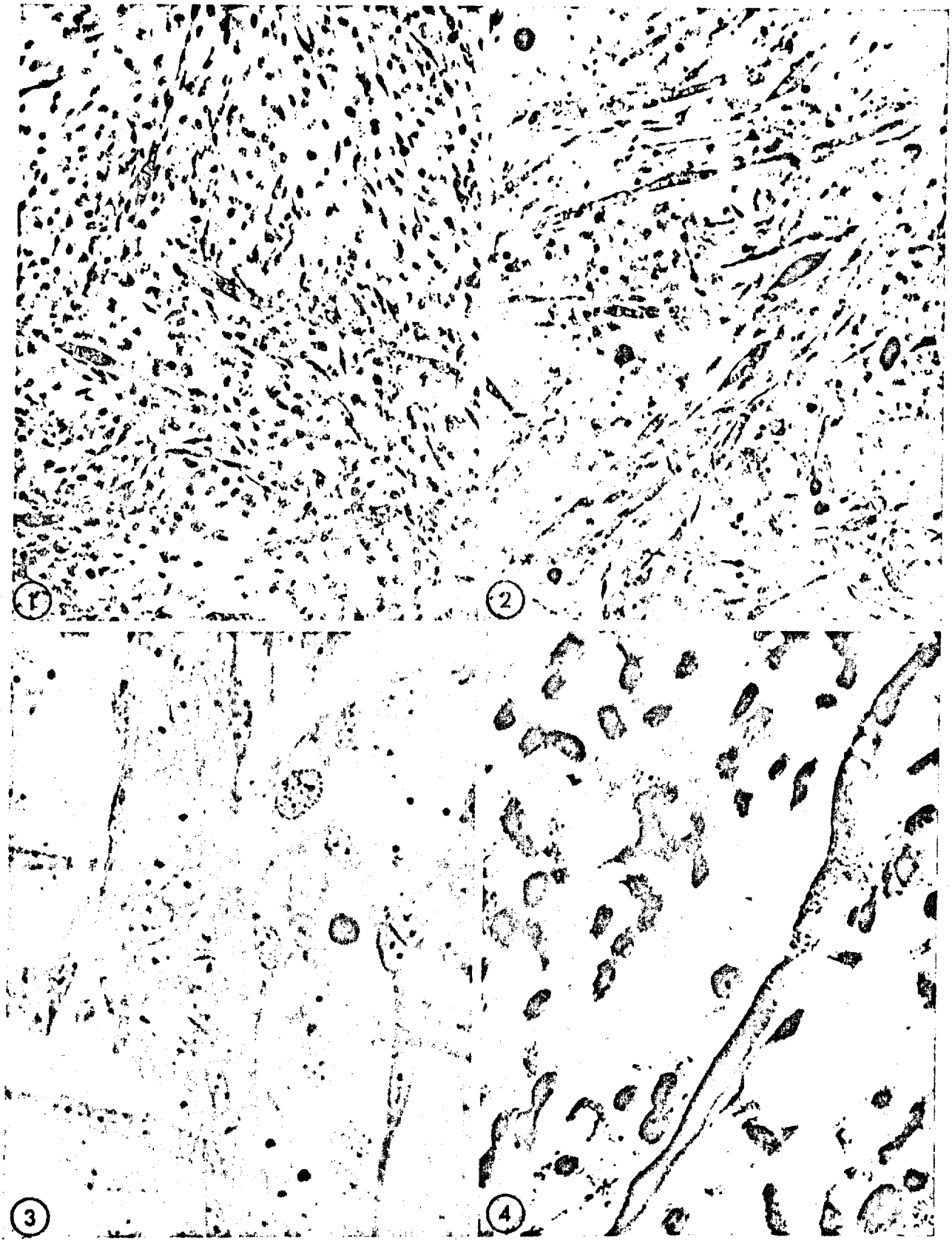


Note: Figures 1 to 4 are of rat embryo muscle cultures showing the normal sequence of growth. May-Grünwald-Giemsa stain.

FIG. 1. 24-hour-old culture. Most cells are fibroblast-like. Note also a few multinucleated myoblasts. ×250.

FIG. 2. 2-day-old culture. Multinucleated elements have become more numerous. ×250.

FIG. 3. 4-day-old culture at higher magnification. Note the wide multinucleate myotube showing longitudinal filaments. ×500.

FIG. 4. 6-day-old culture. Mature muscle fibers with cross-striations are visible. ×450.

noted in all nickel-treated cultures (Table 1) could be considered a concomitant of these effects. An initial suppression of cell division and the induction of abnormal mitoses similar to those observed in the present study have been reported previously with a variety of physical and chemical carcinogens.[9, 10, 12, 19] The occurrence of a large percentage of abnormal mitotic figures exhibiting multipolar spindles, altered polarity, unequal segregation of nuclear and cytoplasmic material and C-metaphase-like stages indicates that nickel, one of the known metal carcinogens,[16-18, 20] may be interfering with the spindle mechanism. Although it is possible that the abnormalities resulting in laggards are due to damage to chromosomes, or specifically to the point of attachment to the spindle fiber as suggested by Bloom, Zirkle, and Uretz,[10] it is apparent from the preponderance of multipolar spindles and the upset in spindle polarity that the centrioles responsible for the normal functioning of mitotic spindle[26-28, 30] are affected. Mazia[28] has demonstrated that mercaptoethanol, which inhibits the reproduction of centrioles, also produces multipolar spindles and abnormal mitoses in sea urchin eggs when the treated eggs are returned to normal environment.

The purpose of the histochemical procedures in the present study was to test whether nickel is affecting the chemical make-up of spindle proteins. Mercury orange, an azo dye that combines with sulfhydryl through its phenyl mercury group to form a colored mercaptide, has been claimed to be a specific stain for —SH groups.[8] The DDD reagent reacts with sulfhydryl groups of fixed proteins at alkaline pH to form an insoluble complex which subsequently forms a colored azo dye by coupling with a diazonium salt.[2, 3] With both these reagents, only the mitotic cells were stained in the present study. Kawamura and Dan[22] have demonstrated a marked increase in stainable —SH groups that corresponded with the appearance of the spindle fibers at the early stages of mitosis. Cells in control cultures in the present study showed a similar —SH stainability with a maximal intensity at metaphase and the subsequent decrease in post-anaphase stages.

The difference in the affinity of chromosomes for mercury orange and DDD in the present study is of interest since both stains are reported to be specific for sulfhydryl proteins. Even though the mitotic chromosomes exhibit a strong positive reaction with mercury orange, they are totally unstained with DDD. The

TABLE 1. CELL COUNTS AND MITOTIC INDICES OF UNTREATED AND NICKEL-TREATED RAT EMBRYO MUSCLE CULTURES

Age of cultures at harvest	Untreated		Treated		
	No. of cells per field	Mitoses	Duration of treatment[a]	No. of cells per field	Mitoses
hr.	*mean*	%	*hr.*	*mean*	%
48	46	2.4	24	52	1.3
72	76	2.9	48	36	1.4
96	120	3.1	72	55	1.3

[a] Cultures were 24 hours old at the commencement of nickel treatment.

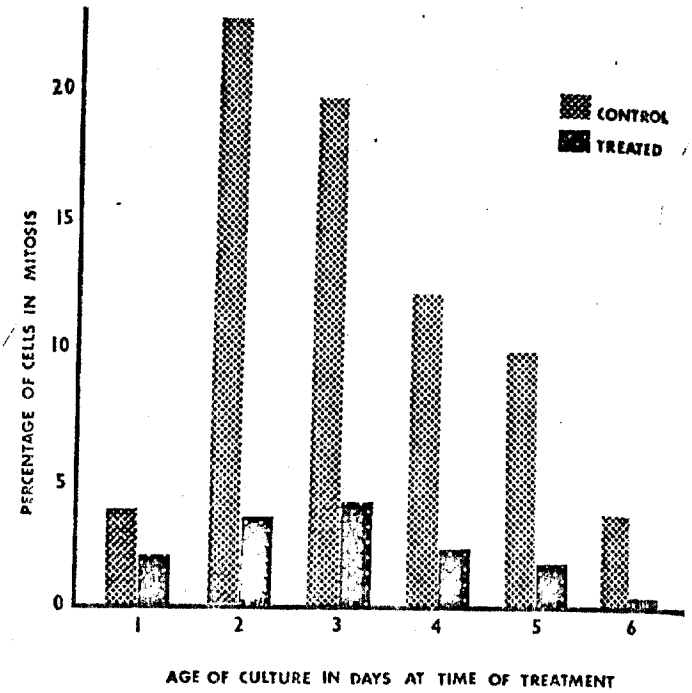
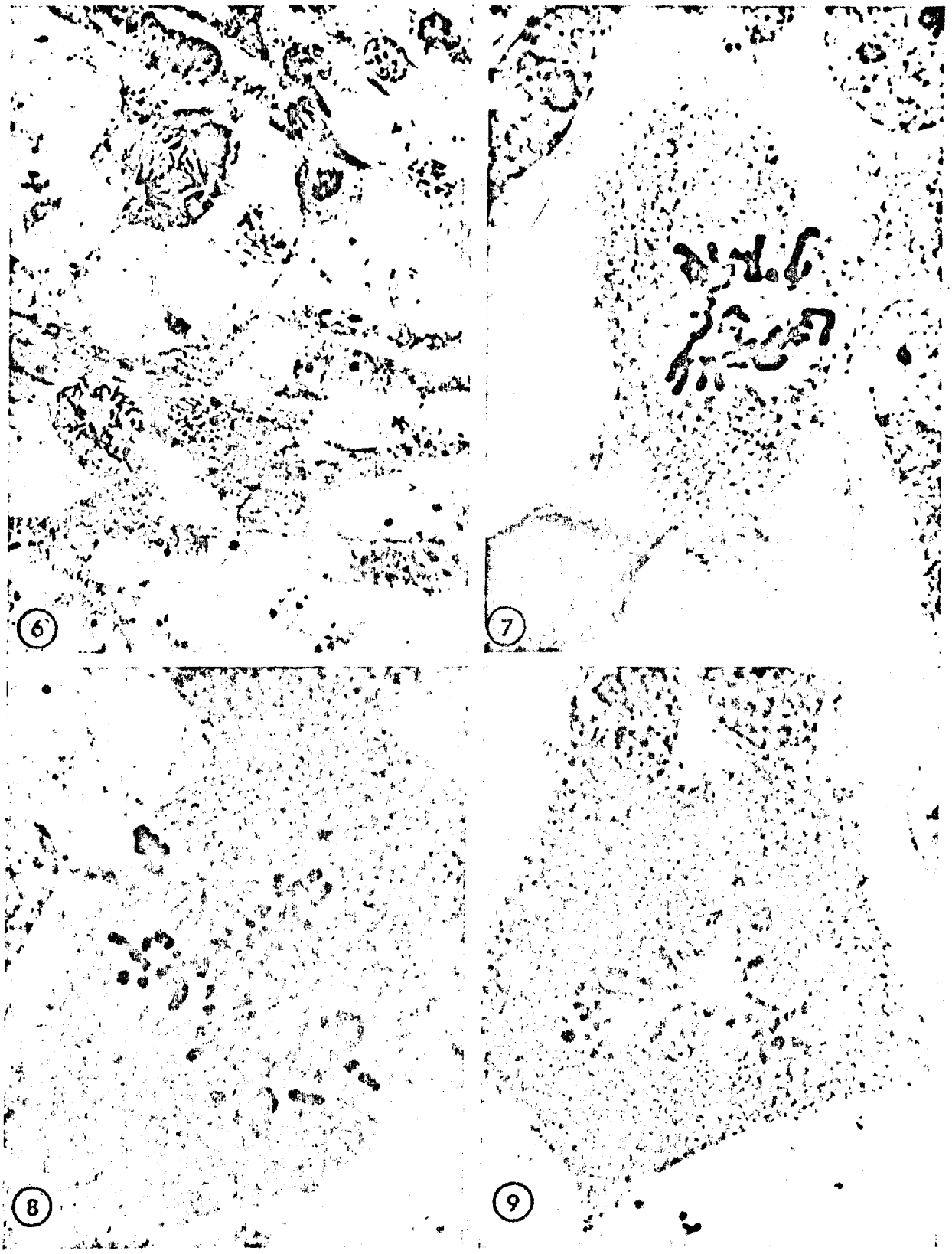


FIG. 5. Histogram showing the mitotic index in nickel-treated and control rat embryo muscle cultures. Each set of cultures was exposed to nickel starting from the first day of explantation for 24 hours, including 6 hours with colchicine.

chromosome stainability with mercury orange may be due to the lipid content of the chromosomes[13] although repeated washings with alcohol should have removed free lipids in the present instance. Lipoprotein thiols have been reported to be concentrated in the vicinity of mitotic chromosomes in dividing cells and in the nucleopolar region of nondividing cells.[37] The deep color of metaphase chromosomes noted with mercury orange was believed to be due to the presence of sulfur in disulfide linkages in the chromosomes during the later stages of nuclear division.[35] If this assumption is valid, both the reagents reported to be specific for cystine and cysteine amino acids in the protein[2-4, 8] should have produced a positive reaction in chromosomes. Kawamura and Dan,[22] who had tested several procedures for —SH groups, noted chromosome staining with mercury orange but made no reference to DDD in this respect. Since mercury orange has been shown to be more specific for —SH than DDD,[22, 24] it is possible that the stainability is due to sulfhydryl groups probably bound to lipids as lipoprotein thiols in the chromosomes. However, the lack of stainability of interphase and early prophase chromosomes with mercury orange is not easy to explain. It is conceivable that enzymes rich in —SH groups are involved in the process of chromosome despiralization which generally starts after metaphase and continues until the middle of interphase.[29]

The intensity of stain in nickel-treated cultures was strikingly greater than in controls, especially in cells at telophase and post-telophase stages. The possibility exists that the presence of nickel in treated cultures may have influenced the reaction with the mercury orange method, which depends upon the ability of heavy



Note: Figures 6 to 9 show abnormal mitoses in rat embryo muscle ltures treated with nickel. Toluidine blue-molybdate stain.

FIG. 6. A culture treated with nickel for 24 hours showing a tripolar spindle and a C-metaphase. ×500

FIG. 7. A mitotic apparatus oriented at right angles to the long axis of the cell. ×1000.

FIGS. 8 AND 9. Multipolar spindles in cultures treated with nickel for 48 hours. ×1000.

TABLE 2. FREQUENCY OF ABNORMAL MITOSES IN UNTREATED AND NICKEL-TREATED RAT EMBRYO MUSCLE CULTURES

Age of cultures analyzed	Duration of treatment	Types of abnormal mitoses				Total abnormal mitoses
		Tripolar	Tetra-polar	C-mitoses	Laggards	
hr.		%	%	%	%	%
48	Control	0	0	1.3	8.4	9.7
96	Control	1.1	0	1.0	7.0	9.1
48	24 hr.	7.1	2.0	3.0	13.5	25.6
96	48 hr.	12.5	0	0	18.0	30.5

metals to form insoluble colored mercaptides with —SH groups in the cells. However, the fact that the cover slip cultures were rinsed with phosphate-buffered saline to remove traces of nickel prior to fixation and that the intensity of reaction is confined to certain stages of division in treated cultures, seems to eliminate this possibility. Since glutathione and other free sulfur-containing amino acids will not be demonstrable with the acid alcohol fixation employed in the present study, it would appear that the stronger reaction with DDD and mercury orange in the nickel-treated samples is attributable to an increase in the protein bound —SH groups.

The mitotic apparatus has been demonstrated to be composed mainly of a single species of protein, each molecule of which has approximately 15 potential —SH groups for forming —S—S bonds.[27] The exact nature of the protein-bound sulfur in the mitotic fibers is not known. The present concept on the chemical nature of the mitotic spindle is based on three groups of data: the staining behavior of the spindle in metaphase,[21, 22] estimation of —SH groups in cells at different stages of division,[33] and the action of disrupting agents on the mitotic apparatus.[27, 28]

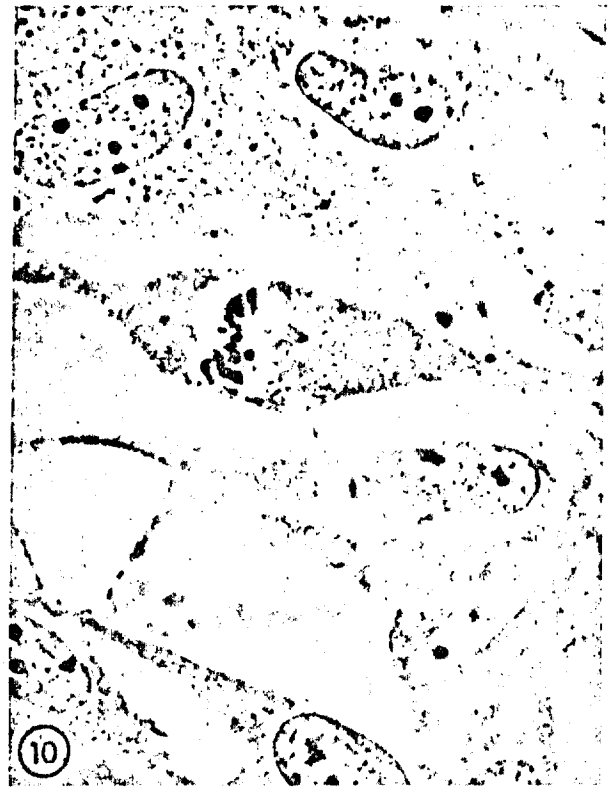
The reactions with nonspecific metal stains such as toluidine blue-molybdate[25] and with reagents reported to be specific for cysteins or cystine[2-4, 8] will favor any one of the modes of bonding for spindle proteins: disulfide, sulfhydryl, or sulfhydryl-chelated via a metal.

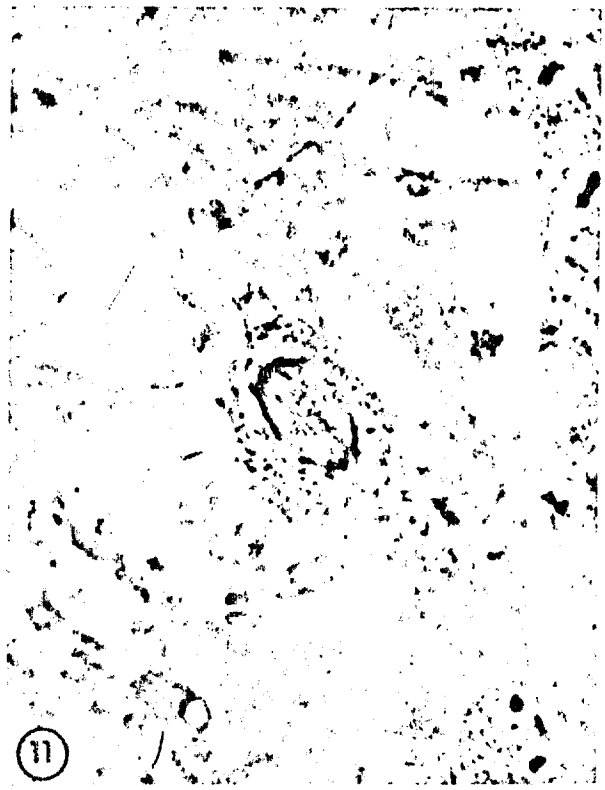
The fluctuations in the soluble —SH contents noted during the mitotic cycle[33] alternating with that of bound —SH have been originally interpreted as evidence for the formation of the mitotic spindle from —SH proteins facilitated by an oxidation-reduction cycle of glutathione.[26] Since then, it has been demonstrated that the level of soluble —SH compounds is constant throughout the division stages and that the mitotic apparatus may not be held together by —S—S bonds.[34] Kawamura's[21] quantitative determinations of —SH and —S—S groups in dividing sea urchin eggs showed that the amounts of —S—S groups in the centrosphere, aster, and spindle are insignificant since the stainability and the architecture of the spindle do not change before or after reduction of —S—S—groups by BAL. On the basis of these observations, Kawamura[21] has suggested that a major part of the mitotic apparatus may be composed of —SH proteins which arise from the —S—S—proteins in the cytoplasm.

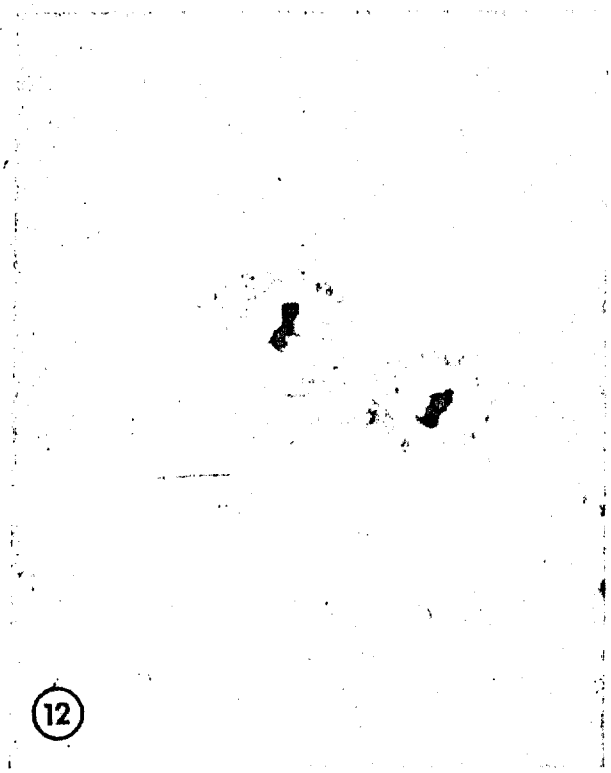
The ability of organic mercurials such as *p*-chloromercuribenzoate and salyrgan to solubilize the mitotic apparatus[27] has been interpreted as evidence for the polymerization of spindle proteins through disulfide bonds since heavy metals are known to attack the —S—S—bonds.[27] On the other hand, the disruption of the spindle in KCl and mercaptoethanol[28, 30] could be interpreted as evidence that the fibers are held together by weaker hydrogen-bonded sulfhydryl groups. It is also conceivable that the dissolution of the mitotic apparatus by mercurial compounds is through attack on a metal that might be present as a chelate holding the sulfhydryls in proteins. Mazia[28] has demonstrated the requirement for calcium in maintaining the architecture of the isolated mitotic apparatus. The present observations seem to indicate that a metal chelate, probably one that will easily be replaced by nickel,[28] may be involved in the integrity of sulfhydryl-rich spindle protein. The retention of intense —SH-specific stain after anaphase in nickel-treated cultures might suggest an interference with the mechanism involved in the cleavage of —S—S—bonds to —SH in the cytoplasmic protein involved in the dissolution of the spindle after anaphase.[21, 22]

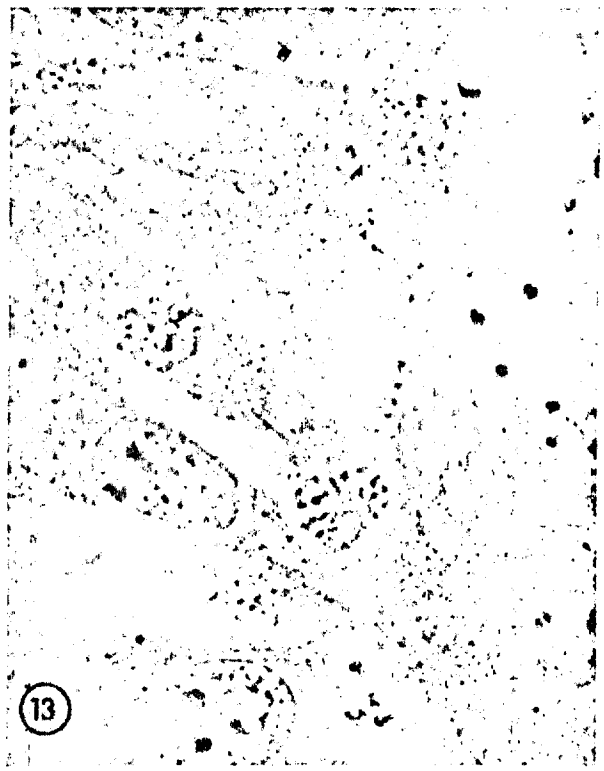
Although nickel may be interfering specifically with the interconversion of the SS—SH system or with the process of denaturation of spindle protein which is an antecedent of cell division, the retention of —SH stainability in the nickel-treated cultures may be a secondary effect resulting from the interference of nickel with —SH groups of glycolytic enzymes such as glyceraldehyde 3-phosphate dehydrogenase, lactic dehydrogenase, and aldolase, all of which are known to have —SH groups as active centers.[36] Glycolysis is generally regarded as the key pathway to mitosis although it is well known that the entire division cycle is not characteristically anerobic.[28] Stern[35] has demonstrated that the levels of —SH proteins and the glycolytic activities during mitosis follow an inverse course. Two stages in the cell cycle which are aerobic respiration-dependent have been demonstrated to be the one preceding and the one following the mitotic cycle, as evidenced by the simultaneous peak concentrations of ascorbic acid and glutathione at these stages.[35] Previous investigations with tritiated thymidine on nickel-treated rat embryo muscle cultures[6] and the present studies have indicated that the stage preparatory to S phase and the post-telophase stage are sensitive to the action of nickel, suggesting that glutathione or —SH proteins of enzymes operative at these stages may be inactivated by nickel.

Another explanation for the —SH-positive reaction of post-telophase stages may be found in the type of cells in mitosis in these cultures. It is well known that —SH groups play important roles in the binding of adenosine triphosphate to myosin, in the area of contact between actin and myosin, and in the interactions of nucleotides and other compounds with actomyosin.[1] Myotubes of normal muscle cultures and strap cells of nickel-induced rhabdomyosarcomas generally show intense reaction with —SH reagents.[7] Although it has been well documented that myoblasts in the course of









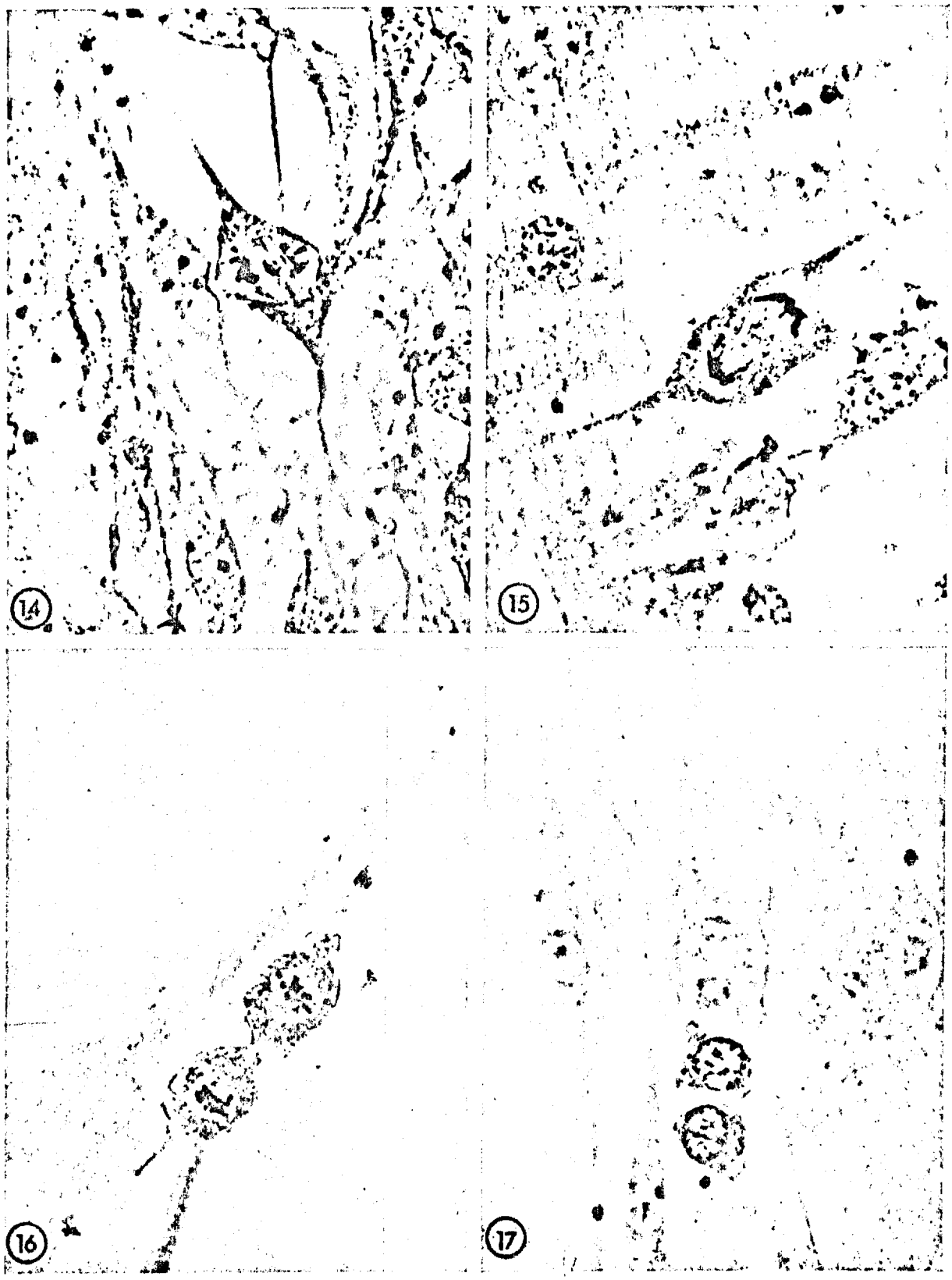
Note: Figures 10 to 13 are of rat embryo cultures stained with mercury orange. ×750.

FIG. 10. A culture showing a metaphase and several interphase cells. Note the intense mercury orange reaction in interphase nuclei, the centrioles at both poles, and in metaphase chromosomes.

FIG. 11. An anaphase showing mercury orange reaction in the area of the mitotic spindle.

FIG. 12. A telophase showing relatively less stainability of the cytoplasm and intense stain in chromosomes.

FIG. 13. Post-telophase, a stage at which cytokinesis is nearly complete. Note the spindle remnants in the cell in post-telophase stage. The cytoplasm is indistinguishable from that of interphase cells.



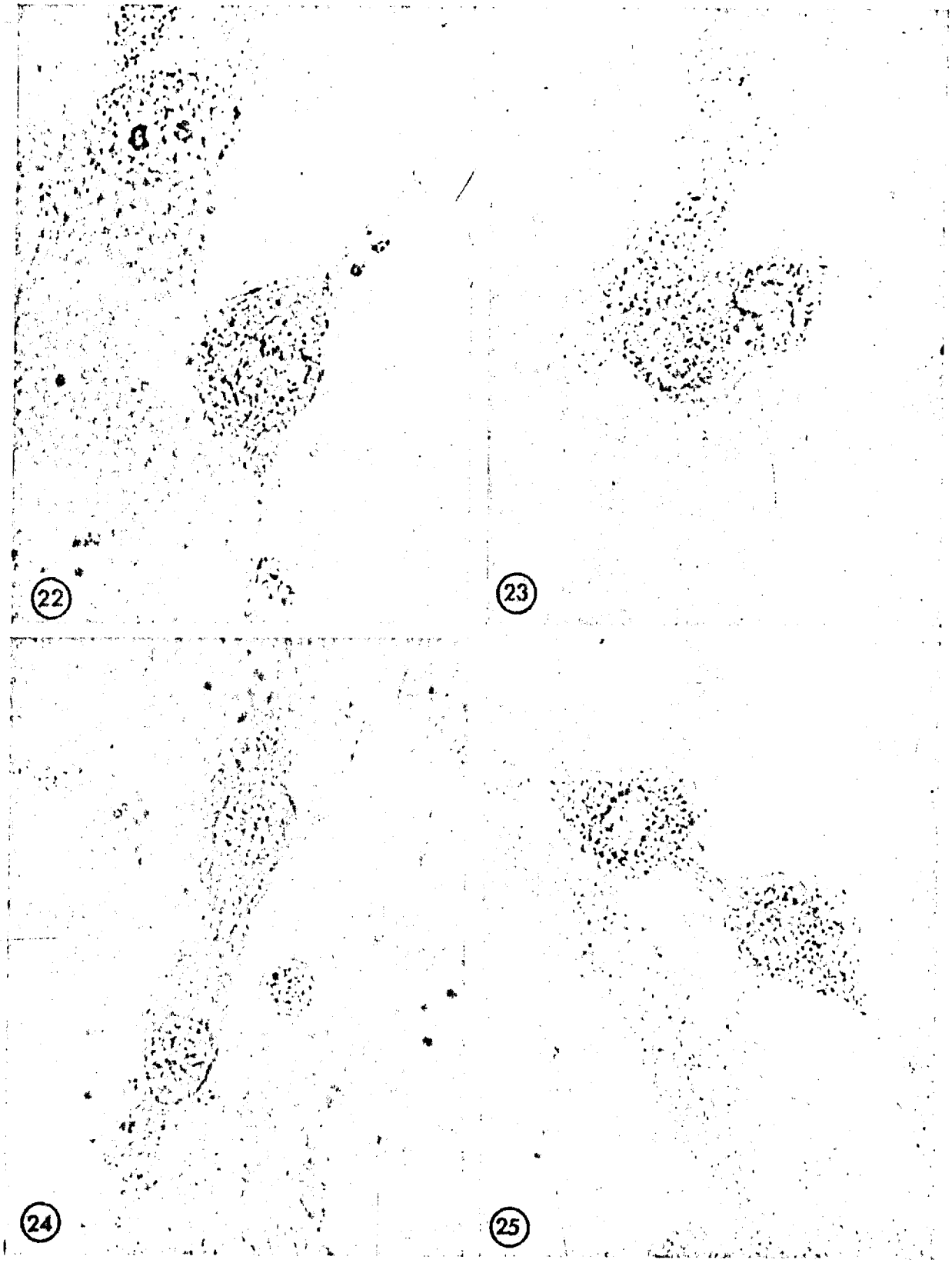
FIGS. 14 TO 17. Cells from nickel-treated cultures stained with mercury orange. Note that the mitotic stages, especially the post-telophase stages (Fig. 13) are more intensely stained than controls.

Note: Figures 18 to 21 are from cultures treated with nickel for ours.

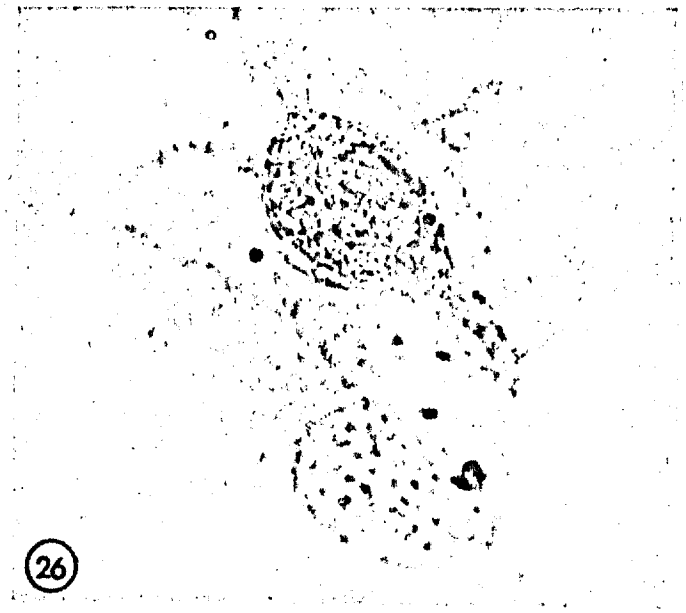
IG. 18. Mitotic cell of a nickel-treated culture oriented at right angles to normal axis of cell. ×450.

FIG. 19. A late anaphase from a culture treated with nickel for 48 hours. Note the unequal division of cytoplasm and the laggards. ×450.

FIGS. 20 AND 21. Post-telophase stages showing strongly positive mercury orange reaction at the midbody and in the cytoplasm. ×450.



FIGS. 22 TO 25. Cells at various mitotic stages in control cultures stained with dihydroxy dinaphthyl disulfide. Note that the chromosomes and the interphase cells are unstained. ×750.



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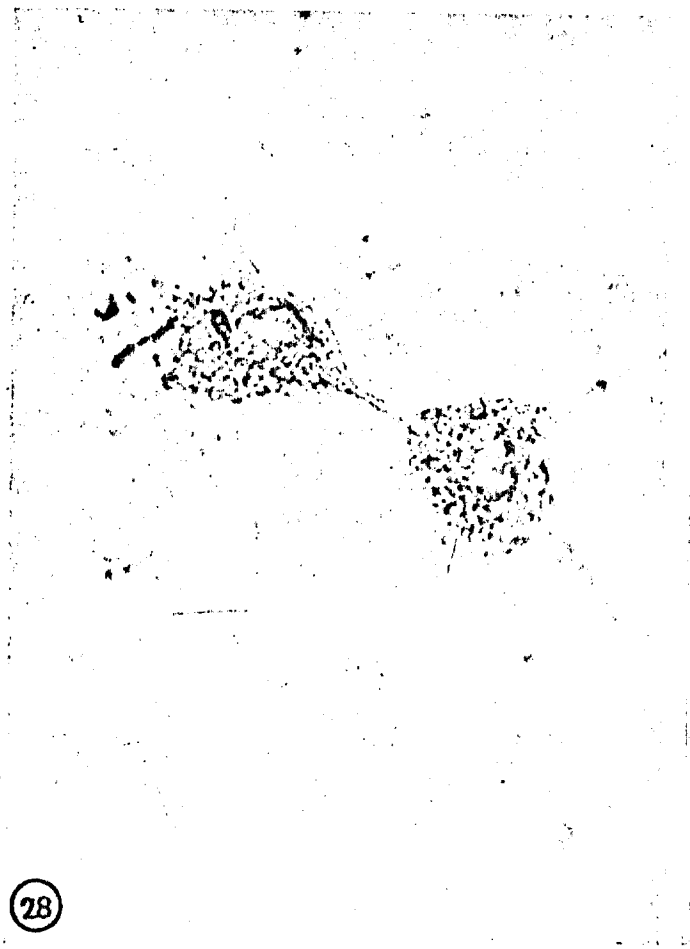

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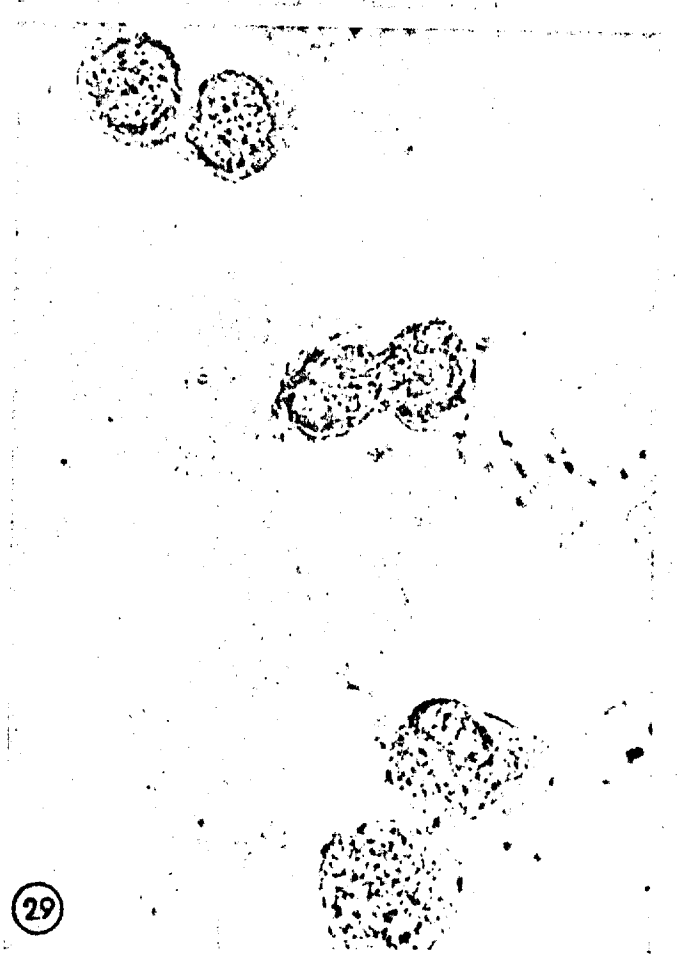

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Note: Figures 26 to 29 are of cells from nickel-treated cultures stained with dihydroxy dinaphthyl disulfide (DDD). All mitotic stages are stained more intensely than controls, especially the telophase and post-telophase stages. ×750.

FIG. 26. A metaphase showing intense DDD reaction.

FIGS. 27 TO 29. Telophase and post-telophase stages. Note the greater intensity of reaction as compared with the controls at corresponding stages (Figs. 22 to 25).

normal differentiation do not undergo mitosis,[32] it is possible that the cells retaining intense —SH reaction in nickel-treated cultures are "committed" myoblasts, which in the presence of nickel, have reverted to mitosis.

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In Vitro 6:377 (1971)

response to murine oncogenic viruses will be discussed.

22. Ultrastructural Changes in Cultured Rat Embryo Myoblasts Exposed to Nickel Sulfide.

A. K. Sykes* and P. K. Basrur (Department of Biomedical Sciences, University of Guelph, Guelph, Canada).

Rat embryo muscle cultures exposed to nickel sulfide exhibit drastic reductions in mitotic index and cell survival. The targets of insult are not known although intramuscular injections of this compound consistently induce muscle tumors in rats. An industrial plastic "Melinex" which facilitates flat-face electron microscopic preparation was used for growing cultures and studying the ultrastructural features of nickel-treated and untreated myoblasts. Myogenesis in the untreated cultures during the first 72 hours included active proliferation of fibroblast-like cells followed by myoblast fusion and the longitudinal orientation of contractile elements in close proximity to microtubules and elongated mitochondria. Exposure of cultures to nickel sulfide for 24 hours caused destruction of a majority of mononucleate cells while the surviving cells exhibited a variety of alterations in cell organelles. Myotube formation continued although the disorientation of microtubules and the alterations in the fine structure and alignment of mitochondria caused derangement of the contractile elements. At the cell periphery, cytoplasmic blebs containing clusters of free ribosomes appeared to form and dissociate from the myotube while cellular organelles aggregated in the center. The blebbing and elimination of cytoplasm may be related to the dedifferentiation generally occurring in the reactive zones where muscle cells and straps are noted to be reverting to mitosis during nickel tumorogenesis.

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ACTION OF INORGANIC IONS ON THE EFFECT OF EPINEPHRINE

By

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(Received June 27, 1958)

The adrenolytic effect of cadmium, manganous, stannous, nickelous and vanadium ions has been investigated. Of these ions, cadmium proved to be the most effective, inhibiting the epinephrine action on the frog heart, the Trendelenburg frog, the blood pressure and spleen volume of the dog. It has been shown that the inhibitory effect was not due to a blockage of sulphydryl groups, but the decisive role is ascribed to changes in cell permeability.

In previous experiments [1, 2, 3] ferrous ions were found to abolish or markedly to diminish the effect of epinephrine on smooth muscle. We observed a similar antagonism between cobaltous ion and epinephrine, which was, however, by far not so general as with ferrous ions [4]. ANDO *et al.* [5] concluded from a study on 18 cations that manganous, cadmium, ferrous, cobaltous, nickelous and vanadium ions all had an epinephrine-antagonistic effect. Since in our earlier experiments some of these ions had given results contrasting to those obtained by the Japanese authors, a more detailed analysis was now made of the influence of manganous, stannous, nickelous, cadmium and vanadium ions on the reactions of epinephrine and, in some cases, on those of acetylcholine and histamine.

Materials and methods

Isolated frog hearts were used for studying the effect on heart function, and Trendelenburg frogs for studying the effect on blood vessels. The frog experiments were made between February and May, and between October and December. Excised rabbit intestine was prepared according to Magnus and suspended in 20 ml Tyrode solution. Volume changes of the spleen were measured by oncometry, and blood pressure in the carotid artery by means of a mercury manometer, in dogs under chloralose (0.11 g/kg) anaesthesia. The ions under investigation were infused into one saphenous vein, and the drugs were injected into the other.

The following salts were used: $SnCl_2 \cdot 2\,H_2O$; $MnCl_2 \cdot 4H_2O$; $NiSO_4 \cdot 7\,H_2O$; and $CdCl_2$ *(Merck)*. The vanadium compound was prepared by dissolving V_2O_5 in nitric acid and adjusting the solution to pH 7.0 with sodium hydroxide. Under these conditions, vanadium was almost exclusively present as vanadate.

Results and discussion

Isolated frog heart. 0.05 μg cadmium was found markedly to diminish the effect of 0.1 μg epinephrine (Fig. 1). This dose of cadmium had no effect upon the heart function, while 10—30 μg caused standstill. As Fig. 2 shows,

100 μg nickelous considerably depressed the heart function. After the nickelous infusion 0.05 μg epinephrine was far less effective than in the control experiments. Nickelous exerted an adrenolytic effect only in doses high enough to depress heart function. On the other hand, even high doses of manganous, stannous and vanadium ions had no epinephrine-antagonizing action.

The effect of acetylcholine on heart function was not influenced by any of the five ions investigated. In this respect identical results were obtained on both spring or autumn frogs.

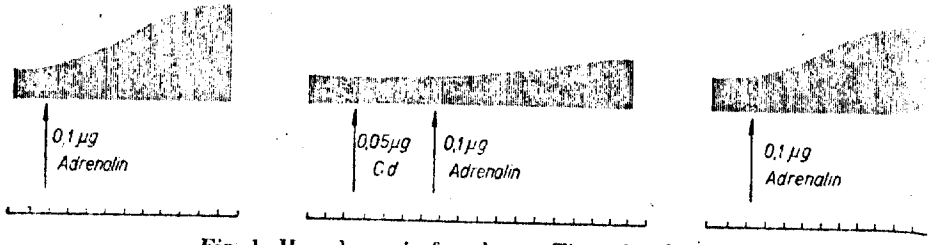

Fig. 1. Hypodynamic frog heart. Time signal, 6 secs

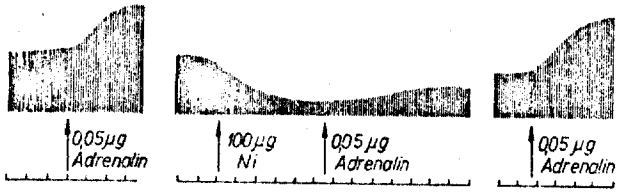

Fig. 2. Hypodynamic frog heart. Time signal, 6 secs

Trendelenburg frog. As seen in Fig. 3, 400 to 600 μg cadmium diminished, and 800 μg cadmium abolished the effect of 30 μg epinephrine. Perfusion with 200 μg/kg/min cadmium antagonized the vasoconstrictory effect of epinephrine. As to the other ions, 800 μg vanadate (Fig. 4) and 1000 μg nickelous or manganous ions were capable of abolishing the effect of 30 μg epinephrine, while stannous ions had no measurable adrenolytic effect, even when given in high doses.

Isolated rabbit intestine. Only stannous ions were weakly antagonistic to epinephrine, and not even in every experiment. All the ions investigated failed to influence effect in this test.

Blood pressure and changes of the spleen volume. Fig. 5 shows that, in the dog, infusion of 300 μg/kg/min manganous ions nearly abolished the effect of 20 μg epinephrine, 10 μg acetylcholine or 10 μg histamine. 90 minutes after the infusion 100 μg acetylcholine was still hardly effective. An almost similar inhibitory action was evoked by the infusion of 200 μg/kg/min manganous.

Fig. 6 demonstrates the effects of 20 μg epinephrine and of 10 μg acetylcholine. These doses were nearly ineffective during the infusion of 350 μg/kg

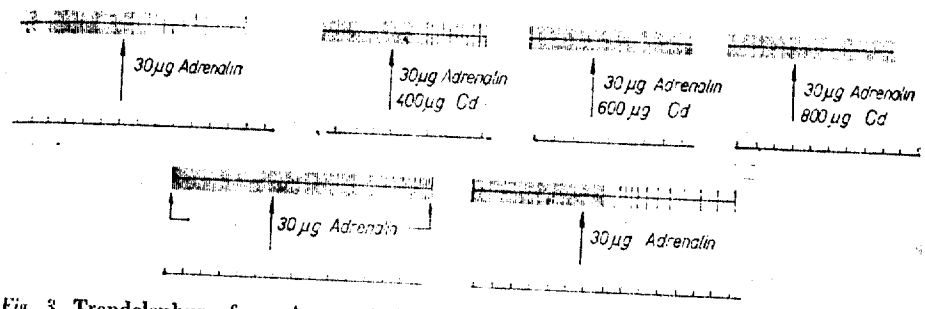

Fig. 3. Trendelenburg frog. Arrows indicate the administration of the drug. Between the ↑_ _↑ arrows, perfusion of 200 µg/ml cadmium. Time signal, 6 secs

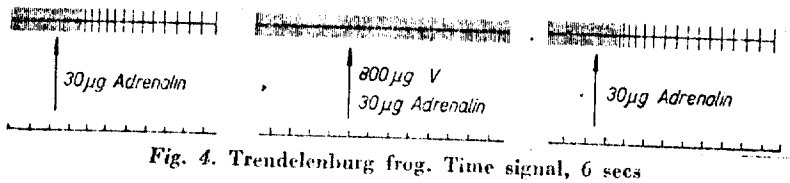

Fig. 4. Trendelenburg frog. Time signal, 6 secs

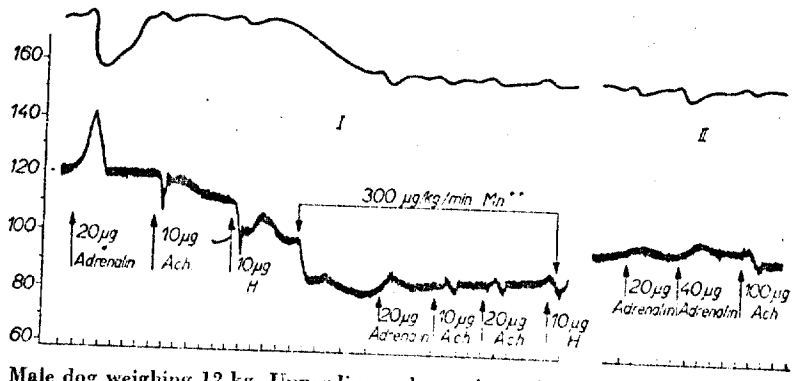

Fig. 5. Male dog weighing 12 kg. Upper line, spleen : lower line, blood pressure. Time signal, 20 secs. Interval between I and II = 90 minutes

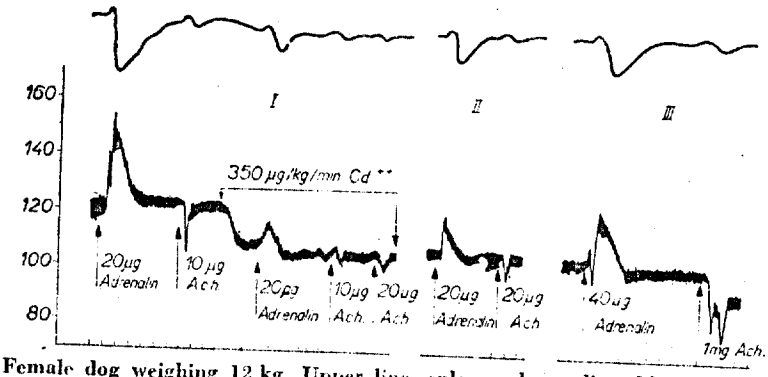

Fig. 6. Female dog weighing 12 kg. Upper line, spleen ; lower line, blood pressure. Time signal, 20 secs. Interval between I and II = 90 minutes ; between II and III = 30 minutes

min cadmium. 120 minutes after the infusion even 40 μg epinephrine was not able to produce the effect 20 μg had exerted before the infusion, and only about 1000 μg acetylcholine was as effective as had been 10 μg. The effect of 240 μg/kg/min cadmium was also marked, but transitory.

Infusion of 955 μg/kg/min nickelous made 20 μg epinephrine nearly ineffective (Fig. 7). This inhibition was invariably present after 30 minutes and began to disappear only 60 minutes after the infusion. 170 μg/kg/min nickelous too, had an adrenolytic effect which, however, did not persist for more than 10 minutes after the end of infusion.

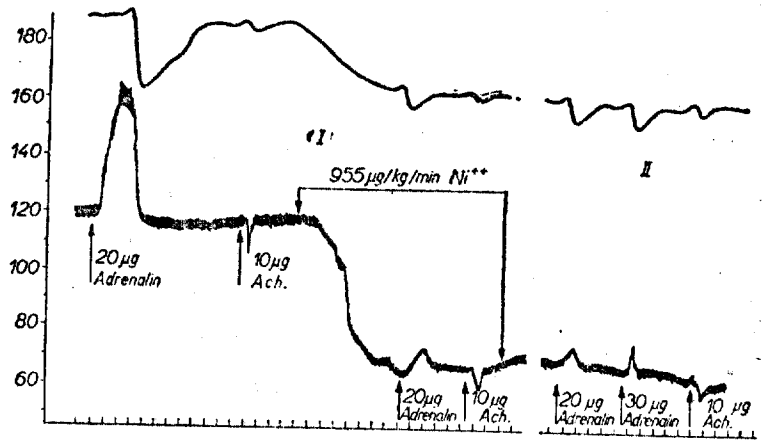

Fig. 7. Male dog weighing 15 kg. Upper line, spleen; lower line, blood pressure. Time signal, 20 secs. Interval between I and II = 30 minutes

Fig. 8 shows the influence of 160 μg/kg/min vanadium on the effect of 20 μg epinephrine, 10 μg acetylcholine and 20 μg histamine. During the infusion, histamine alone was effective. Acetylcholine sensitivity returned 120 minutes after the infusion, but epinephrine continued to act weaker than before the vanadium infusion.

During the infusion of 200 μg/kg/min stannous ions only the epinephrine action was weakened, until 30 minutes after the infusion.

As it can be seen from Fig. 5 to 8, the constricting effect of epinephrine on the spleen diminished considerably during the infusion of the inorganic ions. This inhibitory effect was especially marked and long-lasting with manganous: in this case epinephrine was still hardly effective 90 minutes after the infusion had ceased.

Summing up, the most marked adrenolytic action was exhibited by cadmium. Stannous ions evoked no change except a transitory weakening of the effect on blood pressure and spleen. As it can be seen from the Figures, blood pressure was markedly lowered by all the ions tested. This supports the view

that the paralysing effect on the musculature of the blood vessels is primarily responsible for the adrenolytic action of the ions. LAGNADO and SOURKES [6] observed that *in vivo* neither cadmium nor manganous ions influenced the amino-oxydase activity of the tissues, while *in vitro* both had an inhibitory effect. It is thus obvious that neither of these ions can enhance the inactivation of epinephrine. ANDO *et al.* [5] explained the adrenolytic effect of inorganic ions by the fact that, by virtue of their slight ionization, they react with the sulphydryl groups of the receptor proteins, which groups are supposed to play a

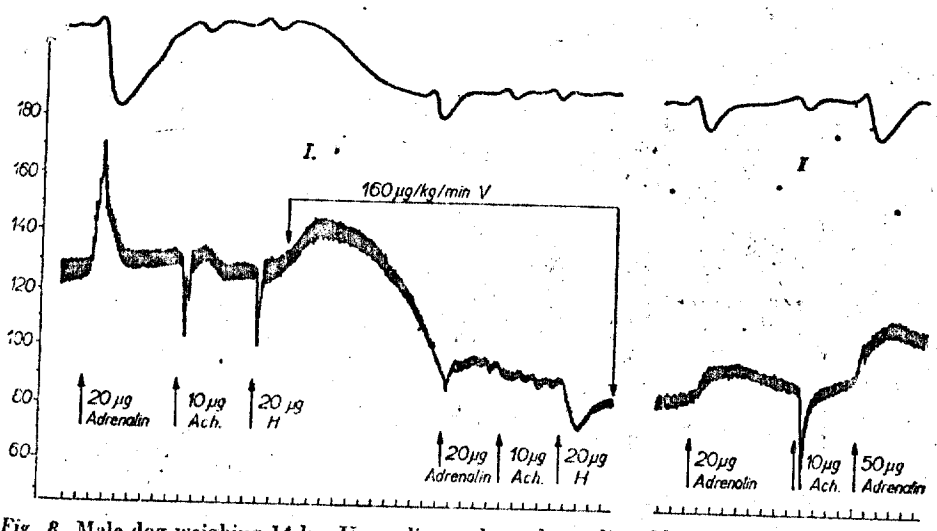

Fig. 8. Male dog weighing 14 kg. Upper line, spleen; lower line, blood pressure. Time signal, 20 secs. Interval between I and II = 120 minutes

decisive part in the epinephrine-induced vasoconstriction. This explanation seems to be most acceptable in the case of cadmium. In order to elucidate this point we made the following experiment. As shown in Fig. 9, the effect of 0.05 µg epinephrine on the isolated frog heart was completely abolished by 20 µg cadmium. This antagonism was not reversed by 1000 µg of cysteine. On the *Trendelenburg* frog 500 µg cadmium completely antagonized the effect of 30 µg epinephrine: here again, not even 2 mg cysteine altered the cadmium effect (Fig. 10). These findings show clearly that cysteine does not influence the antagonism between cadmium and epinephrine, a fact making obvious that [1] the adrenolytic action of cadmium is not due to its blocking the sulphydryl groups; and (2) sulphydryl groups do not play a decisive part in the manifestation of the epinephrine effect.

KOSHTOYANTS mentions in his monograph [7] that 1 µg acetylcholine injected into the isolated rabbit ear, which has only nervous connections, evokes a reflectory fall in blood pressure, which can be prevented by 1 mg (!)

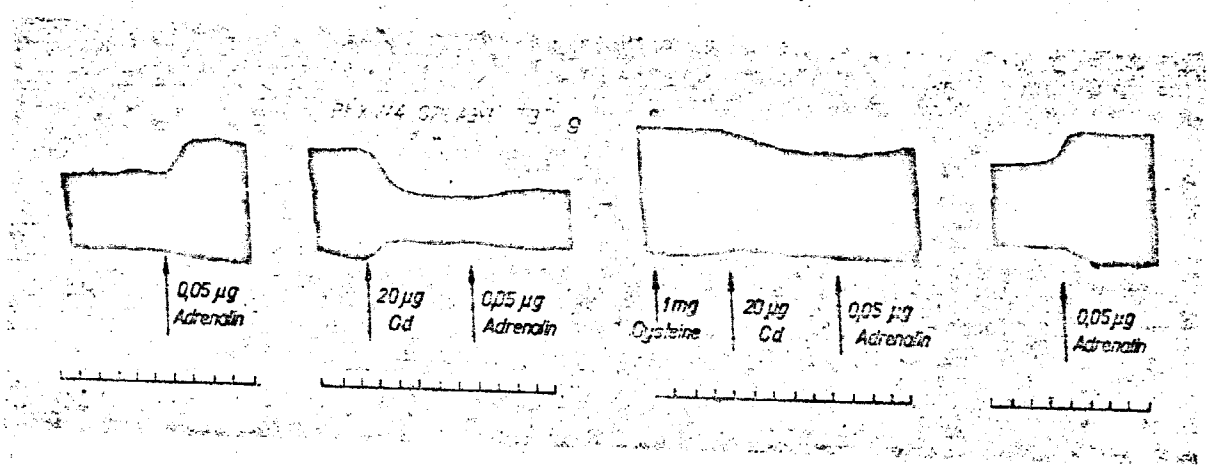

Fig. 9. Isolated frog heart. Time signal, 6 secs

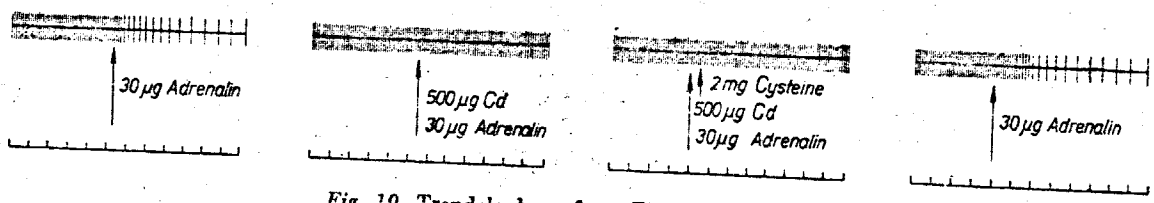

Fig. 10. Trendelenburg frog. Time signal, 6 secs

250

cadmium. This inhibitory effect is abolished by 100 μg cysteine. SMYRNOV, BYZOV and RAMPAN [8] stimulated the cervical sympathetic chain of the rabbit and registered the contractions of the nictitating membrane. They found that $2 \cdot 10^{-4}$ $CdCl_2$ introduced intraarterially was capable of blocking the transmission of the stimuli, meanwhile the effect of $5 \cdot 10^{-4}$ acetylcholine remained unchanged. According to DAMJANOVICH, HALÁSZ and MECHLER [9], the above dose of cadmium inhibited also the acetylcholine effect, while smaller doses blocked only the response to electrical stimulation. They also found that cadmium inhibited cholinesterase activity. According to our own findings, cadmium markedly diminishes the effect of acetylcholine on blood pressure. It must, however, be noted that in our experiments the low initial blood pressure made the evaluation of the results somewhat questionable.

A further assumption of ANDO *et al.* [5] is that the weakly ionizing cations attach themselves to the negative part of the receptor and so prevent epinephrine from attaining the receptor surface; they attribute less importance to the changes in the cell permeability. In our opinion, the latter phenomenon is the essential one, especially when the inorganic ions are administered in high doses, as in our experiments concerning blood pressure and spleen volume.

*

We are indebted to the *Institute* of *Medical Chemistry* for supplying the vanadium.

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Trace Elements and Compounds in Waters

Floyd B. Taylor

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TRACE ELEMENTS AND COMPOUNDS IN

Floyd B. Taylor

The chemical **constituents of drinking water are discussed from the standpoint of limits of standards and occurrence in US water supplies.**

THE CHEMICAL constituents of water with which the water-works operator is concerned, and which are significant from a health viewpoint, are not easily determined. Indeed the sophistication of the procedures and instrumentation now used for chemical analysis of water is terribly impressive when one remembers the relatively simple procedures that were spelled out in the early issues of *Standard Methods*. At one time, that publication was about 100 pages in length, and it didn't take an expert to follow the directions. Now, with gas chromatography, atomic absorption, and emission spectroscopy, few people other than an experienced chemist would attempt a water analysis. Yet this capability and precision is needed because of the vast number of chemicals that are in use in the environment—some of which, in both tiny and large amounts, find their way into drinking-water sources.

It is the author's purpose to examine the situation with regard to the chemical constituents of drinking water. The first thing to be considered is the evolution of drinking-water standards with respect to chemical limits.

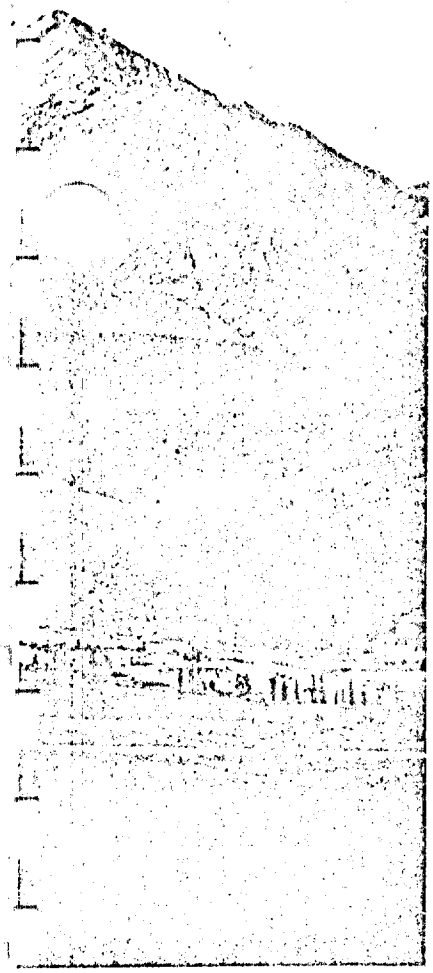
Development of Standards

The first US drinking-water standards were ones promulgated in 1914 by the Surgeon General of the US Public Health Service (USPHS). They were published by the Secy. of the Treasury, William Gibbs McAdoo, and, thereby, they were referred to as the "Treasury Standards." They were established for the purpose of restraining the spread of communicable diseases via water supply used aboard interstate carriers. The 1914 standards contained no chemical limits.

By 1925, the scene was changing and limits were imposed for lead, copper, and zinc. Some recommended limits for other chemical constituents were included at that time, and the document became the "USPHS Drinking Water Standards," under which name successive revisions appeared until the present. The revisers of the 1925 standards added still more chemicals, so that in the 1942 edition there appeared thirteen in all, which were in two categories (mandatory and recommended). The 1942 standards were reissued in 1946. The only change was that an appendix in the 1942 version was published separately under the title of *Recommended Water Sanitation Practice*.

Sixteen years passed before the next revision—years during which the array of chemicals, including many organics for which no limit had theretofore been considered, continued to increase. Thus, it is not surprising that the number of chemical limits in the 1962 "USPHS Drinking Water Standards" jumped to nine in the mandatory category and fourteen in the recommended category. Also, the 1962 revision contained two new concepts for chemical limits for water constituents.

One of these concepts was a general limit for organics called carbon chloroform extracts. It is interesting to note



WATERS

the philosophy advanced by Morris Ettinger when he proposed this standard (200 ppb)—a philosophy that was approved by the revision committee and incorporated in an appendix as follows: "It is proposed as a technically practical procedure which will afford a large measure of protection against the presence of undetected toxic materials in finished drinking water."[1] The application of the CCE standard (and the procedure for its determination) has for nearly nine years borne out the wisdom of this statement.

The second innovation of the 1962 standards was the incorporation (for the first time) of limits for a chemical constituent, which were based on the concept of actually adding a constituent (fluoride ion) until an amount optimum for the improvement of health was present. To be sure, the language was such that the numbers were stated as limits in the recommended category.

The 1962 standards also included a brand new category of chemical—namely, the radiochemicals, of which two were named (radium 226 and strontium 90).

At present the 1962 standards are undergoing revision by an in-house committee of the Div. of Water Hygiene, Office of Water Quality, Environmental Protection Agency, under the chairmanship of Dr. Paul Kabler. It should be no surprise to find that when they appear in print, possibly under the title of "National Drinking Water Standards," they will contain a list of chemicals almost double the number found in the 1962 standards. There will be limits for an impressive list of pesticides, new inorganic chemical limits, and, possibly (under a still different concept than the one described previously), a limit for sodium.

Increased Public Concern

Inclusion of chemical limits in drinking-water standards is sometimes a reaction to public concern.

Long before the appearance of the report of the National Community Water Supply Study, the public was expressing concern, and sometimes alarm, about what it was being subjected to in the way of chemical insults. In all fairness it should be stated that the public was in some instances finally becoming aware of things that the waterworks, public-health, and conservation professionals had been noticing and predicting. First there was the DDT scare, and more recently the mercury flap. The first was brought about largely by Rachel Carson when she published her *Silent Spring*, and it is a fact that DDT has become an ubiquitous chemical, being found in the tissues of nearly all living things the world over. However, as the Mrak Committee noted,[2] there is evidence that, after a certain level of DDT is reached, the human body, by some mechanism, protects itself by discharging new incoming increments, thus maintaining a constant level that is below the acute and, as far as is known, the chronic health threshold. Be that as it may, it is still reasonable to limit, as far as

A paper presented at the Pacific Northwest Sec. Meeting on May 13, 1971, by Floyd B. Taylor (Active Member, AWWA), Region I repr., Div. of Water Hygiene, OWQ, Environmental Protection Agency, Boston, Mass. [Q]

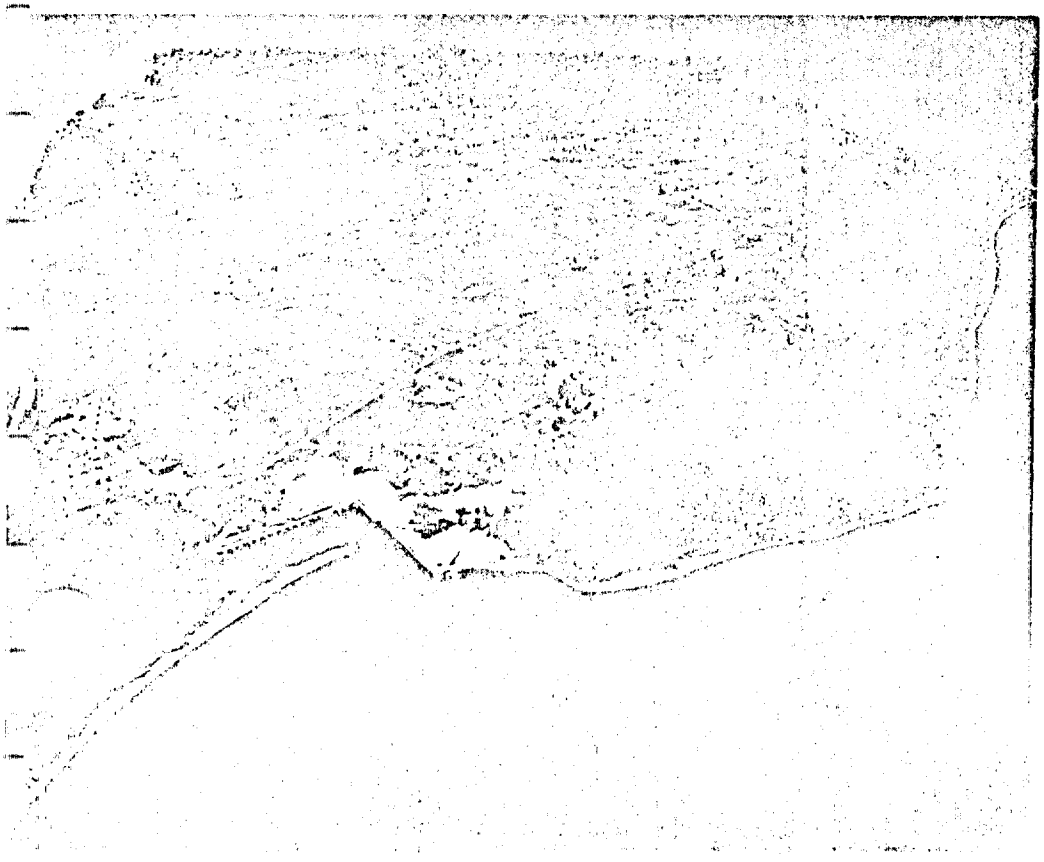
Fish and, hence, fishing are affected by DDT pollution.

TABLE 1*

Number of Water Supply and Distribution Samples Where Average Delivered Water Exceeded Specific Constituent Limits†

Constituent	Constituent Limit	Maximum Concentration Found	Number of Samples Exceeding Limit	Exceeding Limit *per cent*	Number of Systems Exceeding Constituent Limit	All Water Supplies in Study *per cent*	Number of Systems Exceeding One or More Additional Constituent Limit
Recommended							
ABS	0.5 mg/l	0.41 mg/l	0	0	0	0	0
Arsenic (As)	0.01 mg/l	0.10 mg/l	10	0.4	2	<1	2
Boron‡ (B)	1.0 mg/l	3.28 mg/l	20	0.8	9	1	9
Chloride (Cl^-)	250 mg/l	1,950 mg/l	31	1.2	9	1	9
Color	15 units	49 mg/l	17	0.7	7	1	5
Copper (Cu)	1.0 mg/l	8.35 mg/l	42	1.6	11	1	4
CCE	0.200 mg/l	0.56 mg/l	33	1.2††	12††	1	2
Cyanide (Cn^{-2})	0.01 mg/l	0.008 mg/l	0	0	0	0	0
Fluoride (F^-)	Varies	4.40 mg/l	118	4.5	52	5	35
Iron (Fe)	0.3 mg/l	26.0 mg/l	223	8.6	96	10	52
Manganese (Mn)	0.05 mg/l	1.32 mg/l	211	8.1	90	9	45
Iron and manganese					159	16	56
Nitrate (NO_3)	45 mg/l	127 mg/l	53	2.1	19	3	15
Radium-226	3 pc/l	135.9 pc/l	16	0.6	6	1	5
Strontium-90	10 pc/l	2 pc/l	0	0	0	0	0
Sulfate (SO_4)	250 mg/l	770 mg/l	47	1.8	25	3	25
TDS	500 mg/l	2,760 mg/l	219	8.5	95	10	62
Turbidity	5 units or 1 unit§	53 units	60	2.4	26	3	17
Zinc (Zn)	5 mg/l	13.0 mg/l	8	0.3	1	<1	1
Mandatory							
Arsenic (As)	0.05 mg/l	0.10 mg/l	5	0.2	2	<1	1
Barium (Ba)	1.0 mg/l	1.55 mg/l	2	<0.1††	1	<1	0
Boron‡ (B)	5.0 mg/l	3.28 mg/l	0	0	0	0	0
Cadmium (Cd)	0.01 mg/l	3.94 mg/l	4	0.2	3	<1	3
Chromium (hexavalent)**	0.05 mg/l	0.079 mg/l	5	0.2	4	<1	4
Coliform organisms	1/100 ml	2,000/100 ml	228	8.8	120	12	34
Cyanide (CN^{-2})	0.20 mg/l	0.008 mg/l	0	0	0	0	0
Fluoride (F^-)	Varies	4.40 mg/l	55	2.2	24	2	17
Gross beta activity	1,000 pc/l	154 pc/l	0	0	0	0	0
Lead (Pb)	0.05 mg/l	0.64 mg/l	37	1.4	14	1	6
Selenium (Se)	0.01 mg/l	0.07 mg/l	10	0.4	5	1	5
Silver (Ag)	0.05 mg/l	0.03 mg/l	0	0	0	0	0

* Adapted from Community Water Supply Study—analysis of national survey findings.
† Constituent in 2,595 distribution samples. Constituent in 969 water-supply systems.
§ Treated by more than disinfection.
** During this study total chromium was measured and compared to the standard.
†† These constituents were evaluated only on selected samples. The remainder were assumed not to exceed the limit.
‡‡ Measured in only 110 selected supplies, including 94 surface supplies.



possible, this persistent organic chemical in the environment. It and other chlorinated hydrocarbons are certainly harmful to wildlife.

The public concern about mercury all started in Michigan when fish from Au Ste. Clair were found to contain rather large amounts of this heavy metal. Then the Canadians confirmed the same thing in fish from lakes on the New England border, and this announcement started a search for mercury in the waters of Lake Champlain. It didn't take long to find it, for almost the very first fish was found to contain mercury. Before long the Vermont Comr. of Health had placed a ban on all commercial fishing in Lake Champlain. Then the author's department began looking for mercury in the overlying waters and in the bottom sediments; in the silts rather large amounts were found, but in the overlying waters the concentrations were very low. This situation supposedly might have been expected from compounds of a heavy metal like mercury. For the most part, mercury concentrations in the surface-water supplies of Vermont have not exceeded 0.5 $\mu g/l$ (ppb). As of Jul. 31, 1970, some 95 water-supply samples had been analyzed for mercury by the states and over 60 by the federal laboratories, and all were below the 5.0-ppb limit adopted by the USPHS. The maximum found was 3.3 ppb, but the great majority of samples contained less than 1.0 ppb mercury.

These critical situations should teach everyone a lesson—namely, to be alert for other trace-element contaminants that have not surfaced as yet but that may be equally significant. Information on two (cobalt and nickel) was gathered during the National Community Water Supply Study conducted by the Bureau of Water Hygiene in 1969.

The 1969 National Community Water Supply Study

The National Community Water Supply Study gave the water-works and public-health professionals an extensive evaluation of the quality of wa-

Burton Island State Park—one of the areas in Vermont surveyed.
(Courtesy Vermont Development Dept.)

ter being consumed by the public. Water supplies in one standard metropolitan statistical area (SMSA) in each of eight USPHS regions (II–IX) and a complete state (Vermont, in Region I) were surveyed in depth, and many samples for both chemical and bacteriological testing were collected from the distribution systems of the 969 water supplies visited. No survey was made in the Pacific Northwest. This study added a new dimension to a study that had been done by the USPHS in 1961–62 when 139 cities were surveyed,[3] but in which the samples had been collected almost entirely from the plant effluents.

Although the results of the 1969 work have been widely discussed and reported upon, some of them that are pertinent to the subject of this article have been selected for further attention. Table 1 describes the number of water-supply systems and the number of distribution-system samples that exceeded the trace-element and compound limits of the 1962 "USPHS Drinking Water Standards." Several points are of interest.

First, there was a considerable exceeding of the USPHS standards, which the water-works profession had become accustomed to thinking of as "minimum." If the results of the National Community Water Supply Study had been compared with the criteria or goals[4] of AWWA, the degree of noncompliance would have been much greater.

Second, the sampling of the 969 supplies was on a "grab" basis and unannounced. This factor indicates that the obtained results were probably indicative of the quality of water being consumed at the time, but, of course, the data were limited to one point in time. What would be shown if follow-up surveys were made?

Third, the results, when compared with ones obtained in 1961–62, indicate experimentally that there is deterioration of water quality after water leaves a plant and enters a distribution system.

TABLE 2

Health and Aesthetic Significance of Trace Elements and Compounds and Their Occurrence in US Water Supplies

Trace Element or Compound	Health or Aesthetic Effect	1962 USPHS Drinking-Water Standards Limit *mg/l*	Occurrence in US Water Supplies 2,595 Distribution Samples (1969) *mg/l*	
			Average	Range
Alkyl benzine sulfonate (ABS)	USPHS limit gives a safety factor of 15,000; not highly toxic	0.5*	0.05	0–0.41
Arsenic (As)	Serious systemic poison—100 mg usually causes severe poisoning, is cumulative, and may cause chronic effects. Late evidence indicates tiny amounts may be beneficial.	0.01* 0.05†	0.0001	<0.03–0.10
Chloride (Cl^-)	Limit set for taste reasons.	250.*	27.6	<1.0–1,950
Copper (Cu)	Body needs copper at level of about a mg/day for adults; not a health hazard except when large amounts are ingested.	1.*	0.13	0–8.35
Carbon chloroform Extract (CCE)	At limit stated, organics in water are not considered a health hazard.	0.2*	0.11	0.008–0.56
Cyanide (CN^{-2})	Rapid fatal poison, but limit set provides safety factor of about 100.	0.01* 0.2†	0.00009	<0.1–0.008
Fluoride (F^-)	Beneficial in small amounts; above 2,250 mg, dose can cause death.	0.7–1.2* 1.4–2.4†	0.32	<0.2–4.40
Nitrate (NO_3)	Excessive amounts can cause methemoglobinemia (blue baby) in infants.	45*	6.3	<0.1–127
Sulfate (SO_4)	Above 750 mg/l, usually has laxative effects.	250*	46	<1.0–770
Zinc (Zn)	Zinc is beneficial in that a child needs 0.3 mg/kg/day; 675–2,280 mg/l may be an emetic.	5*	0.19	0–13.0
Barium (Ba)	Fatal dose is 550–600 mg as the chloride; it is a muscle (including heart) stimulant.	1.0†	0.034	0–1.55
Cadmium (Cd)	13–15 ppm in food has caused illness.	0.01†	0.003	<0.2–3.94
Chromium (Cr^{+6})	Limit provides a safety factor. Carcinogenic when inhaled.	0.05†	0.0023	0–0.079
Lead (Pb)	Serious, cumulative, body poison.	0.05*	0.013	0–0.61
Selenium (Se)	Toxic to both humans and animals in large amounts. Late research suggests small amounts may be beneficial.	0.01†	0.003	0–0.07
Silver (Ag)	Can produce irreversible, adverse cosmetic changes.	0.05†	0.008	0–0.03
Radium (Ra-226)	A bone-seeking, internal alpha emitter that can destroy bone marrow.	3 pc/l‡	2.2 pc/l	0–135.9 pc/l
Strontium (SR-90)	A bone-seeking, internal beta emitter.	10 pc/l‡	<1.0 pc/l	0–2.0 pc/l

* Not to be exceeded where other more suitable supplies are, or can be made, available.
† Excess constitutes grounds for rejection of the supply.
‡ Consult 1962 "USPHS Drinking Water Standards" for interpretation.

Water pollution often can be observed from the air—as noted in this view of Boston harbor.
(Courtesy US Army Corps of Engrs.)

Significance of Trace Elements and Compounds

The guiding principle by which a limit for a trace element or chemical compound is determined and then published in drinking water standards is, with one exception thus far, on the basis of deleterious effects. These effects are evaluated from two viewpoints—namely, health and aesthetics. A comprehensive evaluation of the national, or any, situation with regards to trace elements and chemical compounds must include consideration of the following: (1) name of element or compound, (2) its effect upon health and well-being, (3) the limit for the element or compound published in drinking-water standards, (4) the concentration of the trace element or compound in the water, and (5) a comparison of the values of analytical results with the limits published in standards.

The term "standards" here is used in a broad sense, because, although the standards that largely govern American water-works practice are found in the 1962 "USPHS Drinking Water Standards," there are other criteria or standards that have been adopted unofficially by the Div. of Water Hygiene and others in the interim period and that are more or less generally known. There are also the "International Standards for Drinking Water" and the aforementioned "Quality Goals for Potable Water" of AWWA. Some concept of how this problem may be approached is given in Table 2 wherein are listed the trace element or compound, the limit set for it in the 1962 "USPHS Drinking Water Standards," a brief statement of the public-health or aesthetic rationale behind the limit, and data on the occurrence of the chemical in US public water supplies as learned from the National Community Water Supply Study.

Table 3 gives information on trace elements and compounds for which no limit was published in the 1962 standards, but for which limits have been unofficially adopted by the Div. of Water Hygiene in the intervening period. Also included in Table 3 are some elements for which a standard has not yet been adopted, but for which one is being considered by the committee revising the 1962 standards.

Exciting Possibilities

The topic of future possibilities may bring to mind a picture of dire condi-

TABLE 3

Health and Aesthetic Significance of Trace Elements and Compounds in Finished Drinking Water (Limits Not Found in 1962 "USPHS Drinking Water Standards")

Name of Ingredient	Health or Aesthetic Effect	Unofficial Limit mg/l	Occurrence in US Water Supplies mg/l	
			Average	Range
Substance				
Antimony (Sb)	Similar to arsenic but less acute. Recommended limit not to exceed 0.1 mg/l; routinely below 0.05 mg/l; over long-time periods below 0.01 mg/l.	0.05		
Beryllium (Be)	Poisonous in some of its salts in occupational exposure.	None		
Bismuth (Bi)	A heavy metal in the arsenic family—avoid in water supplies.	None		
Boron (B)	Ingestion of large amounts can affect central nervous system, and protracted ingestion may cause borism.	1.0	0.069	0–3.28*
Cobalt (Co)	Beneficial in small amounts; about 7 μg/day.	None	0.002	0.0–0.019*
Molybdenum (Mo)	Necessary for plants and ruminants. Excessive intakes may be toxic to higher animals; acute or chronic effects not well-known.	None		
Mercury (Hg)	Continued ingestion or large amounts can damage brain and central nervous system.	0.005	0.000	0.000–0.033†
Nickel (Ni)	May cause dermatitis in sensitive people; doses of 30–73 mg of $NiSO_4 \cdot 6H_2O$ have produced toxic effects.	None	0.005	0.0–0.072*
Sodium (Na)	A beneficial and needed body element, but can be harmful to people with certain diseases.	20‡	40 per cent above 20 mg/l	0.4–1,900§
Tin (Sn)	Long used in food containers without known harmful effects.	None		
Uryanyl ion (UO_2)	May cause damage to kidneys.	5.0		
Vanadium (V)	Some evidence that vanadium may be beneficial with respect to heart disease.	None		
Pesticides				
Aldrin	One or all of these complex organic compounds have severe, acute, adverse health effects when ingested in large amounts. Small amounts accumulate, and long-range effects are generally unknown.	0.017**	160 samples in NCWWS showed lower values than the limits.	
Chlordane		0.003**		
DDT		0.042**		
Dieldrin		0.017**		
Endrin		0.001**		
Heptachlor		0.018**		
Heptachlor epoxide		0.018**		
Lindane		0.056**		
Methoxychlor		0.045**		
Toxaphene		0.005**		
Organic phosphorous plus carbamates, such as Parathion, Malathion, Carbaryl, and others	The organic phosphorous and carbamate pesticides are severe acute poisons affecting the central nervous system; ingestion of small amounts over time can harm the central nervous system.	0.1††	Same as for pesticides	
Herbicides				
Algicide	A group having toxic properties of a generally lower order than the above pesticides. However, they should not be used without great care and should not be found in drinking water.	1.0		
Copper key-El		1.0 (Cu)		
Cuprose		1.0 (Cu)		
Cuttine		1.0 (Cu)		
Diquot		0.2		
Dalapon		0.1		
2,4-D		1.0		
2,4,5-T should not be used.				
Micro-gard PR		0		
Mogul algicide AG-470		1.0		
Radapon		0.1		
Silvex (2,4,5-TP)		0.2		
Tordon 10K & 101		0		
Twink		0.2		
Urcabor		1.0 (as B)		

* National Community Water Supply Study. 2500 samples.

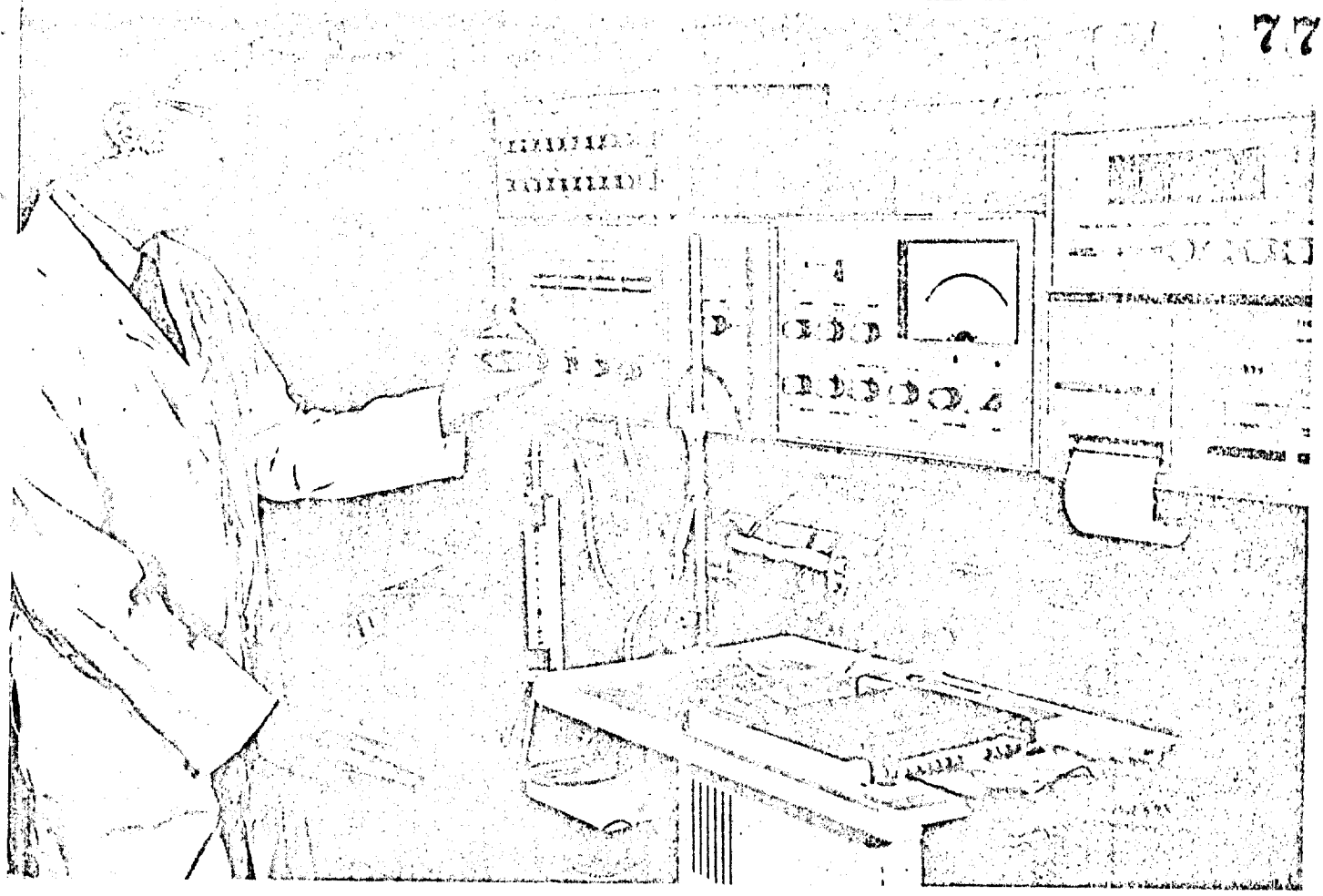
† Late data from BWH, USPHS.

‡ The amount at, and above, which people on strict (500 mgd) sodium-restricted diets must include in their daily sodium-intake calculations.

§ Survey of over 2,000 US municipalities by USPHS in 1963–66.

** Report of the Com. on Water Quality Criteria, FWPCA, US Dept. of the Interior.

†† As parathion equivalent in cholinesterase inhibition.



Technician tests for mercury—one of trace elements, which author says may become a problem

ons if the examples of DDT and mercury are repeated with other trace elements and compounds: The future probably will not be free from this sort of thing. However, the future holds the probability that drinking water may become a vehicle for trace elements in the same way that fluoride ion has been advocated, but for far greater benefits to health.

In the early 1960s, researchers in the US and other countries began to notice what appeared to be a relationship between heart disease and water hardness. Dr. Henry Schroeder of Brattleboro, Vt.—the medical scientist who earlier in the century had pointed to sodium as an element involved, in the bad sense, in congestive heart failure—showed that there was an inverse relationship between water hardness and certain forms of heart ailments. Although one or two studies seemed to contraindicate this theory, further work in England, Sweden, and Japan confirmed it. Dr. Schroeder was not long in concluding that the causative factor in the observed relationship was not water hardness per se, but rather some element that was present in hard water (or perhaps absent in soft water) or even some harmful element being dissolved by soft water. Right now, researchers in various parts of the world are searching for the causative agent; when it is found, the way may be open to helping millions who struggle with debilitating and deadly heart disease.

Another researcher [5] has been doing a great deal of work with the toxic element selenium and has been published on the subject of the relationship of selenium to cancer, postulating an inhibitory effect.

In Summary

The trend continues in the importance of trace elements and compounds in drinking water. Whereas the first drinking-water standards published by the federal government contained no limits for chemicals, it appears that the list of them in the forthcoming revision of the 1962 "USPHS Drinking Water Standards" will be nearly double that in the 1962 standards.

What the author has said thus far has been concentrated on the subject of trace elements and compounds from the standpoint of limits of standards and occurrence in US water supplies. The question may be asked, "What about removal?" The answer is that conventional treatment practices do not notably reduce most of the trace elements and compounds discussed in this article.

Although the concern about these substances usually stems from fears of what they may do to a person's health if they are present, one should not overlook the possibility that a few of them are of potential benefit and that there is a likelihood that trace elements and compounds not now known will be found to have both adverse and beneficial effects.

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Nickel Poisoning

V. The Metabolism of Nickel Under Normal Conditions and After Exposure to Nickel Carbonyl

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Most of the previous studies on nickel from our laboratories have been directed toward the toxicity of nickel carbonyl and to the treatment following exposure.[1-3] The present studies are concerned with the metabolism of nickel in dogs before and after exposure to the vapors of nickel carbonyl.

Drinker and co-workers[4] in 1924 found that human subjects who had ingested nickel excreted as much as 30 mg. per day in the feces, whereas only 4 mg. per day was excreted in the urine. These observations are in contrast to those of Kent and McCance,[5] who found under normal conditions that more nickel was excreted in the urine than in the feces. Wase, Goss, and Boyd,[6] in recent experiments in mice, injected radioactive nickel in the form of nickel chloride intraperitoneally and found that in the first eight hours most of the nickel was excreted in the feces rather than in the urine.

With the exception of the results reported by Sunderman and Kincaid,[2] the metabolism of nickel following the inhalation of nickel carbonyl has not been studied. These investigators made clinical observations on 36 persons accidentally exposed to this compound and found that nickel concentrations in urine increased manifoldly above normal after exposure.

The experiments to be described are concerned with nickel metabolism in dogs under normal conditions and following exposure to nickel carbonyl.

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This study was supported in part by contract of Jefferson Medical College with the Atomic Energy Commission.

Methods

The following methods were employed: Female dogs were permitted free access to Purina Dog Chow and distilled water and were episiotomized in order to facilitate catherization and collection of urine. The metabolism studies were divided into three-day periods and performed in a conventional manner, with use of carmine and charcoal to mark the beginning and end, respectively, of each three-day metabolic period.

Nickel balance studies were undertaken before and after exposure to vapors of nickel carbonyl in concentrations ranging from 0.2 to 1.0 mg. per liter for 30-minute periods. Nickel analyses were performed on both the ingesta and egesta during two consecutive three-day periods, according to a modified method of Alexander, Godar, and Linde.[7] A modification of the method of Tobias and Weston[8] was used in studies completed in one dog in which the retention of nickel was measured after the inhalation of nickel carbonyl. Essentially, this method measures the nickel carbonyl retained by determining the difference between the total amount inhaled and exhaled.

Figure 1 is a diagram of the method used to determine nickel carbonyl retention in the dog. The animal was anesthetized lightly with pentobarbital sodium, and a tight-fitting rubber mask which was

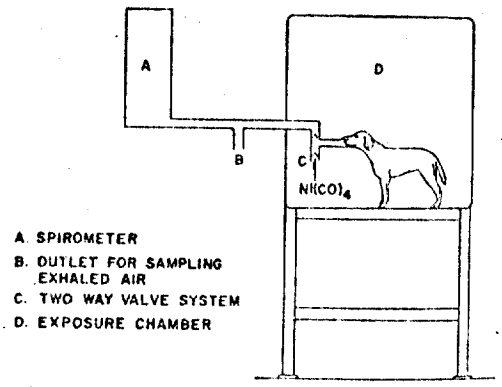

Fig. 1.—Schematic diagram to illustrate method for measuring respiratory exchange during exposure to nickel carbonyl.

TABLE 1.—*Nickel Balance Studies (Before Exposure to Nickel Carbonyl)*

Dog	3-Day Period	Ni in Food, Mg.	Ni in Stool, Mg.	Ni in Urine, Mg.	Balance, Mg.
1	1	0.79	0.72	0.04	+0.03
	2	0.48	0.44	0.03	+0.01
	3	1.34	1.21	0.07	+0.06
	4	0.88	0.83	0.05	0.0
2	1	1.21	1.05	0.07	+0.11
	2	1.39	1.19	0.07	+0.13
	3	1.69	1.19	0.10	+0.40
3	1	1.38	1.14	0.08	+0.16
4	1	1.51	1.36	0.13	+0.05
Average S.D.		1.12±0.16	1.01±0.44	0.07±0.03	

well coated with an unctuous mixture of paraffin and petrolatum was placed over the face. An Ace Bandage was wrapped around the mask to insure the prevention of leakage. Air containing nickel carbonyl vapors was inhaled and exhaled through a two-way valve system. The volume of exhaled air was determined by collection in a spirometer, and the average concentration of nickel carbonyl in the inhaled and exhaled air was determined on aliquots obtained by a continuous sampling method. With this information it was possible to determine the amount of nickel carbonyl retained.

Results

The results of nickel balance studies before exposure to nickel carbonyl are given in Table 1. These data show that nickel intake in the food is virtually equal to the output in the urine and feces. Furthermore, our results indicate that an average of 90% of the ingested nickel is excreted in the stool and only 10% is eliminated in the urine. These observations confirm those of Drinker and his co-workers.[4]

The amounts of nickel excreted after exposure to nickel carbonyl are given in Table 2. It will be seen that there is a striking increase in the excretion of nickel in the urine during the first three-day metabolic period after exposure as compared with the control period. This increased excretion in the urine is highly significant ($P<0.01$). In contrast, there is no significant difference between nickel ingestion and excretion of nickel in the stool during the control period and the first metabolic period after exposure (Table 2, Fig. 2). In the second metabolic period following exposure (Days 4, 5, and 6) the amount of nickel in urine tends to return to preexposure levels.

Nickel retention was measured after both ingestion and inhalation in Dog 5. The results of the studies on nickel balance in Dog 5 are given in Table 2. The total nickel intake was 7.01 mg., and the total output in urine and feces was 6.97 mg. Thus, approximately 99% of the ingested and inhaled nickel was eliminated in the urine and feces within six days.

Comment

Since nickel carbonyl is very reactive and labile in the presence of oxygen, it is not certain whether or not nickel carbonyl per se diffuses across the alveolar membrane in the lungs. Armit[9,10] postulated that it is not absorbed as such but is decomposed by moisture and is deposited as colloidal metallic nickel on the pulmonary epithelium. Unfortunately, there is little experimental evidence to support this view.

The observation that there is a sharp increase in nickel excretion in the urine immediately after acute exposure in dogs is of major practical importance. Clinically, it has been found that in some cases of accidental poisoning symptoms occasionally remain latent for a period of 8 to 10 days. Therefore, the low concentrations of nickel

TABLE 2.—*Nickel Balance Studies (After Exposure to Nickel Carbonyl)*

Dog	3-Day Period	$Ni(CO)_4$, Mg./L.	Ni in Food, Mg.	Ni Inhaled, Mg.	Ni in Stool, Mg.	Ni in Urine, Mg.	Ingesta—Egesta, Mg.	Balance
1	1	0.2	1.40		1.57	2.98	—3.2	
2	1	0.2	0.85		1.50	3.86	—4.5	
	2		1.63		1.65	0.22	—0.2	
3	1	1.0	0.55		1.12	3.71	—4.3	
	2		1.61		1.70	0.27	—0.4	
4	1	1.0	0.78		1.17	3.32	—3.7	
	2		1.96		2.22	0.29	—0.6	
5	1	0.4	2.52	2.25	1.78	2.10	—1.4	+0.9
			2.24		2.90	0.19	—0.9	—0.9

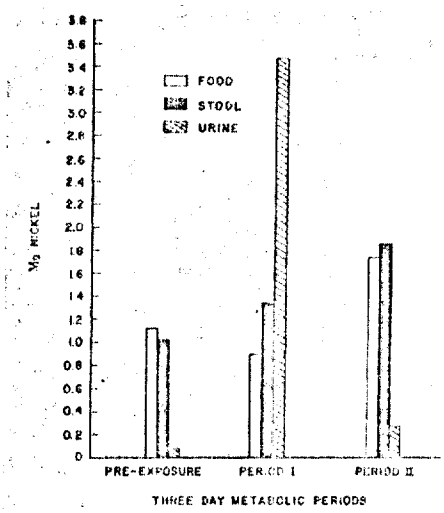

Fig. 2.—Nickel balance studies in dogs before and after exposure to nickel carbonyl.

in the urine should not always be considered as conclusive evidence against nickel carbonyl poisoning. In our studies the nickel content of urine returns to approximately normal levels within six days after exposure.

The high concentrations of nickel in the urine after exposure to nickel carbonyl are of great value in the detection of nickel carbonyl poisoning. Sunderman and Kincaid[2] have emphasized the practical usefulness of measuring nickel in urine among exposed workers. Daily measurements of urine nickel have been shown to serve as a useful index of exposure among workmen in manufacturing plants.

Summary

Nickel balance studies were undertaken in normal dogs. These studies indicate that 90% of the ingested nickel is excreted in the stool and 10% is eliminated in the urine.

After exposure to the vapors of nickel carbonyl the amount of nickel excreted in the urine is strikingly increased. No such increase was found in the feces.

A complete balance study in a dog before and after exposure to nickel carbonyl indicates that virtually all of the nickel is excreted within six days and that there is no significant retention of nickel in the body.

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CONTAMINATION OF FOOD COOKED OR STORED IN CONTACT WITH NICKEL-CHROMIUM-IRON ALLOYS*

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FROM the time of Brearley's discovery of the corrosion protective action of chromium in steel (1), industrial development of these alloys and their application to many important uses have progressively increased. Not the least of these is the recent development of nickel-chromium-iron alloys and their utilization for culinary purposes. The corrosion resistant nature of nickel has long been known and the possible use of pure nickel cooking utensils has been subjected to a searching examination within recent years (2), with results that established the very low intake that could occur and the general harmlessness of these small amounts. The nickel-steel alloys are far more resistant to corrosion than pure nickel itself and would seem ideally suitable for food containers of all sorts. It is necessary, however, from a public health point of view to test these materials in terms of practical usage.

These newer nickel-chromium-iron alloys have the great advantages of noncorrosive properties, of light weight, and of remaining clean and bright under conditions which quickly affect the appearance of other materials. These steels are composed of iron, chromium, and nickel in varying proportions and show a remarkable resistance to corrosion under extreme conditions. Their use for the manufacture of culinary utensils is a logical step and has given impetus to the present investigation concerning their hygienic fitness for this purpose.

Plan of Investigation

The steels that were examined in this laboratory were subjected to conditions which duplicated as nearly as possible the household preparation and storage of food. In order to secure the greatest accuracy, the strip tests were made with accurately weighed and measured squares of metal of uniform surface. Other tests were made with cooking utensils in which the results of actual usage could be observed. The chief dependence, however, was placed upon the analytic data.

The hygienic evaluation of a metal to which foods must be exposed in the course of their preparation involves not only such obvious factors as the length of time the food is exposed to the metal and the temperature at which this occurs, but also other factors such as the hydrogen ion concentration or acidity of the food mixture, salt concentration, dissolved oxygen, and, to some extent, the nature of the food. It would be expected that the degree of contamination would be more or less

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proportional to the degree of acidity. That this is not strictly so in the case of these particular alloys is apparent from the data which follow.

The three alloys investigated each contained chromium, nickel, and iron, the proportion only being varied. One of the alloys contained, in addition, a small amount of molybdenum, which has been found to make the metal more resistant to attack of certain sulphur compounds. The approximate composition of these three alloys is as follows:

	QB %	*QA* %	*A* %
Iron	67	65.3	74
Chromium	18	17.0	18
Nickel	15	14.0	8
Molybdenum	0	3.7	0

It will be observed that all three alloys are quite similar in their chromium content. Alloy A, which is being produced commercially under several trade names, has been manufactured into culinary utensils for some time.

Strips of these metals measuring in general 85 mm. by 135 mm., and 0.02 mm. thick, were polished and bent so that two could be arranged in each beaker in which the food materials were cooked. Each strip was accurately weighed, its area exposed to both liquid and air measured, the length of the line of contact noted, and the temperature and time of contact determined. After cooking, the strips were carefully washed free from adhering food materials and were weighed. The washings were added to the contents of the beakers, evaporated to dryness, charred, ashed, and analyzed for chromium, iron, nickel, and molybdenum. The foods before cooking had been previously analyzed for traces of these metals, and by deducting the latter amount from the former, the amount dissolved from the strips was obtained.

In determining the weight losses of the strips, extreme care was necessary in order to obtain significant figures. Owing to the corrosion resistant nature of the alloy and, furthermore, to the fact that the time of contact during ordinary cooking (one hour) is not particularly long, the weight loss is extremely small. There is also another factor to be considered—the formation of a film of tarnish which may affect the weight of the strip in another sense, causing in fact a weight gain.

Tarnish is of importance principally in evaluating the aesthetic advantage of culinary utensils. It does not indicate that the metal has reacted with the food and has contaminated it necessarily. In fact, it may imply quite the opposite from the latter. *As a test for contamination of this type, it is unreliable.*

Owing to the fact that the weight of the metal was considerable as compared with the loss sustained, the weighings were made with a counterpoise of the same metal and of practically the same weight; thus errors due to moisture absorption, etc., were eliminated. In order to attain greater accuracy and because of the minute differences in weight, the weighings were made on a balance accurate to 0.05 mg. The tarnish formed may be classified as of two kinds—superficial and permanent. The superficial tarnish is that which is removable by very gently rubbing the surface with soapy water, either by hand or with soft gauze. The tarnish remaining after

this treatment is more or less closely adherent and requires the use of abrasives for removal. Both degrees of tarnish were determined in terms of an arbitrary scale, ranging from 1 (plainly apparent) to 4 (barely perceptible).

That there was no relation between the change in weight of the metal strip and the amount of metal picked up by the food was apparent when the latter was submitted to quantitative analysis for the metals involved. The analysis bore no relation to the change in weight. Instead of a weight loss there was occasionally a weight *gain* on rinsed strips only. This was due either to surface oxidation, sulphide formation, or to the formation of an insoluble compound with some constituent of the food, or, possibly, to deposition of some more noble metal present in the food. It was usually most noticeable just at the juncture of contact with the liquid, indicating a possible oxidative effect.

It might be mentioned that in the case of these alloys, tarnish consisted in a barely perceptible film which often required careful illumination to become visible. The weight losses were small, amounting, in many cases, to only a fraction of a milligram for a 50 gm. strip of metal. Tarnish was not directly proportional to acidity.

The weight changes and the degree of film formation in these metals are shown in Table 1. Table 2 shows the weight changes per 4 square decimeters per hour of contact at boiling point (that is, in terms of the amount

TABLE 1.—WEIGHT CHANGES AND FILM FORMATION UNDER NORMAL COOKING CONDITIONS

(Total surface in contact with food is 2.5 sq. dm., surface above liquid is 1.5 sq. dm., and line of contact is 53 cm. There are 250 c.c. of food material.)

FOOD	ALLOY QB Fe = 67%, Cr = 18%, Ni = 15%			ALLOY QA Fe = 65.3%, Cr = 17%, Ni = 14%, Mo = 3.7%			ALLOY A Fe = 74%, Cr = 18%, Ni = 8%		
	Rinsed	Washed	Film	Rinsed	Washed	Film	Rinsed	Washed	Film
	mg.	*mg.*		*mg.*	*mg.*		*mg.*	*mg.*	
Apple 1	−1.4	−1.9	4	−1.2	−2.0	4	...	−1.8	4
Apple 2	...	...	4	...	...	4	...	...	4
Chicken	...	−1.0	1	...	−0.3	1	...	−0.6	1
Gooseberries	+0.2	−1.2	4	+0.1	−0.6	4	−0.2	−0.8	4
Rice	−0.8	−2.1	4	−0.6	−0.9	4	−0.8	−1.3	4
Rice (stored)	+0.3	−0.3	4	+0.5	−0.6	4	+0.4	−0.3	4
Potatoes	0.0	−0.2	3	−0.8	−1.2	3	−0.7	−0.8	3
Flounder	+0.4	−1.0	1	+0.5	−0.7	2	+0.6	−0.2	1
Cauliflower	+0.1	−1.1	2	+0.1	−0.9	2	+0.4	−1.3	1
Corn	...	−1.0	4	...	−0.6	4	...	−0.8	4
Custard	−2.2	−1.6	1	+1.6	−0.5	1	+1.1	−0.4	1

of metal exposed in an ordinary stewpan). This represents about the average surface of metal exposed to food cooked under ordinary domestic conditions. According to these figures, no significant correlation, although the weight loss is somewhat greater for hydrogen ion concentrations below 6.1 than for concentrations above this point.

TABLE 2.—WEIGHT CHANGE PER 4 SQUARE DECIMETERS PER HOUR OF CONTACT AT BOILING POINT

FOOD	ALLOY QB		ALLOY QA		ALLOY A	
	Rinsed	Washed	Rinsed	Washed	Rinsed	Washed
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Apple 1	−2.5	−3.6	−2.4	−3.8	...	−3.5
Apple 2	...	...	...	...	...	...
Chicken	...	−1.6	...	−0.4	...	−0.9
Gooseberries	+0.5	−2.4	+0.2	−1.2	−0.5	−1.5
Rice	−0.5	−1.3	−0.4	−0.6	−0.6	−0.8
Rice (stored)	0.0	0.0	0.0	0.0	0.0	0.0
Potatoes	0.0	−0.6	−1.6	−1.9	−1.7	−1.9
Flounder	+2.8	−6.4	+3.2	−4.8	+4.0	−2.4
Cauliflower	+0.4	−3.4	+0.4	−2.8	+1.4	−4.2
Corn	...	−9.6	...	−6.0	...	−7.2
Custard	+1.7	−1.3	+1.3	−0.4	+0.9	−0.3

TABLE 3.—EFFECT OF VARIATION IN HYDROGEN ION CONCENTRATION UPON WEIGHT CHANGES

P_H OF FOOD	WASHED WEIGHT LOSS PER SQ. DM.		
	Alloy QB	Alloy QA	Alloy A
	mg.	*mg.*	*mg.*
3.0	0.6	0.4	0.4
3.3	0.9	0.9	0.9
6.0	1.6	1.2	0.6
6.1	0.9	0.7	1.0
6.4	0.4	0.0	0.0
6.4	0.0	0.1	0.2
6.6	0.02	0.7	0.5

there is no marked difference between the various alloys. The weight losses of the three alloys compared with the variation in hydrogen ion concentration of the various foods is shown in Table 3. As indicated above, there is

The point of greatest importance from an hygienic point of view is the quality rather than the quantity of material that contaminates the food. In all cases, therefore, the foods were carefully analyzed after they had been in contact with the metal. These analyses showed that the resistance of the alloy to corrosion is very high, for only a trace of the total metal dissolved could be found in the food and this trace consisted chiefly of iron. The analytic determinations as compared with the strip weight determinations therefore constitute a valuable control, for they not only show accurately the degree to which the metal dissolves, but also show the important fact that the metal which goes into solution from these alloys is principally iron.

The analytic procedure adopted was

as follows: The ash from the completely ashed food was dissolved in hydrochloric acid and water, and was made up to a given volume. Portions of this solution were separately analyzed for iron, chromium, and nickel, and in several cases for molybdenum. These constituents were determined colorimetrically—the iron as the thiocyanate (3), the chromium as diphenylcarbazide (4), the nickel as dithiooxalate (5), and the molybdenum as

TABLE 4.—AMOUNTS OF METAL TAKEN UP BY COOKING 400 GRAMS OF VARIOUS FOOD MATERIALS FOR ONE HOUR IN CONTACT WITH 4 SQUARE DECIMETERS OF ALLOY

FOOD	ALLOY QB			ALLOY QA				ALLOY A		
	Iron	Chromium	Nickel	Iron	Chromium	Nickel	Molybdenum	Iron	Chromium	Nickel
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Apple 1	1.80	....	0.12	4.20	....	0.24	0.02	2.30	....	0.24
Apple 2	1.00	0.03	0.12	0.80	0.25	0.12	0.00	0.80	0.30	trace
Chicken	1.40	0.04	0.04	1.10	0.35	0.07	0.007	0.47	0.04	0.02
Gooseberries	1.00	....	0.18	0.90	....	0.02	0.05	1.30	....	0.12
Rice	0.32	0.01	0.01	0.24	0.01	0.02	trace	0.36	0.02	0.02
Rice (stored)	0.01	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00
Potatoes	0.76	0.06	0.00	1.60	0.06	0.17	trace?	1.40	0.06	0.06
Flounder	1.90	0.16	0.08	1.90	1.40	0.00	0.00	2.90	....	0.00
Cauliflower	0.86	0.08	0.10	0.52	0.08	0.04	.....	1.40	0.08	0.04
Corn	2.10	....	0.00	2.10	....	0.00	0.00	3.80	....	0.00
Custard	0.03	0.02	0.00	0.01	0.25	0.12	0.00	0.08	0.02	0.00

TABLE 5.—COMPOSITION OF COOKED FOOD, PER KILOGRAM OF MATERIAL

RAW MATERIAL	AMOUNT	SALT	SUGAR	VINEGAR	TIME OF COOKING
	gm.	*gm.*	*gm.*	*c.c.*	*min.*
Apple	440	....	400	...	50
Chicken	240	....	...	...	60
Gooseberries	320	....	428	...	50
Rice	116	13.2	...	...	150
Rice (stored)	236	26.8	...	...	40
Potatoes	200	....	...	...	40
Flounder	200	24.0	...	7.2	15
Cauliflower	200	....	...	...	30
Corn	256	....	...	...	10
Custard	(4 eggs, 800 c.c. milk)		80	...	120[1]

[1] 50°–80°.

thiocyanate (6). The amounts that were taken up by the various foods are summarized in Table 4. In these experiments the food was prepared as in the usual household cooking in triplicate and each metal constituent in the food determined for each alloy. The data given in Table 4 represent averages of all the determinations that gave satisfactory checks. Table 5 indicates the amounts of materials that entered into the composition of the cooked food. Similar experiments in which the food was cooked directly in the utensil and then analyzed are shown in Table 6.

TABLE 6.—FOOD COOKED FOR ONE HOUR DIRECTLY IN METALLIC CONTAINERS OF ALLOY A

(Surface exposed to liquid is 4 sq. dm.)

FOOD	P_H	IRON	CHROMIUM	NICKEL
		mg.	*mg.*	*mg.*
Chicken	...	...	0.05	0.02
Apples (stored 1 hr.)	3.3	0.2	0.01	0.00
Apples (cooking)	3.4	0.3	0.20	0.17
Plums 1	1.7	4.0	0.40	0.08
Plums 2	1.7	...	0.20	0.04
Cottage cheese	...	0.0	0.00	0.00

It is of interest that the acid foods, such as apple and gooseberries, are not necessarily the type which are largely contaminated on contact with the metal. Corn and fish, which are less acid, are more or less uniformly contaminated. Of the three alloys, QB appears to be the best. The average degree of contamination produced by this metal is appreciably less than that of the others.

Comparison of these tests with those made with utensils manufactured from alloy A indicated that the amounts of metal taken up from the cooking utensils were of the same order of magnitude for similar surfaces and lines of contact as shown by the strip tests. The amounts were, if anything, somewhat less, as indicated in Table 7.

TABLE 7.—COMPARISON OF UTENSIL AND STRIP TEST COOKED FOOD

(Both of Alloy A: Fe 74%, Cr 18%, Ni 8%)

	UTENSIL COOKING	STRIP TEST COOKING
Area of metal exposed	4 sq. dm.	4 sq. dm.
Area of metal exposed to vapor	3.8 " "	4 " "
Line of contact (liquid)	45 cm.	45 cm.
Iron dissolved	0.30 mg.	0.80 mg.
Chromium dissolved	0.20 "	0.30 "
Nickel dissolved	0.17 "	slight trace

Discussion

The results obtained in this investigation indicate that, while certain nickel-chromium-iron alloys are affected by ordinary cooking operations to the extent that traces of the constituents are determinable by the exceedingly delicate tests that are available, the amounts of metal are so minute that these quantities have no hygienic importance. In the case of

alloy QB (67 per cent. iron, 18 per cent. chromium, and 15 per cent. nickel), the average amounts of metal entering foods of various kinds cooked for one hour were (computed per kilogram of food material):

	Mg.
Iron	2.56
Chromium	0.12
Nickel	0.16

These foods covered the range of acidity between that of gooseberries and that of chicken. In no case could any change be noted in the appearance or taste of food so cooked or cooked and stored for seventy-two hours in a refrigerator in contact with the metal.

These amounts are very small compared with the amounts of metal that dissolve from ordinary cooking utensils in common use. Järvinen (7) recently found that 5 per cent. solution of sodium chloride boiled for three hours in various utensils dissolved the following amounts of metal per kilogram of solution:

	Mg.	*Metal*
Iron pot	104.0	iron
Copper kettle	70.0	copper
Tinned copper kettle	7.0	tin
Nickel kettle	4.0	nickel
Aluminium kettle	9.0	aluminium
Brass kettle	2.0	zinc
	1.2	copper

When sweetened fruit juices were boiled in these utensils, Järvinen found that in many cases ten times the foregoing quantity of metals entered into solution.

A recent investigation by Hunziker, Cordes, and Nissen (8) showed that Allegheny metal (a nickel-chromium-iron alloy) had a high corrosion resistance. Their results, while based upon strip weight changes and not upon analytic findings and for other reasons not strictly comparable with the results obtained in this study, show a marked advantage of this type of alloy. The strip weight experiments of Mrak and Cruess (9), in which various metals including chromium-iron alloys were subjected to the action of fruit juices, show a higher degree of corrosion than was obtained in our experiments with nickel-chromium-iron alloys. The latter type of alloy appears to be peculiarly resistant to corrosion.

The difference between the amounts of metal taken up from nickel-chromium-iron alloys on the one hand and other metals in common use on the other hand indicates that these alloys are hygienically safe for cooking purposes.

Summary

Various nickel-chromium-iron alloys were subjected to an extensive investigation concerning their hygienic fitness for use in contact with food. Determinations were made of the weight changes and the character of film formation that occur in the cooking of various foods of different degrees of acidity in contact with the metal, and of the degree to which traces of the constituent metals enter the food. The results show that the total dissolved metal is exceedingly minimal; iron is the chief metal found while nickel and chromium occur in considerably smaller amounts. For the foods investigated, the contamination at a hydrogen ion concentration of 3 was no greater than at a concentration of 6. An average for various foods cooked for one hour in contact with the 15 per cent. nickel–18 per cent. chromium

alloy showed a contamination of 1.01 mg. of iron, 0.05 mg. of chromium, and 0.06 mg. of nickel for 4 square decimeters of surface in contact with the food.

This investigation was carried on in co-operation with the Bureau of Chemistry and Soils of the United States Department of Agriculture and with the Development and Research Department of the International Nickel Company. It is a pleasure to record the fact of this co-operation and particularly to acknowledge the criticism and interest of Dr. F. C. Blanck and of Mr. R. J. McKay in this connection.

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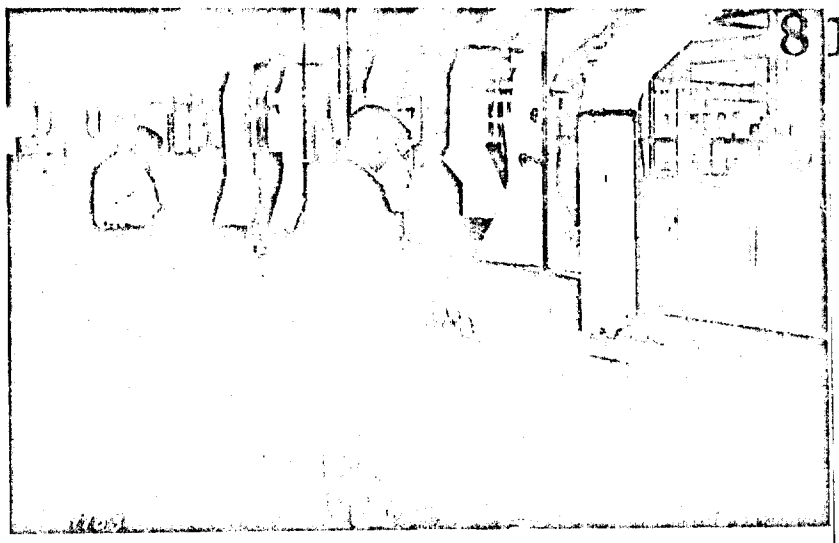


(Courtesy National Safety Council)

Overhead ventilation in the plating room.

metal Finishing 45(8) 55-60, 67 (9) 79-83 (10) 75-81 (1947)

Toxicity of Chemicals in Electroplating

By P. M. Van Arsdell, *Benson & Associates, Chicago 4, Ill.*

METALS and metallic salts, basic factors of electroplating processes, are being used in greater quantity and variety as a result of the expansion of the industry. They are the most poisonous materials known to man. Since the discoveries of Volta, Wollaston, Davy, and others, continued intensive research has gradually increased and widened the field in the types of solutions used and in the number of metals plated or purified by the electrolytic method.

The expansion of the industry increased the number and types of health hazards. Absorption of many of the metals is dangerous. The metallic salts present an even greater health hazard to the human organism. For example, such metals as lead, cadmium, gold, etc., are toxic when they enter the blood stream. All nitrates, sulfides, and cyanides commonly used in an electroplating bath are extremely toxic. Metals and their salts enter the body by three routes, inhalation, absorption through the skin and ingestion.

The ease with which metallic compounds enter the body depends upon their water solubility and also upon their eventual solubility in body fluids. As most electroplating depends upon the solubility of the salts, the anion generally is the determining factor. Because the cyanides, nitrates, chlorides, and certain double salts insure good solubility, they are employed in many baths. Unless great care is exercised, these may be extremely hazardous to the health, comfort, and the life of the operators as the salt concentrations are usually high, and the temperatures at which they are used range from cold to boiling.

A number of factors exist in the environs of some electroplating processes which tend to enhance the toxic reactions. These factors are dampness, heat and inadequate ventilation. All three affect the physical condition of the body, making absorption easy. Many metallic compounds show more marked toxicities for persons who are perspiring. The dampness naturally tends to hold the salts in a more active state upon the skin; inadequate ventilation causes a rise in concentration of toxic materials in the atmosphere.

There are a number of fairly typical physiological reactions to metallic salts when they are present in the working area. Metallic salts cause approximately 79 per cent of the dermatitis reactions from electroplating processes.[13] Many of these salts also cause nasal difficulties, depending upon the susceptibility of the individual, which range from simple irritations to perforation of the nasal septum as is sometimes encountered in chromium plating.[17]

The following discussion of toxic reactions to metals and metallic salts points out the particular reactions which have been reported when these compounds have been used. The metals and salts used will be given in alphabetical order and the particular bath in which they are used will also be indicated.

Aluminum and Its Salts

Aluminum salts have found application in certain types of baths, particularly the acid copper, where they are used as the sulfate.

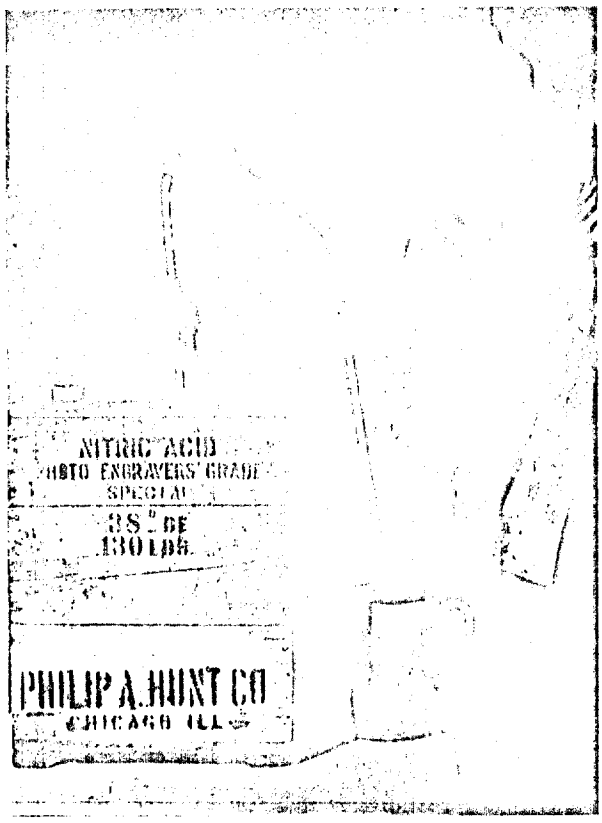
Metallic aluminum is relatively non-toxic,[10] although

certain individuals may be hypersensitive to it. The soluble salts, that is the chloride, nitrate, and sulfate may have a decidedly injurious action, particularly the chloride in dry form or in concentrated solution. This action is due to the release of acid from the molecule and the protein precipitant reaction of aluminum ions.

Inhalation of aluminum chloride, nitrate, or sulfate as sprays or vapors from concentrated solutions tend to withdraw water from the nasal, throat, and lung tissues. Acid as well as aluminum ions are liberated on the mucous tissue. The former are corrosive and irritant and the latter a protein precipitant. Should these compounds be inhaled in any quantity, the individual should be immediately removed from such atmosphere and a physician promptly called to treat the symptoms as they arise. None but professional first aid should be given. Masks and respirators should be worn to prevent respiratory contacts, particularly when dusts from these compounds are present in the working area.

Most aluminum salts in solution have an astringent effect on the skin. Should the hands be immersed in such solutions for any period a tanning reaction is apt to occur. In alum solutions, the hands receive a deposit of hydrated aluminum oxide which hardens and tans the skin leading to cracking.[61] Care must then be exercised to prevent secondary infections arising from the invasion of bacteria in the open fissures.

First aid for skin contacts depends upon the physical state of the salt—in dry form it should be wiped dry from the area with a cloth and then plenty of water flushed over the area to wash away any particles which may still adhere. For concentrated or dilute solutions, thorough water washing is sufficient to remove the material. If any reaction such as burning occurs after first aid, use soothing ointments to restore the oil to the skin. Should dermatitis develop, a physician should be consulted.



(Courtesy National Safety Council)

One of the many safe methods of transferring acids from carboys. Worker should be wearing longer gloves.

Dusts or solutions in the eyes should be removed by washing with copious amounts of water. If a burn has occurred, an eye specialist should be immediately consulted. Goggles should be worn in all atmospheres containing dusts or sprays from such compounds.

Ingestion of aluminum salts in concentrated form causes an astringent or puckering action in the mouth, stomach upset, vomiting, diarrhea, and intestinal catarrh.[8, 46, 65] Stomach washing by administration of plenty of water to prolong the vomiting reaction will relieve the body of much of the poison. Laxatives such as epsom salts in purgative doses, i.e., one tablespoonful to a glass of water, are recommended to rid the body of the poison after the stomach washing. A doctor should be consulted if untoward or prolonged symptoms persist.

Ammonium Hyroxide and Ammonium Salts

Ammonium hydroxide or ammonium salts are used in the baths for brass or bronze alloys, cadmium and also in anodizing operations.

Inhalation of ammonia fumes causes irritation of the nasal, throat and upper bronchial tracts. When a concentration as low as 0.15% is present in the atmosphere violent sneezing, lacrimation, reddening of the eyes, conjunctivitis, burning of the throat, and difficult breathing may result. The higher the concentrations, the more severe are the symptoms. When very high concentrations are inhaled, the initial shock may cause fainting and death as a consequence of the spasm of the larynx.

The gas is very destructive to mucus tissues; immediate symptoms on inhalation of medium concentrations are severe pains from mouth to stomach followed by vomiting. The lips and tongue may swell and speech be impaired; asphyxiation is steadily progressive. Even after treatment, the after effects may be hoarseness, or perhaps loss of speech which may persist for a considerable period. When ammonia gas contacts the nose and throat, boric acid should be snuffed into the nostrils and the mouth thoroughly rinsed. If possible, large amounts of ½ percent solution of citric acid or lemonade should be swallowed.[71, 2, 72]

A doctor should be consulted as soon as possible and medical treatment continued under his care. In all instances, a careful regime instituted by him should be rigidly followed. Fortunately this gas is easily detected, and its odor is familiar to almost everyone.

The following table shows the physiological effect of various concentrations.

TABLE I

PPM	*Effect*
50	Least detectable odor
698	Least amount causing eye irritation
408	Least amount causing throat irritation
1720	Least amount causing coughing
100	Maximum concentration allowable for an 8 hour day
300-500	Maximum concentration allowable for ½ hour
2500-4500	Dangerous for a short time
5000-10000	Rapidly fatal

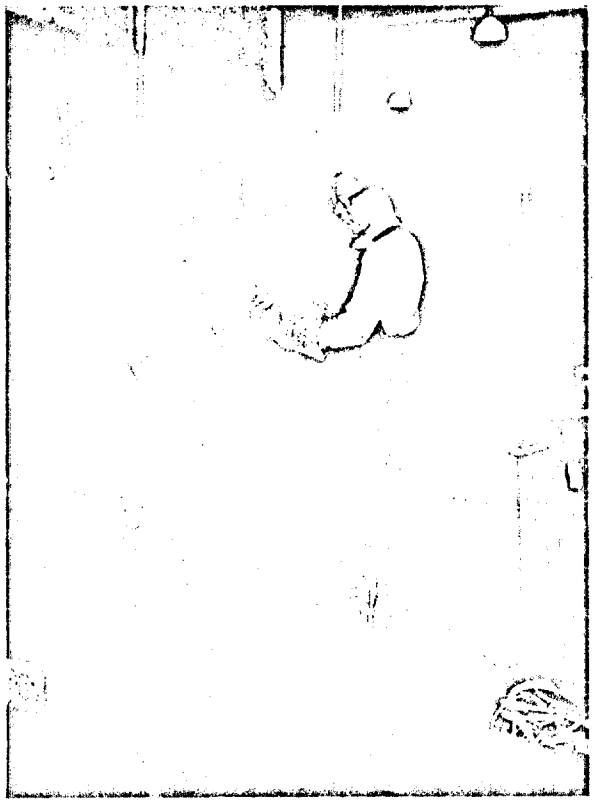
(PPM = Parts per million)

When ammonia gas strikes the eye tissues, severe irritation results as caustic ammonium hydroxide is formed on contact with the moisture of the eyes. When high concentrations strike the eyes, the cornea may be injured followed later by loss of vision due to the infiltration of ammonia into the corneal tissues. The first aid for eye injury or contact should be flushing the eyeballs and lids with plenty of water for five minutes, then an eye wash of 4% boric acid is recommended by some to be instilled into the eye area. Certain authorities also recommend placing 2 drops of liquid petrolatum on the eyeball to decrease the burning;[71, 20] however, such measures should be left to the discretion of the physician in charge. If difficulty results and it often may, a specialist should be consulted and a history of the exposure given in order that as much discomfort as possible be avoided.

Protective measures should be adhered to at all times. Respirators or chemically approved masks and goggles should be worn when a preponderance of ammonia fumes are present.

Ammonium hydroxide acts as a caustic on skin contact and the burning effect will depend upon the individual susceptibility. Dermatitis caused by constant contact with ammonia is characterized by an unbearable itching and redness. Because of its stifling and irritating odor, it is practically impossible to swallow large amounts. Rubber gloves should be worn when using strong solutions of ammonia. If there is danger of other types of bodily contact with the caustic, additional protection should be used. The dermatitis, if not too severe, may be allayed by the use of a lanolin ointment.

When the neutral ammonium salts such as the chloride or sulfate are contacted, even in the crystalline or highly concentrated form, little or no reaction occurs. Eye contact of course should be cared for immediately by thorough washing and if any free ammonia is liberated by contact with the eye fluids, it should be treated in the same manner as outlined previously. When swallowed in large quantities these salts may cause a severe stomach upset, but there are no chronic effects as the ammonium salts are freely excreted in the urine.[65] The treatment for such an upset consists of diluting with plenty of water and the administration of a laxative. The sulfide, nitrate, or cyanide depend upon the anion for their toxic effects.



(Courtesy National Safety Council)

Special gauntlet gloves, wooden soled safety shoes and special goggles protect operator at this hand acid dipping unit.

Antimony and Its Salts

Antimony pentoxide and hydrogen chloride are used in tin stripping operations.

The salts of antimony, the chloride and oxide may cause difficulty on inhalation, although with the exception of the chloride, no cases have been reported in the literature. Antimony trichloride has been shown to cause piercing of the nasal septum.[65] This action is probably caused by the release of hydrochloric acid and metallic antimony in the nasal mucous tissue and the corrosive action caused by both elements.

A further factor to consider in toxic reaction due to the inhalation of antimony salts is the fact that vapors or dusts may lodge in the naso-pharynx, and gradually pass to the digestive tract in the exudate which is usually drained into the throat. In this entrainment it passes to the stomach giving rise to severe digestive disturbances. The diagnostic symptoms of this upset are as follows: metallic taste in the mouth, nausea, vomiting, burning heat over the body, constriction or choking in the throat, sore mouth and throat with peeling of the mucous tissue, colicky pains in the stomach and violent purging. Cramps in the legs and arms appear with cold, clammy perspiration, congestion of the head and face, weak pulse, and short painful respiration as the poisoning progresses, or depending upon the amount absorbed by inhalation. A tetanic spasm sometimes occurs, and a pustular small pox type rash may also be present.[49]

First aid must be given *promptly*. If vomiting is

not present it should be induced by mustard water or zinc sulfate emetics. Plenty of water must be given even when vomiting is present to aid the stomach in ridding itself of the poison. The patient should be kept warm and after the stomach has been thoroughly washed large amounts of strong tea or coffee may be given.[49] If these are not available, whites of eggs or barley water may be given. A doctor should be summoned, but the first aid should be given until his arrival.

Because of the vomiting reaction, acute poisoning is rare, but smaller amounts such as those ingested from mucous entrainment may be the cause of chronic poisoning or the sub-acute type. The symptoms of this type of poisoning are weakness, general fatigue, some diarrhea, and the pustular skin eruptions.[6] Should these symptoms arise, a physician should be consulted and the history of possible contacts given. Respirators should be worn to prevent inhalation of dusts and vapors.

The chlorides of antimony on contact with the skin liberate hydrogen chloride and antimony ions. The corrosive action of hydrogen chloride produces irritation allowing for an easier absorption of free antimony which in turn produces eczematous skin eruptions resembling small-pox and leaving much the same type of scarring. Absorption of antimony into the system eventually causes generalized poisoning. Antimony metal, oxides and tartrates will also cause this type of dermatitis, but the reaction is not so acute as with the chloride.

As a first aid measure any skin contacts should be immediately cleaned with plenty of water, and if any skin breaks are present, great care should be used to make sure that no antimony compounds are absorbed by the body. Rubber gloves and aprons should be worn to prevent any contact of antimony salts either dry or in solution with the body.

Arsenic

Arsenic is used as component of the brass and bronze alloy plating bath.

(Courtesy National Safety Council)

Well ventilated plating tank. Worker's eye protection lacking.

Arsenic enters the body by inhalation, skin absorption, or ingestion.

The physiological reaction on inhalation of the inorganic arsenic compounds may consist of irritation of the nasal lining which may progress to perforation of the nasal septum. The reaction generally seems to be mildly and slowly corrosive showing lesions in the mouth lining as well as in the nasal and air passages. Eczematous eruptions may develop around the nose, mouth, and folds of skin from inhalation or contact.[53] For such symptoms, a physician should be consulted, protective mesaures however, should be the rule and the use of proper respirators mandatory.

Arsenic compounds are absorbed through the skin the trichloride being especially dangerous in this respect, as enough can be absorbed to cause death. According to certain authorities, arsenic injuries the capillaries causing congestion which interferes with normal blood circulation, later causing clotting with a complete starvation of the tissues;[55] however, others attribute its action to that of a general cell poison which disturbs oxygen exchange, albumen absorption and carbohydrate metabolism.[55] A darkening of the skin tissues is an early symptom of arsenic absorption together with inflammation. Horny growths or tissues occurring on the palms of the hands are the first reaction; later they develop into skin tumors. When a skin reaction appears a physician should be consulted at once to institute procedures to forestall more serious difficulties; as the reaction progresses loss of hair and fingernails may also take place. Extreme care should be exercised to prevent contact with arsenic compounds by the use of protective gloves and any other means necessary to prevent skin absorption.

When arsenic compounds contact the eye tissues, general redness and conjunctivitis result.[48] According to other reports, the cornea is not injured due to the fact that no capillaries are present in the tissue. However, any symptoms should be cared for immediately upon their appearance as considerable difficulty can then be averted. Goggles as well as respirators should be worn at all times when the arsenical compounds are present.

Acute arsenical poisoning produced by swallowing causes inflammation of the digestive and intestinal tract, which is characterized by sore throat, great thirst, vomiting and profuse and painful watery diarrhea. The injury to the capillaries produced by the arsenic irritation may produce bleeding in the stomach or intestine and lead to bloody vomiting and diarrhea. The kidney tissues also become involved, the symptoms being bloody, albuminous and scanty urine. The nervous symptoms include dizziness, headache and pain in the arms and legs. Acute poisoning is shown by inflammation of the stomach and intestines which may not occur until several hours to a day after ingestion. Sub-acute poisoning is shown by inflammation of the stomach and intestines which may not be as severe as in acute poisoning. Other symptoms are inflammation of other mucous membranes appearing as conjunctivitis, nasal catarrh,

sore mouth, considerable salivation and pharyngitis. Skin eruptions may also appear if sub-acute poisoning is of long duration. Arsenic accumulates in the body tissues; thus more acute symptoms may appear as the accumulation increases.[65]

First aid for acute poisoning consists of giving emetics, washing the stomach with warm salt water, followed by castor oil or equal parts of oil and lime water to allay intestinal irritation,[19] and summoning the doctor. In the meantime the patient should be kept warm. A specific arsenic antidote has also been recommended which consists of 40 cc. of ferric sulfate diluted with 125 cc. of water to which has been added 10 grams of magnesium oxide and the entire diluted to 750 cc. The patient is given 4 oz. at a time.[65]

For the sub-acute type of poisoning, a physician should be consulted and the history of any contacts given in order that whatever reactions appear may be treated as they arise.

Boron Compounds

Boric acid and sodium perborate are used in nickel baths.

The compounds of boron, borax, boric acid and the perborates have been extensively used by the general public for many years as antiseptic eye washes and mouth washes and for cleaning wounds; however, it has not been widely recognized that there may be toxic reactions arising from contact with either intact or broken skin tissues. Boric acid diffuses to a certain extent through whole skin, but it is absorbed very readily when the epidermis is broken.[7] Treatment consists of thorough washing to remove the compounds.

Inhalation of the dry compounds might give certain drying as well as delayed toxic effects, but no instances have been recorded in the literature. Mild solutions of boric acid have been recommended as nasal washes when ammonia fumes have been inhaled without record of ill effects.[2]

Ingestion of borax or boric acid is more serious; small quantities taken over a long period or accidentally swallowed in large amounts create disturbances of the appetite, digestion and general health. The initial toxic symptoms upon ingestion of large quantities consist of dry cough, nausea, severe vomiting, diarrhea and considerable stomach irritation. As poisoning develops, irregular pulse, raised temperature, considerable redness of the body and possibly a skin eruption may appear.[35, 58] Animal tests have shown that the development of diarrhea is the initial symptom on either injection or ingestion.[35] Treatment of ingestion consists of administering copious quantities of water and inducing vomiting if it is not present. The seriousness of the reaction depends to a large extent upon the ease with which the kidneys as well as the bowels excrete the boron compounds.[7]

Cadmium and Its Salts

Cadmium plating has been used commercially since 1919. The cyanide bath in plating is used almost

(Courtesy National Safety Council)

Complete guard and ventilating system on buffing wheels.

exclusively; however in winning cadmium from zinc ores an acid sulfate bath is used.

Industrially cadmium poisoning results from inhalation and not ingestion;[66] however, the use of the plated articles used in food storage or preparation has been the cause of a number of poisonings from ingestion which are worthy of mention.

The toxic reaction following the inhalation of cadmium containing vapors shows the following symptoms—a distressing cough, followed later by headache, dizziness and eventually a constricted feeling in the chest.[38] Accompanying the early symptoms are often throat dryness, and sometimes nausea, diarrhea, colic and vomiting. Chest pains which are quite severe follow the early symptoms and accompanying the pain there is also shortness of breath and difficulty in breathing. Later reactions are diffuse and patchy broncho-pneumonia which spreads and an edema which obliterates the alveolar spaces in the lungs making it impossible for the blood to receive the necessary oxygen. Generally the inhalation symptoms of cadmium poisoning are delayed for about four to eight hours. The broncho-pneumonia symptoms do not appear until twenty to thirty-six hours have elapsed and this generalized pneumonia is fatal in about fifteen per cent of the industrial cases. Death occurs between the fifth and seventh day after exposure. Recovery occurs within seven to eleven days after exposure and the chest is free from symptoms.[66] Because of the increasing use of cadmium and its salts in industry, the American Standards Association has adopted a permissible limit for working atmospheres of 1 mg. per 10 cubic meters of air.[1]

According to other reports, cadmium electroplaters also have ulcerations of the cartilage of the nasal septum as well as inflammation of the nose and pharynx which is thought to be caused by the absorption of small amounts of cadmium in the body.[50] These signs appear more frequently in the winter months than in summer and are believed to result from the lack of adequate ventilation. Respirators should be worn by all persons contacting cadmium in any form.

When inhalation symptoms occur a physician should be summoned immediately and the person removed from the contaminated atmosphere. No first aid measures are adequate and all treatment should

be directed by the doctor who has been thoroughly acquainted with the history of the exposure.

Localized skin contacts cause irritation and inflammation.[57] Rubber gloves should be worn, and constant cleanliness mandatory.

Cadmium plated articles used in food preparation or as containers for fruit juices have caused a number of outbreaks of cadmium poisoning from ingestion.[65] These symptoms appear within fifteen minutes to one hour after ingestion of the contaminated food and are ushered in by severe colic, vomiting associated with acute abdominal pains. Diarrhea commences within about fifteen minutes after the onset of vomiting, and in some cases collapse has been noted. During collapse, paleness and profuse sweating are also present. All patients exhibit some degree of shock with subnormal temperatures and thready pulse. The vomiting and diarrhea are continued at intervals and diminish after two to three hours following the ingestion. The vomiting is copious at the beginning consisting mainly of bile and mucous. The diarrhea is watery in type and the stools also contain mucous shreds. Headache and dry throat accompany the other reactions. These symptoms abate in most patients after six to eight hours, but in certain cases the diarrhea may be continued for twenty-four hours.[37]

A survey of apartment houses in one area experiencing a rather widespread outbreak of cadmium poisoning showed that about twenty-five per cent of the refrigerator trays from one manufacturer were cadmium plated. Further inquiry also revealed that refrigerator shelves and broiler grids had also been cadmium plated.[63]

First aid treatment consists of giving large quantities of water to insure thorough washing of the stomach until the arrival of the physician, who will then treat the case symptomatically.

As further precaution for workers in cadmium plating, *no food should be consumed or stored near the plating baths, and the hands should be thoroughly clean before any food is handled or eaten to insure that no cadmium is ingested by this means.* Acid type foods such as fruit or fruit juices are easily contaminated by very small amounts of cadmium, hence they should never be allowed to touch any article containing cadmium.

Chromium and Compounds Used in Plating

Hexavalent chromium (CrO_3) necessarily used to secure the best results in electroplating operations exhibits toxic properties on inhalation, contact, or ingestion.

Inhalation of sprays, or dusts of chromic oxide, chromic acid, or alkaline chromates or dichromates cause nasal irritation, inflammation of the nasal mucous tissues, nose bleed, and with continued exposures, perforating ulcers of the nasal septum.[12, 65, 46, 3] The throat and mouth are also affected on inhalation and the following symptoms appear progressively—many small ulcers appear on the soft palate and posterior surface of the tongue and floor of the mouth. Drying and cracking of the back of the tongue with enlargement and reddening of the buds with drying and crusting in the tonsillar region are also present after massive exposures or those of long duration.[3, 42] The earliest symptoms of chromium toxicity are sneezing, excessive secretion of tears and a profuse and watery nasal discharge. Other symptoms which may follow are labored breathing, choking sensations, dizziness, weakness and headaches. According to recommendations published in 1943 and adopted by a number of states, chromic acid or hexavalent compounds of chromium should not exceed 0.1 mg. per cubic meter of air in any working area.[12, 54, 55]

(*To be continued next month*)

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(*Continued on page* 67)

advice on a certain item and he spent a full morning on the phone calling just about everyone in a local office of the Department and also the district office. Finally he contacted an old-timer and got his information in two seconds.

Many of the present tax laws lie not in the internal revenue code but in the decisions of tax cases which have come up in the past. This we call "precedent" and truly becomes a most important part of the tax set-up. This is chiefly important for items on which the internal revenue code is not very explicit. We refer to such unexplained items as business expense, ordinary income and the like. Many cases have come up where a taxpayer claims an item to be a business expense which the collector of internal revenue may not allow without a fight. Although in some cases the law tries to be very explicit, still these items come up, not once a year, but every day in the year; so often that the "Tax Court" set up for this very purpose is one of the busiest in the land.

If the internal revenue officer should wish to make an audit, be as courteous to him as you can. Try your best to get the auditor or accountant of your firm to assist the gentlemen as much as possible. As a general rule he will have all his proper credentials with him, as not a case is known where someone was accused of impersonating an internal revenue man. Have the books in good order and do not hesitate to give the man access to them and the copies of past returns, together with copies of supporting invoices, etc.

Conclusion

As a general rule, if you have run your business on a conservative basis, there should be enough cash in the bank at the end of the year to pay the proper income taxes that are payable. Unless considerable funds have been diverted to equipment, inventories, etc., any situation in which a proper tax cannot be paid should be investigated. Some error may be found in the accounting and another audit may be advisable. Besides being accurate, the books should make sense. If the firm has had a great loss or a great gain in the past year, then it should show in lower or higher values in assets or dividends or drawings. And believe it or not and contrary to common opinion, income tax returns really do make sense, especially to those who understand them.

In three cases out of every four in the past year it has been more profitable to make separate returns for husband and wife, especially where there is a high income and where the wife is an income producer.

Although most executives in the metal finishing industry will be wise enough to engage counsel in tax matters, one should not hesitate to cross bats with the counsel at any time it is felt something is wrong with the tax return. Although a person may be an expert on tax matters, it is entirely possible that he may not be entirely familiar with your particular set-up. No matter how settled tax laws and tax case precedents are, there is always possibility of new situations arising.

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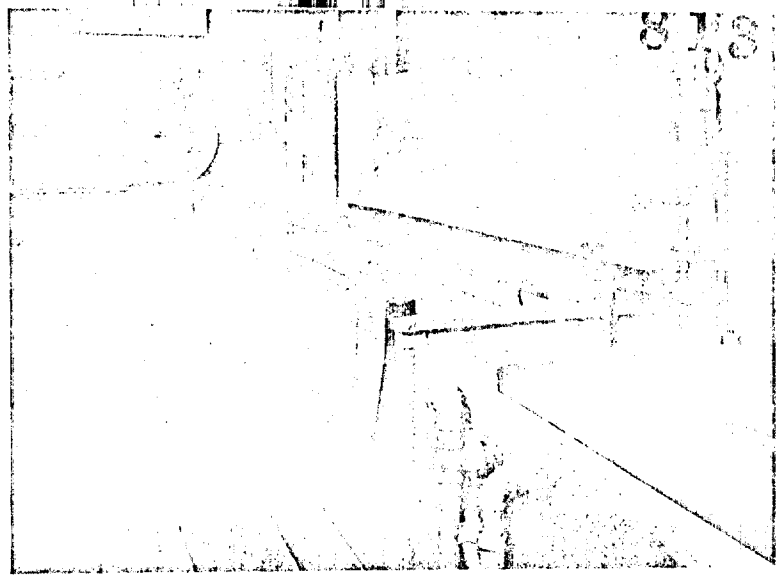


(Courtesy National Safety Council)

Slot type ventilation for plating tanks.

Toxicity of Chemicals in Electroplating

Continued

By P. M. Van Arsdell, *Benson & Associates, Chicago 4, Ill.*

A NUMBER of symptoms have been observed when even low concentration of chromic acid (5%) have been used in anodizing solutions. These consist of ulceration of the nasal septum, particularly in those persons having spurs or deflections of this structure, and skin rashes. No one escapes some involvement. The ulceration and perforation of the septum proceeds with very little disturbance and usually the case may be far advanced before aid is sought. Bleeding, scabbing, dryness, and nasal irritation are the only symptoms which may be delayed for some time in their appearance. The hydrogen evolved in this type of bath is responsible for carrying the chromic acid into the atmosphere. Adequate exhaust ventilation, 134 feet per minute per square foot of tank area, is the best means of eliminating this hazard.[27, 79] However, as a temporary means of guarding against possible nasal injury, a heavy ointment consisting of 75 per cent petrolatum and 25 per cent lanolin may be used if applied to the nostrils several times daily when only temporary contacts of the acid containing atmospheres are necessary. When the contact is prolonged, respirators of an approved type for chromium containing atmospheres should be used.

When the hands and other exposed portions come in contact with the plating solutions, ulcerations known as "chrome holes" slowly develop which are deep and painful. The skin may be entirely removed, particularly at the knuckles and at the base of the nails. The tissues show scars when the scab is sloughed. In addition to the dermatitis, certain individuals have been known to develop allergic reactions to the chromates; these consist of asthma, fever, nephritis, and the like.[38]

Chromic acid has proved especially dangerous on skin contact with concentrated solutions. It has been reported that workers receiving splashes in the face have died as a result of the exposure.[23] Chrome ulcers should be treated immediately as they may spread and continue for long periods; they are likely to become the focal points of secondary infection, and every skin break should be carefully treated to prevent the entry of chromium. With care these heal completely, but persistent scars remain.[65] For proper cleansing after contact with any chromium compounds, a wash of five per cent sodium thiosulfate, "hypo." solution in water should be used. As a protective measure, rubber gloves, aprons and boots should be worn. Protective cream on the hands before putting on the gloves will aid in preventing dermatitis reactions in the event a glove is punctured during operations.

Ingestion of chromates may occur accidentally—a splash entering the mouth. The symptoms of such type of contact are sore throat, disagreeable taste, vomiting, pain, diarrhea, collapse and unconsciousness with dilated pupils; slow respiration and muscular cramps. Less than 0.5 grams (dry) of potassium dichromate may cause serious poisoning; while the

lethal dose is below eight grams. Six grams of chromic acid may be fatal. Depending upon the amount swallowed, the reactions may appear as follows: kidney damage with blood and albumen appearing in the urine, nervous symptoms such as headache, stiffness at the back of the neck and muscular cramps. In serious poisoning through this route, hemorrhages in stomach, intestines and respiratory tract are also present.[65]

First aid treatment consists of prompt evacuation of the stomach by such quick acting emetics as mustard water, or ipecac, or zinc sulfate (15 to 30 grains in a glass of water) and washing the stomach thoroughly. The individual should be kept warm and a doctor promptly summoned.[49]

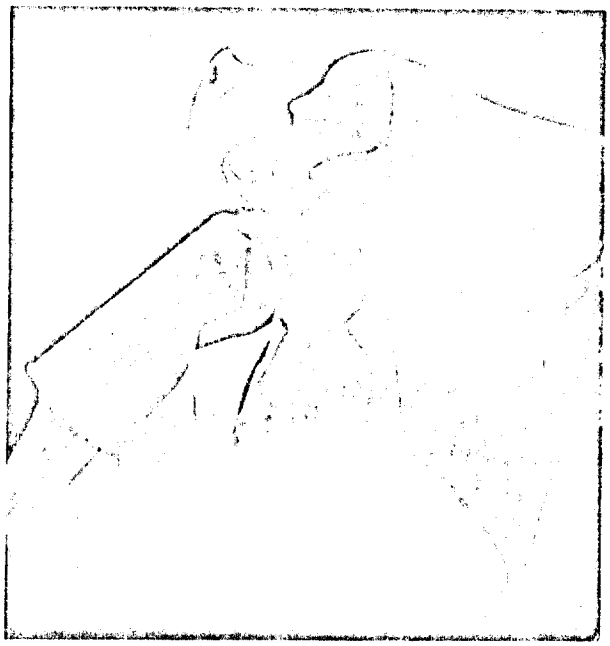
As a further precaution in handling chromium vapors, the exhaust ventilating systems should periodically be thoroughly cleaned with water to remove the dry chromium compounds which collect. These are oxidizing agents and are apt to cause serious fires when lint or organic vapors contact them. The entire plant can be seriously endangered from a fire in the exhaust systems.

Cobalt Compounds

Chemically the salts of cobalt resemble those of iron and nickel, but are not nearly so toxic.

Salts of cobalt may cause an allergic dermatitis which may be ascribed to a chemical reaction with some specific complex molecule in the skin cells.[61]

On inhalation, cobaltous acetate salts produce nausea, vomiting, colic and fever. The stomach may show sensitiveness on pressure and the blood and urine show some slight symptoms of damage. However, removal from the atmosphere is usually sufficient to bring about recovery.[28] Respirators should be worn when dusts or fumes of cobaltous or cobaltic salts are present.



(Courtesy National Safety Council)
What not to do—heading for an eye injury.

Ingestion of cobalt compounds in concentrated form causes nausea and vomiting.[28] Muscular jerking which is uncontrollable may also be present.[41] According to some opinions, the toxic action on ingestion would seem to be more on the central nervous system than on the motor nerve endings. It is believed that nickel and cobalt paralyze to some extent the fore-brain. The action is more nearly referrable to that of barium and platinum than to iron which is distinctly different.[61]

Treatment consists of washing the stomach thoroughly and administering laxatives. A physician should be consulted if symptoms persist.

In comparison with nickel, cobalt is much less toxic in its effect. Fatal doses in animal experiments have shown that nearly three times more cobalt than nickel are necessary to produce like results.[15]

Copper and Copper Salts

Copper sulfate and copper cyanide are the two most extensively used salts in electroplating. However, occasionally the chloride, nitrate, carbonate, or acetate may be encountered. With the exception of the cyanide salt which is a violent poison, these salts act only as astringents and may be somewhat caustic on contact with the mucous linings of the nose and throat. Absorption of copper by the body is low and the effects are generally not cumulative.

Skin contacts with copper sprays with the exception of the cyanide react in the folowing manner—eczema-like eruptions appear in susceptible individuals[21] and copper itch[64] has been reported but its incidence is questioned by most observers. Should severe dermatitis develop, a doctor should be consulted. The best means of prevention of skin reactions are constant attention to cleanliness and the use of protective gloves and aprons.

Upon ingestion copper salts produce a metallic taste, constriction in the throat and gullet, griping pains in the abdomen, nausea, vomiting, purging with much straining, partial suppression of the urine and jaundiced skin. The breathing may be rapid and difficult; the pulse small and quick. When poisoning is severe there may be some kidney damage, great weakness, intense thirst, cold perspiration and marked coldness of the arms and legs, headache, giddiness, coma and death. Authorities differ on the questions of cumulative toxicity of copper on ingestion, however should chronic copper poisoning develop, a green line may appear on the gums, and there may be muscular weakness and cramps.[19]

Copper sulfate is usually a prompt and effective emetic; its action is preceded by a short nausea.[25] It is extremely irritating to the digestive tract and should the emetic action fail to appear, immediate vomiting should be induced. The stomach should be washed to insure the removal of the compound.

First aid procedure on ingestion of copper salts consists of inducing vomiting as quickly as possible if it is not already present by the use of mustard water or ipecac,[19] keeping the patient warm and calling the doctor.

When purifying copper electrolytically contamin-

ants such as selenium gold or silver are often encountered. Of the three, selenium is the more dangerous as it combines with the hydrogen forming hydrogen selenide. This compound is somewhat analogous chemicaly to hydrogen sulfide in its toxic action, although from reports, it does not seem to be so rapidly fatal. This compound has the odor of garlic. In animal experiments, it has been noted that there is olfactory fatigue when the concentration is of the order of 0.001 mg. in the atmosphere.

For workers, when the concentration is 0.005 mg. per liter of hydrogen selenide or over, the eye and nose irritation is too severe for working. When accidental inhalations occur, findings have shown that this compound is sufficiently damaging to cause pneumonia which may persist in an acute form.[19] Inhalation sometimes causes pain in the abdomen, vomiting and pain in the lumbar region, but not commonly.[20]

For the more chronic symptoms, it has been found that there is early liver damage marked by fatty deposits which later subside. Spleen changes are also shown which progress to a moderate degree.[19] Persons in contact with the selenium compounds are often bothered with garlicky breath. This is caused by the formation of methyl selenide within the body from any selenium which may be present.[20]

Gold Compounds

Gold plating is usually done with the cyanide or the chloride bath.[75] Either of these salts of gold are poisonous, although the cyanide is much more toxic than the chloride. Because of the economies involved when vapors or sprays escape to the atmosphere and the difficulty of reclaiming from a diffuse area, great care is exercised to prevent any carry out of the solution either by vapors or by objects from the bath.

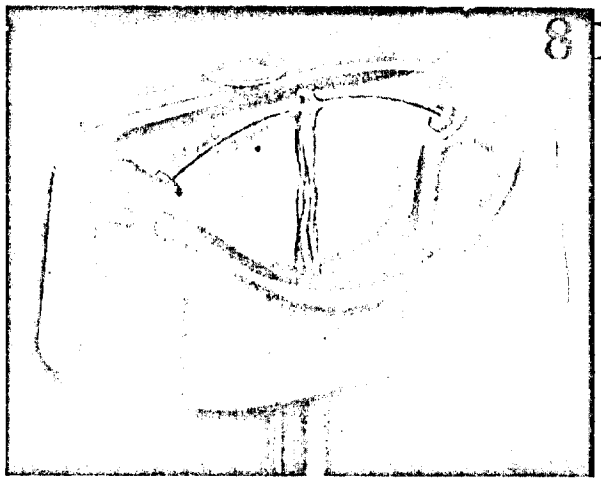
So far as toxic reactions of workers are concerned, little appears in the literature to substantiate or disprove any industrial poisoning which might be claimed. The soluble salts of gold are actively poisonous. Their toxic effects like those of the mercury salts appear to be due to their local irritating action on the alimentary canal and on the kidneys.[77]

The chemical findings when gold salts have been used in the blood stream of rheumatic individuals would lead to the expectation that if gold salts entered the blood stream of workers through chapped hands or open sores, the toxic reactions would reflect in kidney disturbances and others of varied nature. Generally gold salts are poorly absorbed in the digestive tract. Only when they directly enter the blood stream are toxic symptoms shown and because of the nature of these it has been concluded that gold salts act as vascular poisons only.[39, 74]

Iron and the Ferric Salts

Iron plating is limited in its application at present, but electroforming and electrowinning have recently met with renewed interest. The sulfate and chloride salts are generally used when iron plates are deposited.[9]

Physiologically the soluble salts of iron precipitate



(Courtesy Benson & Associates, Inc.)
Eye washing fountain for chemical eye contacts.

proteins and their reaction on contact with living tissue may be astringent, irritant and corrosive. Accidental inhalation of vapors from iron plating baths may have a drying and highly irritating effect on the mucous linings of the nose and throat although no actual cases have been reported.

When ferric chloride enters the alimentary canal, irritation is produced almost immediately with vomiting and diarrhea being the initial symptoms of toxicity. The stomach inflammation may persist. Corrosion of the teeth is also a sign of ferric ion reaction. Ingestion should be treated by the administration of sodium bicarbonate as a stomach wash and the vomiting reaction induced if not present.[65] Persistent inflammation or symptoms should be treated by a physician.

Lead and Lead Salts

Commercially, lead plating is conducted by the use of fluosilicate and fluoborate baths although it has been deposited from a varied number of solutions among which are acid solutions of nitrate, acetate, perchlorate, oxalate, dithionate, sulfamate and alkaline solutions of plumbites and cyanides.[26]

Physiologically, inorganic lead and its compounds are absorbable from all mucous membranes and exposed tissues. These compounds enter the body mainly by inhalation and ingestions and the effects are cumulative. Absorption by the intact skin may also be a mode of entry on prolonged contact as the compounds may enter through the hair follicles or through chapped or scratched skin.

Conjunctivitis may also develop if lead solutions, vapors, or spray contact the eye tissues. All workers handling lead in any form should use rubber gloves, respirators and goggles to prevent contacts.

Inhalation of lead containing vapors is the most common route of poisoning. Lead poisoning may be either sub-acute or acute depending upon the mode of entry, but the following symptoms are fairly common to both inhalation and ingestion. There is a sweet, then a metallic taste in the mouth and dry throat, followed by intensive burning and abdominal

pain within a half hour. Vomiting is usually followed by intensive diarrhea and later symptoms show an involvement of the kidneys as the urine becomes scant and albuminous. Foul breath, coated tongue, headache, pain and cramps in the legs appear in a few hours, and in extreme cases, collapse with coma may also occur. Death has occurred within thirty-six hours when extremely large amounts have been absorbed.

When symptoms of poisoning occur, a physician should be summoned immediately; in the meantime the following first aid should be instituted: Epsom salts or sodium sulfate using 15 grams to 1 pint of water should be given to wash out the stomach with vomiting kept up to insure a complete washing of the stomach. After this has been as thoroughly done as possible the same dosage of the sulfates should be given and allowed to remain in the stomach to aid in the formation of the relatively insoluble lead sulfate which can then be eliminated by the cathartic action of these salts.

When lead containing vapors are inhaled there may be some action on the brain marked by convulsions and followed by stupor combined with other symptoms. The above treatment is also used upon ingestion, as both inhalation and ingestion may occur simultaneously, due to some of the lead compounds, often as much as 70 per cent, being lodged in the naso-pharynx where they are either absorbed or swallowed. Lead poisoning seems to develop by inhalation more quickly than by any other route.[52]

Sub-acute or chronic poisoning occurs when small amounts of lead are absorbed over a longer period and because the symptoms are quite diversified, may be mistaken for some other illness. The symptoms most universally present are weakness and continued fatigue, increasing loss of appetite with subsequent loss of weight, constipation, colic, muscular weakness affecting those muscles most commonly used, tremors of the eye, tongue and mouth and the occurrence of lead colic after the onset of all other symptoms. Extensive psychic changes also occur.[46] A physician should be consulted on the appearance or persistence of any of these symptoms in order that further injury may be averted. A full history of the exposure should also be given him.

Whenever minute quantities of lead are known to be present in the working area, routine urinalyses and blood counts should be made at frequent intervals.

Sub-acute symptoms usually disappear upon removal from the atmospheres containing the lead. There is a predisposition to lead poisoning however, which remains for a considerable period. Certain illnesses tend to cause recurrence of the lead poisoning symptoms as the lead is mobilized in the blood stream by the action of certain toxins or viruses from the disease producing organisms.[52]

Mercuric Salts

Mercuric compounds are absorbed by inhalation, skin contact and ingestion. Some of the plating operations utilize mercuric salts as a prefinishing compound

(Courtesy National Safety Council)

Photograph shows the complete ventilating and exhaust system on a chromium plating tank.

before final plating is done and there is considerable chance of poisoning if great care is not exercised.

Metallic mercury vaporizes even at ordinary room temperatures and whenever it is present there is danger from inhalation. Should large quantities of mercury vapor be inhaled, acute symptoms are experienced which are as follows—foul breath, metallic taste and an increased flow of saliva. Later symptoms include soreness with ulceration in the mouth, blackening of the gums and loosening of the teeth; the tongue may also swell and show ulcers. Still later a toxic kidney reaction occurs which is marked by scanty albuminous urine. When the poisoning is unusually severe, the teeth may be lost and a gangrenous condition of the jaw develop. Sub-acute or chronic mercury poisoning develops slowly and may result from constant exposure to the metal or its compounds, that is, the chloride or the nitrate. The symptoms are a progressive nervousness marked by irritability and muscular tremors and a tendency toward inflammation of the mouth and gums. As an early symptom of chronic mercury poisoning, it has been shown that the handwriting may be affected first. Signs of mercury poisoning are indicated by the omission of a letter either at the beginning or end of a signature, or the substitution of a letter similar in sound. These defects revealed in the handwriting are related to disturbances of the central nervous system and are caused by chronic mercury poisoning either existent or latent.[32]

For acute poisoning by inhalation, the individual should be removed from the contaminated atmospheres as soon as possible (the rescuers should wear gas masks to prevent their own injury) and a physician summoned as quickly as possible. As a pre-

liminary treatment, vomiting should be induced if not present by the administration of a warm mustard water emetic. Plenty of water for dilution and to stimulate kidney elimination and plenty of laxatives to promote bowel elimination are also recommended. Promptness is the keynote in treatment of acute poisoning as the mercury is quickly absorbed and fixed by the mucous membranes. According to one authority, the fate of the individual is practically decided within ten to fifteen minutes after absorption of mercury. Such customary antidotes as raw eggs, milk without cream, calcium sulfide and sodium thiosulfate are considered ineffective after the initial absorption period.[51]

Prevention of mercury absorption should be the rule. This may be accomplished by good ventilation throughout and all operations involving the use of mercury carried out under a hood.[51]

Mercury and its compounds are readily soluble in the body fluids, especially perspiration and also in the skin itself. Sufficient quantities may be absorbed in this way to produce sub-acute or chronic poisoning described under inhalation. When it is necessary that the hands contact either the metal or its compounds, gloves should be worn, but careful washing is necessary even on one contact. The metal salts are corrosive and irritating to the unbroken skin even in dilutions of 1 part in either 2000 or 4000 of water.[51]

Ingestion of mercuric chloride produces acute symptoms almost immediately characterized by a burning sensation in the throat, an astringent metallic taste in the mouth, a greatly increased flow of saliva, great thirst and abdominal pains. Vomiting occurs within a short time with white or bloody mucous shreds appearing in the vomitus. The systemic effects are coagulation, skin irritation and the corrosion of mucous membranes. When the poisoning is especially severe, great weakness, a weak rapid heart, fall in blood pressure, cramps in the legs, dehydration (due to excessive vomiting), stupor and collapse may also occur. As the poisoning progresses the kidneys and large intestines also show corrosion and destruction. Ingested mercury compounds should be evacuated from the digestive tract with all possible speed to prevent widespread tissue destruction.[46]
The emetic and other measures described under inhalation should be immediately used and the doctor summoned as soon as possible.

Clinically there are two different types of mercury poisoning: that of inhalation of volatile mercury compounds causing chronic nervous symptoms, and mercury intoxication with injury to the excretory organs as noted in the case of mercuric compounds and marked by salivation and kidney injury. Volatile organic mercury compounds are dispersed in the blood for a time while the salts undergo a combination with the blood and proteins of the organism.[22]

Nickel and Nickel Salts

Metallic nickel though widely used as a protective covering for many purposes is not considered a toxic element. However some persons are susceptible to toxic reactions upon inhalation of nickel dusts. Throat irritation, usually slight, is the initially appearing symptom and the reaction may not progress further. Other symptoms such as weakness, fever, headache, nausea, muscle and joint pains may appear several hours after the first noticeable throat irritation. Recovery usuallly occurs within twelve to twenty-four hours.[65]

The nickel salts widely used in plating such as the chloride, nitrate and sulfate cause more widespread reaction among workers probably because of their greater solubility and more active state. When these salts or solutions are contacted by the skin for considerable periods, the familiar "nickel itch" or eczema occurs.[18] The irritation is first noticed on the fingers and forearms and considerable redness is the initial sign. Further contact causes some ulcerations with the sores usually appearing on the fingers but they may be present wherever damp clothing rubs the body. This condition is more prevalent in summer than in winter as sweating increases the susceptibility. Careful cleansing of all areas in contact with the solution is the best treatment. As a rule the first attack predisposes to a second, and heavy drinking is also sometimes a predisposing factor in the occurrence of the dermatitis.[64, 76]

Should ingestion of the nickel salts accidentally take place the primary symptom is vomiting. Plenty of water should be administered to aid the stomach to rid itself of the irritant.[65] Epsom salts in purgative doses help to clear the lower digestive tract of any accumulation of the irritant and should be given. If symptoms persist after the first aid treatment a physician should be consulted.

Eye treatment in the event of contact with splashes or sprays consists of copious irrigation with water and consulting a doctor.

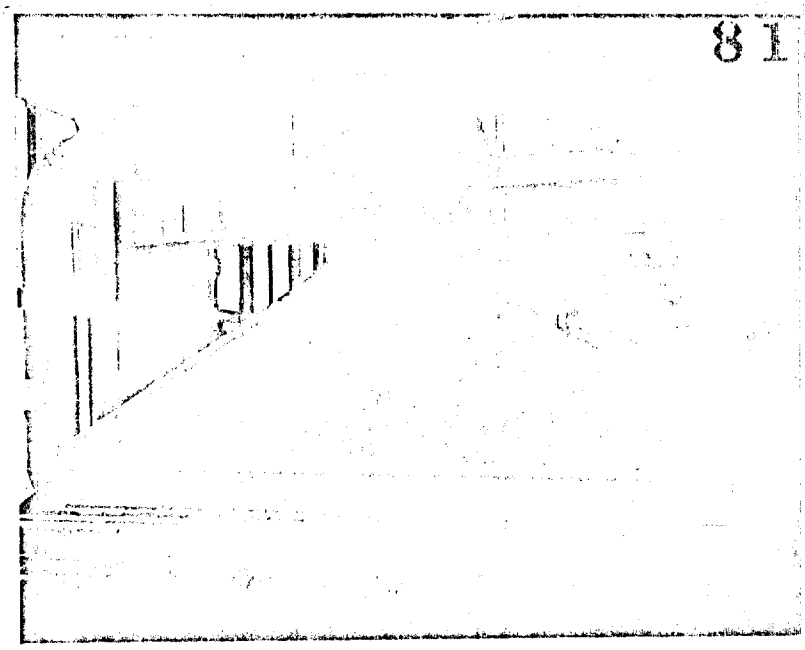
Rhodium, Platinum and Palladium

Rhodium in spite of its rarity and fairly high cost has found a considerable application in the production of costume jewelry. Rhodium chloride is the salt most commonly used in plating operations. The metal is non-poisonous and the solution of the salt is only locally irritating.[78] In animal experiments even higher concentrations on injection have produced no systemic poisoning. Because of the local irritation, however, dermatitis may result when concentrated solutions come in contact with the skin.[79]

Metallic platinum is non-toxic, and never gives rise to occupational injury. The oxide which is rarely encountered in plating operations causes eczema of the hands and forearms and some lesions of the nails.[14] Dust and spray from the complex salts of platinum ordinarily used in plating have been found to cause asthma after continued exposure.[33] The initial symptoms of the reaction begin with repeated sneezing followed by a profuse running of the nose with a watery mucous discharge. Later reactions which may develop are tightness of the chest, shortness of the breath, with wheezing and blue coloration of the face.

(References appeared in the August issue.)

(To be continued)



(Courtesy National Safety Council)
Plating tank exhaust hood to remove toxic fumes.

Toxicity of Chemicals in Electroplating

Conclusion

By P. M. Van Arsdell, *Benson & Associates, Chicago 4, Ill.*

WHEN the operator leaves the work, the symptoms clear with the exception of persistent bouts of coughing in the night which may endure for about one-half hour. When the work is resumed the symptoms recur. Certain typical skin reactions may also develop in some individuals; this is characterized by a scaly red rash. Blood checks, skin tests, and x-rays do not reveal any abnormalities leading to the lesions noted. Precautions lie in minimizing the exposure by adequate ventilation. Treatment consists in removal from exposure and consultation with a physician with full history of the exposure.

Palladium salts commonly used in plating are the chloride and the amino-nitrite. So far as is known the palladium salts do not constitute any threat of injury in industry, but laboratory tests show that when these substances are introduced in animals damage occurs to the heart, kidneys, liver and bone marrow.[47] From these indications it would seem that palladium salts should be carefully watched for chronic and cumulative toxic effects.

Silver and Its Salts

Silver plating is one of the oldest operations in electroplating and the compounds used are extremely toxic. Silver nitrate and silver cyanides have an extremely corrosive and discoloring effect on all tissues they contact. The nitrate salt is astringent and extremely corrosive in high concentrations when it contacts either skin or mucous. The reaction is at first marked by white areas which upon exposure to light turn light then dark gray and finally black by the precepitation of metallic silver.[46, 65]

The cyanide salt in warm solutions releases cyanide to the atmosphere making it almost instantly fatal upon inhalation. All baths containing cyanide solutions should be plainly marked for precautionary measures and good ventilation should be maintained at all times whenever these compounds are present.

In buffing operations following the silver plating procedure, care should be exercised to prevent the presence of any dust in the air from the silver objects as the silver dust is mobile in the blood stream and is deposited in the skin and the eyes of the workers giving rise to a peculiar bluish gray cast of the skin and eyeballs. This is known as argyrosis and is next to impossible to correct once the silver is deposited in the skin and eyeball area. The initial symptoms of silver deposition are a peculiar yellowish gray cast in the whites of the eyes and later the cornea may be colored the same way. The entire area of exposed skin darkens with exposure to light.[30, 68]

Tin and Tin Salts

The wide application of tin plating for the storage of foods has proved that metallic tin is non-poisonous to the greater proportion of the population of the world.[41] However, the tin salts used in tin plating

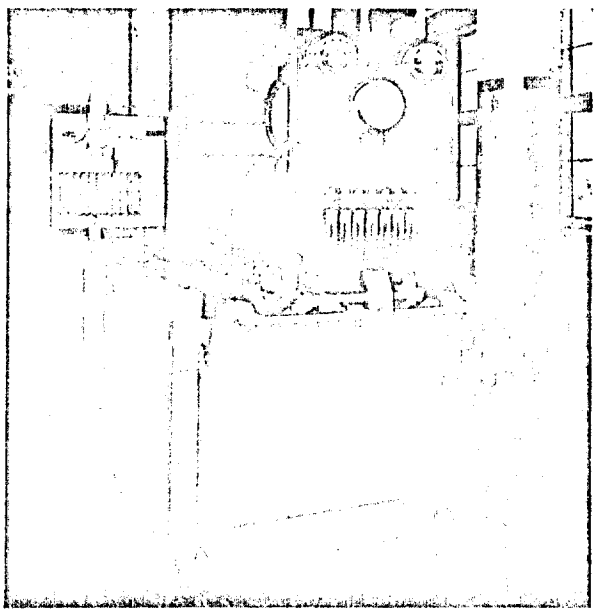
may be the source of some skin reactions due to the release of acid or base ions from the salt, depending upon the type of bath used.[5, 56] These ions act as irritants especially on broken skin, but the tin ion itself is non-toxic.

Zinc and Zinc Salts

Although zinc may be deposited from the salts of many acids, only the cyanide and sulfate baths are of industrial importance. The chloride zinc bath has found a limited application while zinc sulfate is the salt ordinarily used to furnish the metal ions.[43]

Inhalation of zinc compounds may give rise to some respiratory difficulties, however the main danger from zinc in electroplating arises from the effects of the cyanides and caustics and dermatitis is often seen when either the sulfate or the cyanide are contacted frequently. The skin reaction is characterized by reddish blisters which may begin at the hair follicles and develop into boils, ulcers, or eczema. The first stage of this reaction is an unbearable itching. The chloride and cyanide form pit-like holes, with little or no swelling.[45, 64] Treatment consists of washing skin areas with sodium carbonate to precipitate the zinc as the relatively innocuous carbonate when the acid bath is contacted, and when the cyanide bath is contacted hydrogen peroxide and boric acid are more specific treatment. Once the dermatitis subsides, it usually does not recur and only in highly susceptible individuals are lasting effects noted. Rubber gloves and impervious clothing should be worn in such atmospheres.

Conjunctivitis may occur[24] and should be immediately treated as the irritation folows the general order of those symptoms described for the dermatitis. When either the sulfate or the chloride reach the eye, immediate irrigation with water is mandatory; to prevent such entries goggles should be worn. The effect of the caustic or cyanide splash in the eyes is extremely painful. The eyes should be copiously flushed with water and later boric acid may be used.



(Courtesy National Safety Council)
Plating tank exhaust hoods; new improved construction.

Ingestion of zinc chloride causes corrosion of the lips and mucous tissues of the mouth. There is pain and burning of the stomach with incessant vomiting of blood stained fluid. Other symptoms include difficulty in swallowing, accelerated pulse and difficult respiration. In more severe cases of poisoning, convulsions, paralysis, coma and even death occur. First aid treatment consists of administering plenty of warm water containing sodium bicarbonate to wash the stomach. If the bicarbonate is not available, milk and eggs may be given freely with plenty of water, or strong tea may also be given.[49]

Zinc sulfate is usually a prompt and effective emetic, for this reason its use is recommended in many first aid procedures.[45, 65] However, on inhalation or contact, its effect is much the same as for zinc chloride although it is less pronounced.[24]

Anions and Electroplating

Three fairly definite, though not sharply defined, types of aqueous solution are used in electroplating, commonly designated as (a) "acid", (b) "neutral", and (c) "alkaline" baths. These types may be roughly defined in terms of pH respectively; (a) lower than 2; (b) between 2 and 8 and (c) higher than 8. Examples of these three types are (a) acid copper baths, (b) nickel (including low pH nickel); and (c) cyanide baths of various metals.[11]

The most commonly used anions and cyanides, sulfates, and chlorides; these are added either as the acids or as the salts, such as the cyanides. A general resume of the toxicity of these compounds is as follows:

Acids

Concentrated acids are corrosive to the skin causing heat and itching at first contact and within a fairly short time, considerable burning and later blistering. When acid fumes are inhaled, laryngitis, bronchitis and considerable destruction of lung tissue may follow if the contact is of any duration. Chemical pneumonia is a natural reaction to the tissue destruction. Immediate removal to fresh air is imperative.[16, 65]

The first treatment for acid contacts is to use plenty of water to secure the greatest dilution within the shortest possible period.[16, 65] Rapid dilution with water is especially important when acid is splashed in the eyes.

Ingestion of acid causes corrosion of the upper digestive tract with intense pain, vomiting, swelling of the tissues, collapse and even death within a short period. Alkaline carbonates or magnesia may be safely administered for ingestion. The cardinal rule for all serious contacts or ingestions after administering first aid is to summon a doctor immediately.[16, 65]

Generally, in the electroplating operation, acids are used in dilute solutions, although from time to time the concentrated acids must be added to maintain proper concentration in the bath. Because of the nature of the electroplating baths, the hydrogen generated will carry out certain concentrations of vapor to the atmosphere. The acid spray naturally contaminates the atmosphere, and depending upon the

nature of the acid, constitutes a more or less hazardous condition.

Sulfuric acid in the dilutions used in plating is not particularly dangerous, however care must be used when handling the concentrated acid as it constitutes not only a serious health hazard but a fire hazard as well. Splashes in contact with organic material char and burn; this is particularly true when wood or packing materials of a cellulosic type are splashed and no precautions are taken to prevent combustion taking place. Plenty of water should be used to dilute the acid and prevent the liability of fire.[69]

Hydrochloric acid fumes are particularly unpleasant, causing irritation of the skin and mucous membranes. Their reactions follow the pattern outlined above for acids generally.

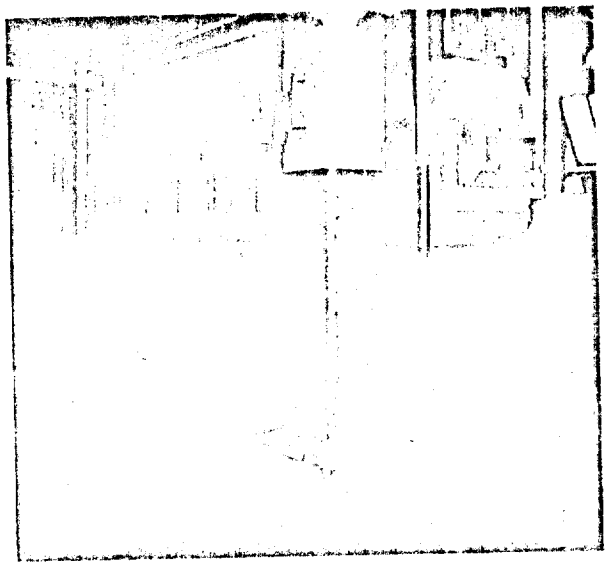
Nitric acid on contact with the skin is extremely irritating, producing the familiar yellow-brown coloration wherever it contacts. Vapors and fumes from this acid may be particularly dangerous as they carry the nitrogen oxides which upon inhalation are responsible for delayed reactions of a particularly insidious nature. Whenever a workman has inhaled any nitric acid vapors or nitrogen oxide fumes, he should be removed to fresh air, and if any untoward symptoms develop he should be hospitalized until the danger period of 5 to 24 hours is past.

Perchloric acid and the perchlorates are hazardous not only from the toxic action on the body, but from an explosion and fire standpoint as well. Organic compounds in the presence of perchloric acid explode violently. When any type of phosphorus containing materials contact perchlorate salts there is a danger of fire and/or explosion.[18] On contact with the skin, perchloric acid causes deep and penetrating sores. Swallowing perchlorates in high concentration cause stomach upset, vomiting and abdominal pain; bluish coloration of the skin, collapse and terminal convulsions result from the change in the blood. Kidney involvement is shown by lumbar pain, albumin and casts in the urine and sometimes cessation of the kidney function.[46,65] First aid consists of washing the stomach with copious amounts of water and administration of hot drinks. A physician should be called as soon as possible in order to treat the symptoms as they arise.[65]

Alkalis

The alkalis, sodium and potassium hydroxides and carbonates are widely used in plating operations. These have a corrosive and destructive effect on the body tissues which may be more damaging than the action of acids. This is particularly true of the dilute solutions. These compounds withdraw water from the tissues and form soaps with the skin and underlying fats which accounts for the prolonged "soapy" feeling experienced after contact with the alkalis has been made.

Caustic burns, as a rule, penetrate deeper than acid burns. This is especially noted in caustic eye burns. When either caustic dust or solution contacts the eyes, it must be removed by washing with plenty of water for at least five minutes, for the cornea is often



(Courtesy National Safety Council)

Enclosure system to confine acid to prevent contamination of air in workroom.

destroyed in this time if immediate washing does not carry the material away.

When swallowed, caustics may remove the entire mucous lining of the digestive tract and mouth. The following treatment should be given promptly—large quantities of water to which vinegar or lemon juice has been added for neutralizing the alkali present. This may be followed by administering vegetable oils, animal fats or milk. A doctor should be summoned to treat the other symptoms as they arise.

A mild ointment with a heavy oily base will help to restore the oils to the skin that have been lost by contact with the alkali compounds.[36, 65, 72]

Cyanides

Cyanide salts are extensively used throughout the plating industry. They are among the most poisonous compounds known today. Copper, zinc, and sodium cyanide are used in the brass and bronze bath; sodium cyanide in the cadmium bath; copper cyanide in the copper bath; gold, potassium, sodium, copper, nickel, and silver cyanide in the gold bath; silver, sodium, and potassium cyanide in the silver bath, and zinc and sodium cyanide in the zinc bath. Most plating shops have a number of cyanide baths continuously in operation.

Cyanide ions are a rapidly acting poison; they enter the body by inhalation, ingestion and skin contact. The salts which free the cyanide ion in the body are extremely toxic.[65] Hydrogen cyanide is given off from a bath containing any of the cyanide in solution.

On inhalation, hydrogen cyanide hinders the normal oxidation processes and is asphyxial in effect. Very low concentrations of cyanide vapor have the following chronic effect: watering and itching of the eyes, headache, pounding of the temples, difficulty in breathing, excessive saliva, stupor and pain in the digestive tract, with possible convulsions and collapse. These symptoms appear in the following order, irritation of the conjunctiva and throat, coated tongue

TABLE II

TOXIC REACTIONS AND FIRST AID PROCEDURES

	TOXIC ACTION			FIRST AID		
	Swallowing	Skin Contact	Eye Contact	Swallowing	Skin Contact	Eye Contact
ACIDS						
Acetic Hydrochloric Nitric Sulfuric Phosphoric	A few cc. of concentrated acid causes intense pain in mouth, with blistering of the tongue, lips, and throat; laryngitis, lung inflammation, vomiting, destruction of digestive tract, feeble rapid pulse, general weakness and collapse.	Extremely corrosive giving acid burns. H_2SO_4 may give white or brown coloration of tissues.	Acid burn.	Administer chalk, lime or soda bicarbonate solutions at once. Follow with milk, eggs or vegetable oils. Call a doctor.	Flush with plenty of water, apply soda bicarbonate paste.	ALL EYE CONTACTS MUST BE FLUSHED PROMPTLY WITH PLENTY OF WATER FOR AT LEAST FIVE MINUTES
Hydrofluoric Fluoboric Fluosilicic	Highly corrosive even in low concentrations. One lungful produces almost immediate death (few minutes to 10 hrs.). Acute irritation of digestive tract, abdominal pain, diarrhea, and vomiting.	Blistering and gray white to purple appearance. Burn may be delayed in action and penetrate very deeply.	Acid burn with blindness from concentrated acid.	Hospitalize at once. Keep inhalation patient prone do not allow any movement.	Wash and use magnesium oxide paste immediately; if action continues, see doctor.	
Oxalic	Readily absorbed, caustic, with difficult swallowing, followed by burning in mouth, stomach, and throat. Rapid collapse preceded by convulsions.	Corrosive, loss of fingernails due to its action.	Acid burn.	Give calcium in any form immediately. Induce vomiting. Call doctor.	Wash thoroughly and often when working with oxalic acid.	
ALKALIS			**ALKALIS**			
Ammonium Hydroxide	Soapy nauseous taste, burning pain in throat, lungs and stomach. Is not as destructive to tissue as NaOH or KOH.	Roughens skin, produces caustic burns in concentrated solution.	Corrosive, causing burning and rapid penetration of deep tissues.	Lemon juice, weak solution of vinegar, oils, animal fats or milk.	Wash with vinegar solution.	
Sodium Hydroxide and/or Potassium Hydroxide	In concentrated solution the entire lining of the mouth and digestive tract is destroyed. Water is withdrawn from surrounding cells which causes deep penetration.	Caustic burns which penetrate deeply because the protective layer is dissolved by alkali action.	Deep penetrating burns, resulting in blindness from action on eye tissue.	Dilute with large quantities of water to which vinegar or lemon juice has been added. Follow with a tumbler of salad oil.	Wash with plenty of water. Neutralize with vinegar.	
Calcium Hydroxide	In dry form or in high concentrations, caustic burns.	Irritating, causing eczema like eruptions with blisters. Skin peels in large flakes.	Small amounts of dust may cause blindness—treat as a caustic eye burn.	Give plenty of water; treat as for other caustic burn.	Cleanse thoroughly and apply boric acid or benzoic acid ointment.	
Barium Hydroxide	Weakness, salivation, and nausea. Vomiting and diarrhea; severe abdominal pains.	Eczema and cyanosis, irritating to sweat glands, may bleach hair and eyebrows.	Acts as caustic.	Administer sodium or magnesium sulfate to precipitate barium.	Wash thoroughly to prevent accidental introduction into mouth.	
COMPOUND						
Chloride Salts of						
Ammonium Cobalt Iron	May be astringent in low concentrations; corrosive and burning in high concentrations HCl may be released in digestive tract. Vomiting and stomach inflammation and diarrhea follow.	Solutions are astringent and drying. Dry salts are hygroscopic and may produce burns.	Irritating; concentrated solutions burn and corrode eyes.	Give plenty of water to wash stomach. Starch or flour water and egg whites halt reaction. Epsom salts flush system, give as a laxative after plenty of vomiting.	Flush with plenty of water; if burn is felt wipe dry and then flush to remove all traces of corrosive action.	
Antimony Nickel Tin	Corrosion and burning in digestive tract; violently emetic; produce metallic taste, some kidney and liver damage when salts remain in system.	Itching and burning, followed by development of severe dermatitis.	Highly irritating.	Give plenty of water to wash stomach. Call a doctor at once to treat symptoms as they occur.	Wash thoroughly; apply lanolin ointment. See doctor if irritation is prolonged.	
Cadmium	Immediately and violently emetic, accompanied by dizziness and shallow breathing and colic with diarrhea—kidney disturbances.	Irritation and inflammation. Ulceration occurs in nasal septum on inhalation.	Irritating.	Give plenty of water to insure dilution, call doctor.	Wash thoroughly and often—do not place hands in mouth or near it.	
Mercuric	Irritating and corrosive to entire digestive tract. Burning sensation in throat, astringent metallic taste, salivation, great thirst, abdominal distress and pain. Vomiting occurs in about 5 minutes, diarrhea in 2 hrs. Death occurs in a few hours with ordinary low concentrations.	1-5% solutions produce irritation, drying, and corrosion. Absorption from intact skin may be sufficient to cause general poisoning.	Irritating.	Remove poison from stomach by administering solution containing sodium bicarbonate; administer milk minus cream mixed with raw egg—this treatment must be given promptly. Call doctor.	Remove promptly with plenty of water. If mercury is absorbed, consult a doctor.	

ACIDS	TOXIC ACTION Swallowing	Skin Contact	Eye Contact	FIRST AID Swallowing	Skin Contact	Eye Contact
CYANIDE SALTS OF ALL METALS USED IN PLATING	If HCN is [illegible] tion, heart palpitation, difficult breathing, watery eyes, headache, giddiness and collapse. Respiratory center is paralyzed.	Skin absorbs cyanide; clothing [illegible] with cyanide solutions should be immediately removed.	Irritating—conjunctivitis may be present.	Administer hydrogen peroxide or potassium permanganate in water. The latter solution should be Burgundy red. Induce vomiting by mustard water emetic. Call doctor.	Wash skin thoroly as soon as contact occurs—watch closely for symptoms listed under swallowing.	ALL EYE CONTACTS MUST BE FLUSHED PROMPTLY WITH PLENTY OF WATER FOR AT LEAST FIVE MINUTES
NITRATE Salts						
Potassium Sodium	Severe abdominal pain followed by muscular weakness, vomiting, frequently bloody stools, irregular pulse; straining to evacuate both kidneys and bowels, convulsions and collapse. Stomach irritation may be so violent as to lead to early and fatal end within 20 minutes to 5 hrs.	Astringent and drying in high concentrations or in crystalline form. May produce dermatitis if contact is prolonged.	Irritating.	Immediately give plenty of water to insure dilution and washing of stomach. Give emetic if vomiting is not present. Starch and flour water, milk and egg whites will absorb some of nitrate. Call doctor.	Wash thoroly—consult a doctor if dermatitis occurs.	
Mercuric	Irritating and corrosive to entire digestive tract. Burning sensation in throat, great thirst, metallic taste, also all symptoms of nitrate poisoning shown above.	Fairly dilute solutions are irritating, drying and corrosive. Mercury is absorbed by intact skin and produces systemic effects shown for swallowing.	Highly irritating.	Give plenty of water immediately with sodium bicarbonate. Give milk minus cream with raw eggs. Call doctor.	Remove immediately, wash with plenty of water. See a doctor if untoward symptoms occur.	
Silver	Astringent, irritating and very caustic. Produces all other symptoms of nitrate poisoning shown under nitrate symptoms above.	Burns skin and mucous tissues turning them black after exposure to light. Silver compounds under skin may cause gray to blue black skin colorations.	Produces burns on eye tissue.	Give 1 teaspoonful of table salt to 1 pt. of water to induce vomiting. Continue treatment until washings fail to show a precipitate.	Wash with salt water and treat as for burns.	
Sulfate Salts of Aluminum Ammonium Cobalt Magnesium (Epsom Salts) Nickel Potassium Sodium (Glauber's Salts)	Diarrhea, vomiting and stomach upset when taken in large quantities. Epsom and Glauber's salts are common cathartics but other cations are displaced by sodium giving cathartic effect.	Astringent and may be irritating causing dermatitis, especially Co and Ni.	Irritating.	Give plenty of water, as sulfates tend to withdraw water from body tissues. See a doctor if other symptoms develop.	Wash with plenty of water.	
Copper Zinc	Promptly emetic in action.	Irritating in high concentrations or on prolonged contact.	Irritating.	Give plenty of water to insure dilution.	Wash thoroly, and if dermatitis appears a doctor should be consulted.	
Cadmium	Immediately and violently emetic accompanied by dizziness, shallow breathing and colic with diarrhea. Kidney disturbances.	Drying and irritating—ulceration of nasal septum.	Irritating.	Give plenty of water and call a doctor.	See above.	
CHROMIC ACID and DICHROMATES	Sore throat, vomiting, diarrhea, albumen and blood in urine. Corrosion of entire digestive tract. Hemorrhages occur in stomach and intestinal tract.	Continued exposure shows ulcers known as "chrome holes." Nasal septum may be pierced. Acid (conc) contacting face has been known to cause death.	Irritating—may produce conjunctivitis.	Give quick acting emetic such as mustard water, or ipecac. Wash stomach thoroly. Keep patient warm; call a doctor.	Use a wash of 5% sodium thiosulfate for all contacts. See a doctor if skin lesions appear. Use petrolatum in nasal passages for brief exposure.	
Sulfides	Irritating and corrosive due to liberation of hydrogen sulfide and free metallic ions.	High concentrations loosen hair and soften skin. On inhalation H_2S is as toxic as cyanide.	Irritating and corrosive.	Give plenty of water—follow with 2% citric acid to neutralize alkali. Call a doctor.	Wash with plenty of water. See a doctor if other symptoms develop.	
OTHER SALTS						
Ferricyanides Ferrocyanides Triocyanates—except from metal poisoning from metallic cation	These are not so dangerous as cyanide salts, as free HCN is not released in the body. Some irritation may result, and gastric upset follow swallowing.	No effect other than drying.	Irritating in high concentrations.	Give plenty of water; follow with a laxative dose of Epsom salts.	Wash thoroly. Use lanolin cream salve to alleviate drying.	
ARSENIC	Sore throat, great thirst vomiting and diarrhea with severe abdominal pain followed by collapse and death.	May be absorbed through skin or by inhaling. Symptoms same as for swallowing, small concentrations produce inflammation and later dark pigmentation with eczema.	Highly irritating to mucous linings of eye area.	Call a doctor promptly. Induce vomiting. Wash stomach with salt water, follow by castor oil or equal parts of oil and lime water. Then wash stomach with ferric hydroxide solution made with 40cc of ferric sulfate diluted with 125 cc. of water to which is added 10 gm of MgO, dilute entire to 750 cc. Give 4 oz. at a time.	When skin reaction appears see a doctor immediately.	

813

and possibly an enlarged and sensitive liver.[10] High concentrations of cyanide vapor paralyze the respiratory center in the brain. While the inhalations have generally proved fatal, there have been a number of reports showing that intravenous injections of sodium nitrate or sodium thiosulfate that have affected complete recoveries.[34] Care should be maintained at all times to provide adequate ventilation as masks are ineffective after a short time. The vapors readily penetrate the clothing.

Cyanide solutions are irritant to the skin, and if the skin is broken there may be danger of absorption. On the unbroken skin, it is thought that the alkali carbonates are formed which may produce a caustic burn. To avoid either absorption of the cyanide or an alkali burn, prompt removal is necessary; if any corrosion has occurred, boric acid ointment or wet dressings should be used. The boric acid acts as a buffering agent and the same effect is produced by using either citric acid or citrates in the same way.[5] Rubber gloves and aprons should be worn to prevent skin contact.

Ingestion of moderate quantities is usually rapidly fatal, although the sodium nitrate or the thiosulfate injections have been used with favorable results.[34] First aid treatment consists of giving large quantities of ferrous sulfate in water, or 1% solution of potassium permanganate as a stomach wash, or hydrogen peroxide. Artificial respiration should be given.[40] Inhalation of ammonium nitrate, which acts as a respiratory stimulant and vasodilator, and exercise to keep the person awake, has also proved helpful as an antidote.

Sulfides

Sulfides and sulfates are frequently used in electroplating operations. Sodium, ammonium and potassium sulfide are the most commonly encountered. These sulfides in contact with hydrogen form hydrogen sulfide which is characterized by the "rotten egg" odor. This gas is extremely poisonous when inhaled in quantity. In certain respects it is even more toxic than the cyanides. Hydrogen sulfide is locally irritating, due in part to its acidity and also because sodium sulfide is formed when in contact with moist tissues. Small concentrations in air produce eye irritation and lung irritation.[21] In small amounts the odor is very noticeable, but in high concentrations, no pronounced odor is noticed by the individual because of paralysis of the respiratory centre. This effect is one of the factors contributing to its high toxicity.[21, 36] The symptoms of acute hydrogen sulfide poisoning are nausea, giddiness, headache and coma occurring in rapid order. Repeated exposures to dilute concentrations of this gas produce a chronic eye irritation, bronchitis, digestive disturbances, continual tiredness and skin eruptions, among numerous other manifestitations. First aid measures for the overcome person consist of artificial respiration and immediate hospitalization. Eye contacts should be treated first with plenty of water and then a drop of olive oil to alleviate the pain.

The alkali sulfides such as sodium and potassium soften the outer layer of the epidermis and remove hair in much the same manner as alkali hydroxides. When accidentally swallowed, the action is irritating and corosive as both hydrogen sulfide and free alkali are freed in the system.[65] Treatment consists of rapidly evacuating the system; inducing vomiting if not present, and securing medical aid.

Toxic doses of sulfides produce the same systemic effects as those shown for hydrogen sulfide.

Sulfates

The sulfate ions act as cathartics, particularly when combined with sodium, potassium, or magnesium.[5] Through their cathartic effect they have a tendency to dry the intestinal tissues and slightly increase gaseous metabolism. These compounds also withdraw water from the body tissues, accounting for the considerable thirst when their laxative use is recommended.

Oxalic Acid

Oxalic acid is used in anodizing. It is highly acid producing local irritation and corrosion, violent stimulation and later paralysis which may be caused by tissues being deprived of their calcium through precipitation.[46] Oxalic acid on the skin is corrosive and loss of the fingernails due to its action is often encountered. The material should be immediately washed from the area; rubber gloves and aprons should be used as protection.

When accidentally swallowed, oxalic acid is readily absorbed. The immediate effects are caustic, with difficult swallowing followed by burning in the mouth, stomach and throat. This is followed by rapid collapse which may be preceded by convulsions. Kidney pains are also present and are a specific symptom of oxalic acid poisoning.

Death by oxalic acid usually occurs much more quickly than with other caustic substances.[65]

The first aid treatment for oxalate poisoning consists of the administration of calcium in any form, i.e., calcium gluconate, lactate, plaster, chalk, lime water and any other readily available calcium source. Quantities of water should be given to prevent the deposition of crystals in the kidneys.[46] A physician should be promptly summoned.

Hydrogen Fluoride; the Fluosilicates and Fluoborates

Hydrogen fluoride is used in the chromic acid plating bath and in pickling solutions. Fluosilicic and fluoboric acid are used in the lead and other baths.

The toxic element in each of these compounds is the fluorine. Fluorine poisoning may be acute or chronic. Inhalation of one lung full of hydrogen fluoride may be fatal immediately; recommendations by investigators show that permissible concentrations should be below 3 parts per million, if fluorine reactions are to be avoided.[62]

Acute symptoms occur with inhalation and ingestion of fluorine compounds. Symptoms of non-fatal

fluorine poisoning consist of acute stomach upset with sudden nausea and vomiting; burning in the abdomen and diarrhea. Pulmonary edema is an outstanding characteristic inhalation symptom.[4] The physiological changes caused by fluorine poisoning are principally blood changes, damage to the lungs, liver and kidneys, and storage of fluorine in the bones. The lungs and liver show the most severe and consistent damage from fluorine absorption.[62]

When sufficient fluorine is absorbed to be fatal, death may occur in a few minutes or be delayed for ten hours or longer. Alternating spasms and paralysis are present prior to death with general weakness and sluggishness between spasms. There is a fall in blood pressure, general collapse and sometimes the skin shows a grayish-blue coloration. Breathing is difficult and may be arrested. The heart action may also be arrested.

When chronic fluorine poisoning is encountered changes in the teeth are most easily recognized, as fluorine is detrimental to dental growth.[62] The bone structure is also changed as a result of chronic fluorine poisoning, as fluorine is stored in the bones and teeth. Movement of the spinal column is restricted and parallels the bone changes, which consists of overgrowth of bone and calcification of the ligaments. Bony fragility is also seen in some of the chronic cases. The bony overgrowth tends to disappear when exposure is stopped.[4]

Loss of appetite, nausea, vomiting and heartburn are common symptoms of chronic poisoning. Rheumatic attacks, shortness of breath and circulatory changes are also experienced.[62]

Skin contacts with fluorine compounds are painful and may lead to deeply penetrating ulcers. The primary difficulty lies in the fact that the fluorine may be on the skin for a considerable period before any of the indications of burning are felt. When this occurs the penetration of the fluorine into the tissue is already deep and the resulting sores continue to eat into the tissue long after the fluorine is supposedly removed from the area, fluorine ion activity being responsible for this reaction.[9] First aid consists of washing the area thoroughly and applying magnesium oxide paste to absorb the fluorine ions. If further difficulty is encountered a physician should be consulted. The eye membranes also react to hydrogen fluoride and fluosilicic acid, showing slow ulceration of the conjunctivae: the initial reaction being one of excessive lacrimation. Chronic eye reactions consist of abnormal sensitivity to light and continuous conjunctival secretion. The eyes must be copiously bathed with water and an ophthalmologist consulted immediately.[16]

Personal protection consists of wearing air supplied respirators, rubber gloves, shoes, coat, or overalls of impervious neoprene coated material. Respirators or goggles should be of material which does not react to fluorine as ordinary glass is etched by the fluorine containing compounds.[62]

Neutral Solutions

The neutral solutions in themselves are not harmful from either a corrosion or fire standpoint, but depend upon the nature of either the metal or the anion present to bring about skin or other type reactions. For instance, the nickel baths derive their toxic reaction from the nickel salts present and not from the carrier.

Conclusion

Table II was compiled as a summary of the foregoing material. The references were not included as it was felt that in a First Aid Chart they were unnecessary and would hinder the quick and efficient use of the material included.

While it has been the purpose of this paper to show the toxic nature of the compounds that are used in the plating industry and the possible effects that may result from inhalation, contact and ingestion, it is advisable that a modicum of common sense be applied in regard to such information. Even after having resorted to the best first aid practices, the patient should promptly avail himself of expert medical advice and attention.

When the hazards generally are known, it makes it less difficult to protect against them at their source. Knowledge of the signs of such contacts helps the operator to help himself before too great damage to his health and life can result. It aids management to provide proper equipment, safeguards and education for the workers. This is the primary aim in placing such information in the hands of many.

Papers are now in the process of preparation for early publication which give a summary of the toxic hazards encountered when degreasing agents, solvents and addition agents are handled.

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Some Effects of Metal Cations on Blood Coagulation*

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Metal salts exert a characteristic effect on proteins. In fact, adding a sufficient amount of metal salt causes precipitation of proteins. The precipitates contain the metal ions, and many people believe that definite chemical compounds are formed.

The aim of this work was to investigate the effect of some metal cations on the blood coagulation. It was supposed that metal, being protein precipitants, should bind to the blood clotting factors, causing an alteration of their physical and chemical properties. It should result in a coagulation defect.

Materials and Methods

1. The *Hellige thromboelastograph*, mod. C was employed and the tracings were recorded on a light sensitive film [Hartert (1)]. Among the various components of the thromboelastogram, *r* and *ma* values were considered.

2. *Preparation of salt solution.* The salts used were: $CdSO_4$, $ZnSO_4$, $NiCl_2$, $Pb(NO_3)_2$, $Al(NO_3)_2$, $CoCl_2$, $FeSO_4$, $SrCl_2$, $MnSO_4$, $BaCl_2$, $LiSO_4$, $HgCl_2$. A 0.10 M solution in 0.145 M NaCl of each salt was made up and the concentration was determined by titration. For use in the test these stock solutions were then suitably diluted as desired. Only the ferrous sulphate solution was prepared from time to time since it oxidises very rapidly in air and $Fe(OH)_2$ precipitates.

3. *Collection and preparation of plasma substrate.* Normal human blood was collected from laboratory staff and selected hospital patients by the two-syringes technique. No attention was paid to the different blood groups. 9.8 ml of blood were drawn into chilled siliconized syringes containing 0.2 ml of 19 per cent trisodium citrate. The plasma was obtained by centrifuging the blood sample at 700 g for 5 minutes in a refrigerated angle-head centrifuge. The plasma was then decanted, centrifuged a second time under the same conditions and immediately used or kept in icewater until using. In some of the experimental procedures normal rabbit plasma was used in order to obtain plasma samples very rich in platelets. Blood from rabbits was obtained from the heart by clean puncture.

The *actual* concentration of plasma separated from a sample of citrated blood and the *final* plasma concentration in a clotting mixture were determined as described by Tocantins (6).

4. *Counting of platelets* was carried out on the plasma by direct method in a Bürker-Türk chamber (0.050 mm deep) under contrast phase microscope.

* This study was aided by a grant from the Italian National Council for Scientific Research.

5. *Buffer solution of Imidazole* (pH range 6.2—7.8). The following stock solutions were prepared: a) 0.1 N HCl (pH 1.08 ± 0.1); b) 0.2 M Imidazole solution (Fluka A.G. Chemische Fabrik Buchs/SG). In order to obtain solutions of the following pH, the corresponding amounts stated below were mixed and diluted with H_2O to a volume of 100 ml.

pH	6.2	6.4	6.6	6.8	7	7.2	7.4	7.6	7.8
ml of stock HCl solution	42.9	39.8	35.5	30.4	24.3	18.6	13.6	9.3	6.0
ml of stock Imidazole solution	25	25	25	25	25	25	25	25	25

After mixture, the pH of each solution should be checked with a pH meter.

6. *Plasma fractionation.* Plasma fractionation was carried out on 4.0 ml volumes of citrated platelet-poor plasma. Globulin and albumin fractions were obtained by salting out with $(NH_4)_2SO_4$, according Perez (5).

7. *Paper electrophoresis.* A micro-electrophoretic technique, using cellulose acetate filter strips (Oxo Ltd., London) was used, as recommended by Kohn (3). An equipment for paper electrophoresis built by the Shandon Scientific Company, Ldt. London was employed. Barbitone buffer, pH 8.6, ionic strength 0.1:

Sodium barbitone	12.76 g
Diethyl barbituric acid	1.66 g
Distilled water to	1 l

The cellulose acetate membrane filter strips were soaked in the buffer for ½—1 hour before use. For filling the tank, the buffer was diluted 4 : 1 with distilled water. The strips were gently blotted and secured on the bridge by means of strip holders lined with filter paper. The plasma was applied by means of a fine capillary pipette at a distance of 4 cm from the cathode end. A constant direct current at 150 volts was applied for 1½ hours. The strips were dried at 56° C for 15—30 minutes and then stained with 0.5 per cent Ponceau S in 3 per cent trichloracetic acid. The strips were washed in 2 per cent acetic acid till the background was clear and then dried at 56° C for 5 minutes.

8. *Starch block zone electrophoresis.* Kunkel's method (4) was adopted with several modifications. The starch (British Drugs Houses, Ltd. London) block (40 × 8.5 × 1 cm) was conveniently prepared by pouring a barely liquid mixture of washed starch and barbitone buffer solution (diluted 4 : 1 with distilled water) into a plexiglas mould. Several layers of thick blotting paper were placed on the top of the block and pressure was exerted with a plexiglass plate to remove further buffer and to provide a flat compressed surface. The citrated platelet-poor plasma to be analyzed was then applied to 1 cm from the edges of the block. The slit was then impressed with a narrow spatula. After resting the plexiglass mould on the electrolyte vessels connected to the electrodes, contact was made with thick filter paper wads covered with cellophane. The electrophoresis was performed at 6° C, after first permitting equilibration for approximately 14 hours without a rise in current as a result of heating during the procedure.

After completion of the run the starch block was readily segmented by slicing the dry appearing block with a thin metal spatula at measured intervals. To obtain aliquots of each segment, saline was added to each segment in a test tube; these were stirred and the starch was allowed to settle. The liquid above the starch contains a proportional amount of the total material in each segment.

9. *Concentration of serum fractions.* The protein eluates from the starch block segments were concentrated by dialysing them in a 15 per cent solution polyvinylpyrrolidone in iso-osmotic phosphate buffer (pH 7.4). The dialysis was carried out for 24—48 hours in a refrigerator at 4° C.

10. *Protein determination.* Since the separation was carried out in barbital buffer, the buffer nitrogen was removed by precipitation of the proteins with Zn (OH)$_2$ and washing the preci-

pitate. A direct estimation of the protein nitrogen of each fraction was thus obtained, using the micro-Kjeldahl technic (2).

Results

A clotting mixture consisting of

platelet-rich plasma (platelets 220 000 per cmm)	0.30 ml
tested metal salt (varying concentrations)	0.18 ml
imidazole buffer (varying pH)	0.18 ml
total volume	0.66 ml

was prepared in a silicone test tube at room temperature. In this mixture the final concentration of plasma and the platelets number resulted 40 per cent and 100 000 per cubic millimeter respectively. The pH of imidazole buffer was adjusted so to achieve a final pH of the clotting mixture over the range 7.2—7.8, which seemed to supply the optimal conditions for coagulation.

0.33 ml of the above mixture were placed in the t.e. graphic cuvet and recalcified with 0.03 ml of 0.125 M $CaCl_2$ (final volume 0.36 ml). The apparatus was started immediately. As a control, 0.85 per cent saline instead of metal salt was used.

No significant differences were noted by using native plasma or adsorbed resin plasma; therefore citrated plasma was used in all experiments. Clotting mixtures incubated for 30 minutes before being tested gave the same results as the non-incubated ones.

1. *Effect of metal salts on blood coagulation.* The results of the experiments are presented in table 1. It is seen that addition of sufficient metal salt to

Table 1: Effect of some metal salts on reaction time and maximal amplitude of t.e. graphic tracing. The cation amount is referred to the 0.36 ml volume reached in the cuvet. For every combination a parallel experiment with saline was carried out; the numbers within brackets correspond to the results so obtained. The mean value of 3 determinations are given

Tested salts	Metal cations γ ions		r values mm	ma values mm	Final pH of mixture
$CdSO_4 \cdot 8H_2O$	Cd^{++}	0.064	60 (12)	1 (47)	7.6
$NiCl_2 \cdot 6H_2O$	Ni^{++}	0.124	75 (11.5)	37 (60)	7.6
$Pb(NO_3)_2$	Pb^{++}	0.217	55 (13.5)	32 (59)	7.6
$ZnSO_4 \cdot 7H_2O$	Zn^{++}	0.254	110 (7.5)	4 (47)	7.8
$FeSO_4 \cdot 7H_2O$	Fe^{++}	0.290	21.5 (9)	43 (60)	7.8
$CoCl_2 \cdot 6H_2O$	Co^{++}	0.339	58.5 (9.5)	10 (56)	7.8
$MnSO_4 \cdot 4H_2O$	Mn^{++}	1.133	32 (8)	47 (60)	7.8
$HgCl_2$	Hg^{++}	0.360	26 (12)	1 (47)	7.6
$Al(NO_3)_3$	Al^{+++}	0.213	16.5 (7.5)	46.5 (47)	7.2
$BaCl_2 \cdot 2H_2O$	Ba^{++}	4.452	16 (11)	46 (51)	7.8
$SrCl_2$	Sr^{++}	0.486	58 (7.5)	40 (47)	7.8
$LiSO_4 \cdot H_2O$	Li^{+}	0.163	10.5 (10)	38 (47)	7.8

plasma gives a delaying in the coagulation process. In fact, by means of t.e. graphic investigations it was demonstrated that all metal salts used in these experiments produce essentially the same *qualitative* effects, consisting in an increase of reaction time and a decrease of maximal amplitude.

Nevertheless, *quantitative* differences were found since Cd^{++}, Zn^{++}, Ni^{++} produce marked effects in small amounts, whereas Fe^{++}, Co^{++}, Pb^{++}, Al^{+++} give the same effects in relatively large amounts and, finally, Mn^{++}, Ba^{++}, Sr^{++} are hardly effective also in very large amounts.

The *quantitative* differences result much more evident when metal cations were used in the same amount. Therefore another experiment was carried out by using 0.2 γ ions of each cation per 0.30 ml of final mixture.

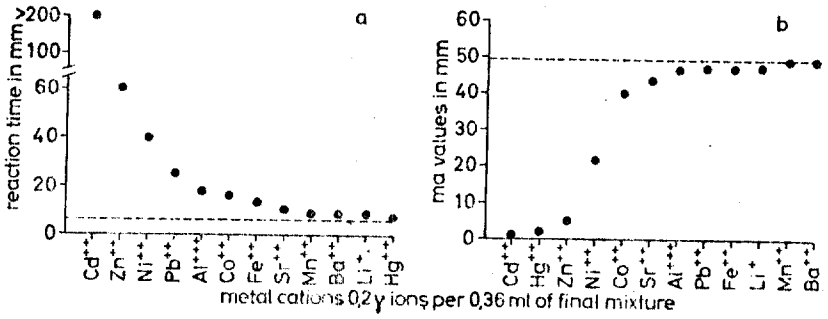

Fig. 1: Increase of reaction time (graph a) and decrease of maximal amplitude (graph b) caused by adding several metal cations to citrated human plasma. The amount employed was 0.2 γ ions per 0.36 ml of final mixture

The results given in Fig. 1 a, b confirmed that the metal salts are not equally effective as well as showed that the modifications of reaction time and maximal amplitude are not of the same degree. In other words, some metals may produce a marked shortening of *ma* and, on the contrary, influence *r* values very slightly. Of the twelve cations studied, only Hg^{++} exhibits an unusual behaviour. By using slowly increasing amounts of mercuric chloride, instead of an *r* increasing, it was observed a gradual *r* decreasing, as long as, when used in the amount of 1 γ ion or else in larger amounts, it dropped to zero (Fig. 2).

Likely this shortening of reaction time is due to the platelet lysis caused by $HgCl_2$ and to an extremely active thromboplastic material thus discharged into the surrounding plasma. When salt concentration reaches high levels, the platelet lysis is complete or nearly complete, the clotting of recalcified plasma occurs in a few seconds and the reaction time is reduced to zero.

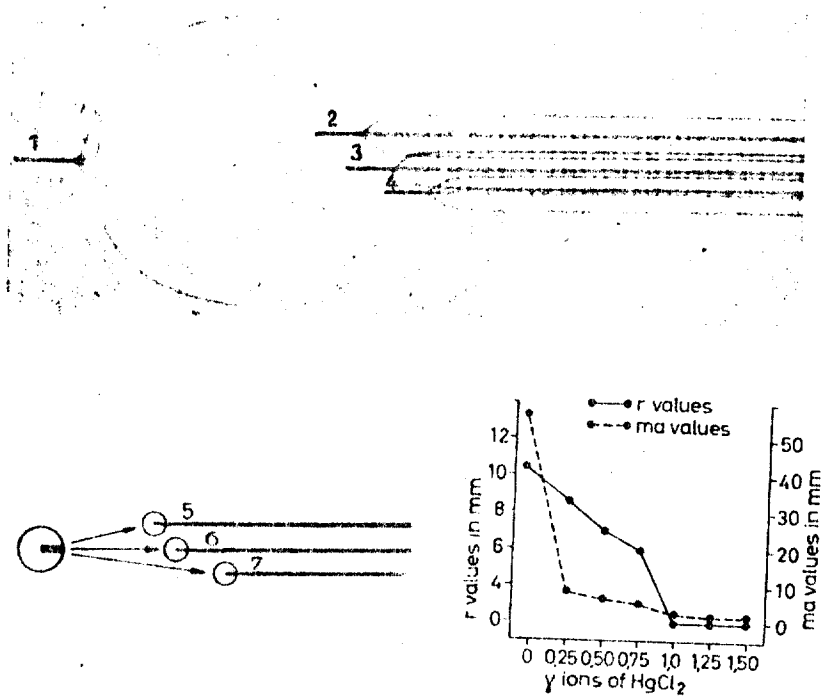

Fig. 2: Increasing amounts of $HgCl_2$ give a gradual *r* decrease and a sharp *ma* decrease; (lower right) the *r* and *ma* changes are plotted against the amounts of $HgCl_2$.

1 — plasma + imidazole buffer + saline (pH 7.4)
2 — plasma + imidazole buffer + 0.25 γ ions $HgCl_2$ (pH 7.4)
3 — plasma + imidazole buffer + 0.50 γ ions $HgCl_2$ (pH 7.2)
4 — plasma + imidazole buffer + 0.75 γ ions $HgCl_2$ (pH 7.2)
5 — plasma + imidazole buffer + 1.00 γ ions $HgCl_2$ (pH 7.2)
6 — plasma + imidazole buffer + 1.25 γ ions $HgCl_2$ (pH 7.2)
7 — plasma + imidazole buffer + 1.50 γ ions $HgCl_2$ (pH 7.0)

The platelet damage is confirmed also by the *ma* behaviour. In fact, *ma* is decreased to such a value that can be explained by a completely abolished platelet activity, being only a function of clot fibrin contents (7).

2. *Effect of salt treated plasma on normal plasma.* In this experiment rabbit plasma (660 000 platelets per cmm) and $Pb(NO_3)_2$ were used. Two samples of this plasma were prepared: one of them (salt-treated plasma) was mixed with salt solution (1.70 γ ions of metal cation per ml) and imidazole buffer, the other one (normal plasma) was mixed with saline and imidazole buffer. The two plasmas contained exactly the same number of platelets (300 000 per cmm).

A series of mixtures of salt-treated plasma and normal plasma were then made in the proportions indicated in table 2 and t.e. graphic determinations were performed as usual. The plasma mixed with metal salt, even though in a very small amount modifies the properties of the normal plasma as regards the modifications in *r* and *ma* values. Plotting the results on a chart as in Fig. 3, it can be demonstrated that there is relationship between changes of *r* and *ma* values and amount of salt-treated plasma.

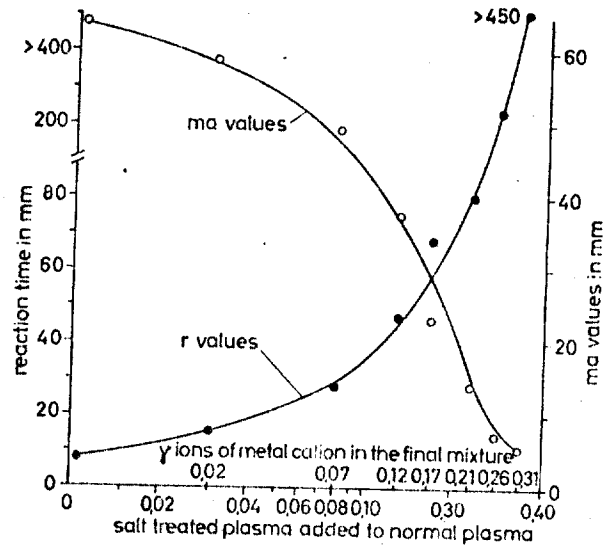

Fig. 3: Reaction time increase and maximal amplitude decrease are caused by adding salt-treated plasma to normal plasma. $Pb(NO_3)_2$ was used in the amount of 1.70 γ ions per ml. The pH in the final mixture was 7.4. A relationship is demonstrated between *r* and *ma* modifications and salt-treated plasma amount

3. *Effect of metal salts on plasma fractions.* For these experiments albumin and globulin fractions achieved by salting-out with ammonium sulphate as well as those isolated with the potato starch supporting medium were used. Since there is no significant difference in the results, only those obtained using electrophoretic fractions are reported.

The nitrogen contents of plasma fractions recovered from starch segments were determined by micro-Kjeldahl method; thereafter they were brought exactly to the same nitrogen content by suitably diluting with normal saline. Mixtures consisted of 0.30 ml platelet-rich plasma, 0.13 ml plasma fractions, 0.05 ml metal salt solution and 0.18 ml imidazole buffer. T.e. graphic determinations were performed on each mixture before and after adding metal salt. The effect exerted by the metal cations on the plasma fractions was expressed as the per cent increase of reaction time and the per cent of maximal amplitude, compared with the *r* and *ma* values observed before adding metal salt.

Table 2: Scheme showing how final mixtures are obtained for different amounts of salt-treated plasma and normal plasma. The plasmas contained 300 000 platelets per cmm. The pH of final mixture was 7.4

Normal plasma ml	Salt-treated plasma ml	$CaCl_2$ 0.125 M ml	Total volume ml	*r* values mm	*ma* values mm
0.33	0.00	0.03	0.36	9.50	63.50
0.30	0.03	0.03	0.36	14.00	59.00
0.25	0.08	0.03	0.36	28.00	54.50
0.20	0.13	0.03	0.36	31.50	49.00
0.15	0.18	0.03	0.36	72.00	5.00
0.10	0.23	0.03	0.36	90.00	1.50
0.05	0.28	0.03	0.36	270.00	0.50
0.00	0.33	0.03	0.36	> 270.00	0.00

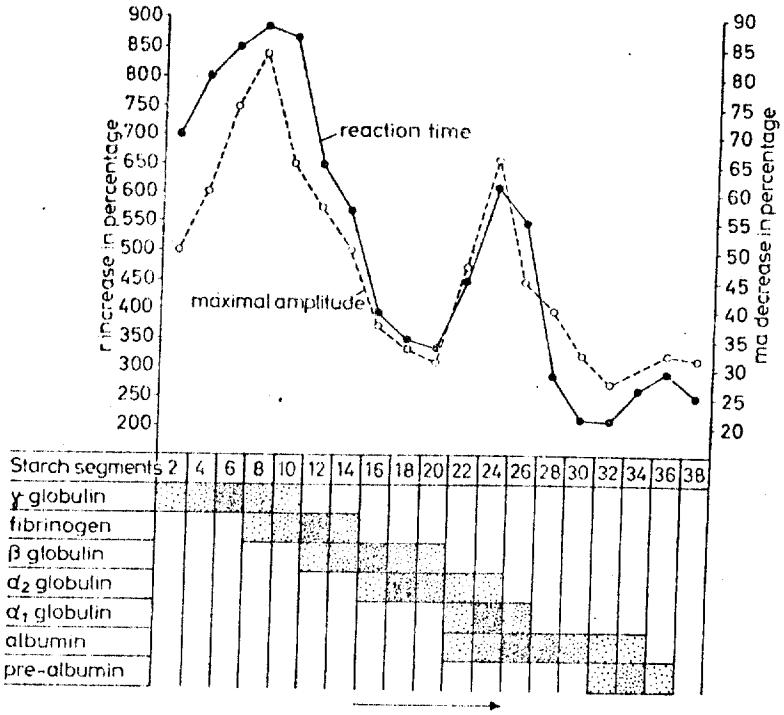

Fig. 4: Effect of activity exerted by 0.65 γ ions of Pb^{++} on plasma fractions recovered by elution after electrophoretic separation. The pH was 7.6. The arrow indicates the direction of migration. The per cent *r* increase (solid line) and the per cent *ma* decrease (dashed line) were much higher when metal cation was tested with globulin than with albumin fraction

Fig. 4 shows, as an instance, the curves obtained when 0.65 γ ions of Pb^{++} were added to the plasma fractions displaced from the starch segments.

Although not all fractions but γ-globulins are highly purified, the behaviour of the two curves indicates clearly that metal exerts a very high activity on γ- and α_1-globulin and fibrinogen, while its activity on albumin fraction is very low.

4. *Reversal of metal cation effects by chelating agents.* The chelating agents have the property of combining with the metal cations and forming complexes in which cations are no longer present as free ions.

An experiment was designed to establish whether chelating agents were able to counteract the effect of metal cations on blood coagulation.

Stock solution (0.10 M) of Na_2EDTA was prepared; this was diluted to 0.025 M using 0.145 M sodium chloride. The mixture was composed of 0.30 ml platelet-rich plasma (220 000 platelets per cmm), 0.09 ml salt solution, 0.09 ml EDTA, 0.18 ml imidazole buffer. After incubating plasma and metal salt for 5 minutes, EDTA and imidazole buffer were added.

Cations γ ions per 0.36 ml of final mixture	r values in mm	ma values in mm	Thromboelastograms
Cd^{++} 0,065	17,5	36,5	7,4
Cd^{++}0,065+EDTA	9,5	64,5	7,0
Zn^{++} 0,23	30	22	7,4
Zn^{++}0,23+EDTA	8	64	7,0
Ni^{++} 0,13	25	59	7,4
Ni^{++} 0,13+EDTA	8	64	7,0
Pb^{++} 0,35	17	62	7,4
Pb^{++}0,35+EDTA	9	64	7,2
Al^{+++} 0,35	13	63	7,0
Al^{+++}0,35+EDTA	13,5	62	6,8
Co^{++} 0,30	18	59	7,4
Co^{++}0,30+EDTA	11	63	7,2
Fe^{++} 0,29	11	59	7,4
Fe^{++}0,29+EDTA	8 5	65	7,2
Hg^{++} 0,36	6	9,5	7,4
Hg^{++}0,36+EDTA	7	9,5	7,0
Control (plasma+saline)	9,5	65	7,4

Fig. 5: Thromboelastogram changes due to metal salts (with and without the addition of 2.25 μmoles EDTA to the mixture). EDTA gives a reversal of *r* and *ma* changes. The numbers adjacent to each thromboelastogram refer to the final pH of mixture

and the final pH of the mixture was determined with a glass electrode. No significant difference in response was noted when EDTA with added salt were incubated for 5 minutes and then mixed with plasma.

As shown in Fig. 5, delaying in blood coagulation induced by free ions is reversed by the addition of excess of EDTA. In fact, the presence of complexing agents can normalise the *r* and *ma* values. Decreasing of EDTA concentration is associated with incomplete reversal of action of metal ions. An example is given in Fig. 6.

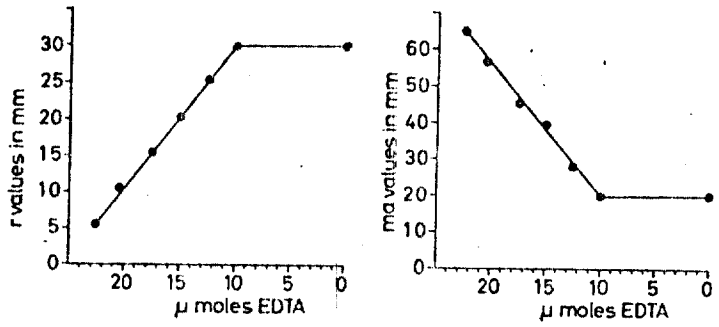

Fig. 6: Decreasing the concentration of EDTA added to the plasma containing 0.65 γ ions of Pb^{++} causes reappearing of the inhibiting action exerted by the free ion

It is to be noted that EDTA did not reverse the effect of aluminium; this observation suggests that EDTA specifically blocks the physiological activity of divalent metal ions.

Also the *ma* reduction produced by mercuric chloride was not reversed. Probably it may be explained on the basis of complete platelet lysis; in fact, when platelets lose their identity, reversal of their function with a chelating agent is impossible.

Conclusions

1. Addition of metal cations to plasma caused t.e. graphic changes, consisting in an increase of *r* and a decrease of *ma* values.

2. The same t.e. graphic modifications were obtained by adding plasma treated with metal cations to normal plasma.

3. From the data obtained one would deduce the following order of cation activity: Cd›Zn›Ni›Pb›Al›Co›Fe›Sr›Mn›Ba›Li›Hg.

831

4. Only mercury presented a different behaviour since it is very active on *ma* but inactive on *r*. Furthermore, it was observed that higher concentrations of Hg^{++} caused progressively decrease of *r* value.

5. When plasma fractions rather than whole plasma were studied, metal cations resulted much more active on globulin than on albumin fractions.

6. The action of metal cations is probably due to the formation of complex and reversible compounds, which obeys to the laws of chemical equilibrium between protein and metal. In fact, addition of a chelating agent complexed the metal and the normal t.e. graphic patterns were again restored.

Summary

The action of some metal cations on blood coagulation was studied by means of t.e. graphic tracings. They exerted a delaying effect on coagulation process, as shown by the *r* increase and *ma* decrease. It is probably due to the formation of complex and reversible compounds. Adding a strong chelating agent, EDTA, caused perceptible reversal of the cation activity.

Résumé

On a étudiée au moyen du thrombélastographe l'action de quelques cations sur la coagulation du sang. Ces cations ont un effet retardateur sur le processus de la coagulation comme l'indiquent l'augmentation de *r* et la diminution de *ma*. Cela est dû probablement à la formation de combinaisons complexes et réversibles. L'addition d'un agent de chélation fort, comme l'EDTA, a une action antagoniste perceptible sur l'effet du cation.

Zusammenfassung

Die Wirkung einiger Metallkationen auf die Blutgerinnung wurde mit Hilfe des TEG untersucht. Sie übten einen verzögernden Effekt auf die Gerinnung aus, charakterisiert durch eine Verlängerung von *r* und eine Verminderung von *ma*. Dies ist wahrscheinlich auf die Bildung von komplexen oder reversiblen Verbindungen zurückzuführen. Zugabe eines starken gelierenden Agens, EDTA, hob die Wirkung der Kationen auf.

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INHIBITORY EFFECT OF COMMON ELEMENTS TOWARDS YEAST GROWTH

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The inhibitory effect towards yeast growth and fermentation of traces of copper has been recognized for many years and several studies on this subject have been made, mostly under brewery conditions. The initial aim of the present investigations was to examine this toxicity for all elements used in construction of fermentation plant, but in view of the occurrence in small quantities of less common elements in natural materials it was decided to examine the toxicity of all the common elements to yeast growth. Of 50 common elements examined by a standard growth method in a defined synthetic medium, six (cadmium, copper, silver, osmium, mercury and palladium) have been found to be extremely toxic. Eleven others are moderately toxic, but the remainder have little poisonous action towards yeast in the concentrations normally encountered in practice. The toxicity of cadmium is reduced by addition of zinc to the medium. The toxicity of the most poisonous group of elements is considerably reduced when part of the sugar of the synthetic medium is replaced by malt or molasses wort, this effect probably being due to a reduction of ionization.

Introduction

For a number of years it has been realized that copper can exert considerable toxic action towards yeast growth and fermentation. R. Guillemet (*Bull. Soc. Chim. biol.*, 1936, **18**, 1125) showed that the addition of copper sulphate to a glucose solution reduced the rate of fermentation by yeast when the solution contained 0·5 p.p.m. of copper, and fermentation was completely prevented when the copper concentration reached 2·0 p.p.m. M. H. van Laer (*Ann. Brass. Dist.*, 1934, **32**, 225–229, 241–248) investigated the effects of copper in brewing in considerable detail, and showed that synthetic media using glucose as the sugar source were far more sensitive to copper additions than was natural malt wort; it was also shown that the toxicity of copper was increased as the temperature was reduced, and that different yeast strains showed variable resistance to the action of the metal. C. A. Elvehjem and E. B. Hart (*J. biol. Chem.*, 1931, **91**, 37) found that small amounts of copper up to 0·1 p.p.m. stimulated yeast growth, but that larger amounts inhibited growth except in the presence of protein and non-sugar extracts which tended to inactivate the copper. R. Koch (*Woch. Brau.*, 1935, **52**, 37) showed that 1·5 p.p.m. of active soluble (ionized) copper affected fermentation.

Very little information appears to be available upon the toxicity towards yeast of other elements. G. Hagues (this *Journ.*, 1931, 366) found that nickel was corroded at the point of contact with the yeast-head in nickel-lined fermentation vessels, and that the yeast contained 140 p.p.m. of nickel; the fermentations, however, were apparently normal.

The normal presence of many elements in the ash of yeasts grown in various media has been reported by O. W. Richards and M. C. Troutman (*J. Bact.*, 1940, **39**, 739), who analysed spectroscopically the ash of many yeasts and found traces of Al, Ba, Bi, B, Ca, Cr, Cu, Au, Fe, La, Pb, Li, Mg, Mn, P, K, Pt, Si, Ag, Sr, Tl, Sn and Zn. Recent work by B. H. Olsen and M. J. Johnson (*J. Bact.*, 1949, **57**, 235) has shown that traces of iron (0·2 p.p.m.), copper (0·01 p.p.m.) and zinc (0·2 p.p.m.) in the nutrient medium are essential to the growth of yeast. These authors have found no justification for the belief of O. W. Richards (*J. Amer. chem. Soc.*, 1925, **47**, 1671) that yeast growth is stimulated by traces of thallium.

The present authors have for some years had experience of interesting examples of yeast poisoning due to copper. The present investigation was aimed at examining the toxic action towards yeast growth of all the

other elements likely to be met in practice. A growing number of elements is being used in the composition of alloys which may at some time or other be employed in the fabrication of plant for the brewing and yeast industries, and an examination of the toxicity of salts of the following common metals was first undertaken: copper, tin, iron, aluminium, chromium, nickel, zinc, cobalt, manganese, lead, molybdenum, tungsten and cadmium. As recent work has shown that some of the less common elements can occur to a considerable extent in natural materials [*e.g.*, the titanium content of hay can be as high as 30 p.p.m. (R. L. Mitchell and J. Tosic, *Proc. Soc. gen. Microbiology*, 1949, **3**, xvii)], it was decided that a knowledge of the effect on yeast growth of as many elements as possible would be of considerable interest and the original list was extended to include strontium, barium, mercury, silver, arsenic, antimony, boron, selenium, uranium, vanadium, lithium, caesium, lanthanum, thorium, rubidium, thallium, beryllium, titanium, cerium, tellurium, palladium, osmium, zirconium and finally the halogen elements fluorine, chlorine, bromine and iodine. The importance of traces of copper, iron and zinc as essential factors for yeast growth has been recognized in the basic synthetic medium used in this and previous yeast nutrition work. It should be emphasized that requirement is quite a different problem from that of toxicity. The level of copper requirement for full growth (0·01 p.p.m.) is only one-tenth of the minimum amount of copper required to show incipient toxic action towards yeast growth.

Experimental

Growth methods.—Growth was carried out in aerated synthetic wort using a technique precisely similar to that employed for assay of nutrilites in raw materials (White and Munns, this *Journ.*, 1950, 141). The standard medium was composed as follows, the figures representing the weights of nutrients per 1,100 ml. of solution:

Sugar (as sucrose)	50·0	grm.
Ammonium sulphate	4·0	,,
Disodium phosphate ($Na_2HPO_4.2H_2O$)	2·0	,,
Magnesium sulphate	0·50	,,
Calcium chloride	0·10	,,
Ferric ammonium [illegible]	0·02	,,
Zinc (as sulphate)	0·2	mgrm.
Copper (as sulphate)	0·01	,,
D-Biotin	1·0	μgrm.
Aneurin (vitamin B_1) hydrochloride	200	,,
Riboflavin (vitamin B_2)	100	,,
Nicotinic acid	5,000	,,
p-Amino benzoic acid	300	,,
Pyridoxin (vitamin B_6)	1,000	,,
Calcium D-pantothenate	500	,,
Meso-inositol	50,000	,,

Buffer solution:—

10 ml. of a solution consisting of 800 ml. of 50 *per cent.* potassium lactate + 75 ml. A.R. lactic acid. (This also supplies the necessary potassium.)

The yield of yeast in control experiments was 19·4 grm., the original seed yeast being 0·3 grm. (at 27% dry matter).

The elements under examination were added as the requisite amounts of suitably soluble salts, additions being calculated as p.p.m. on the volume of the solution. Yeast crops were assayed after 24 hr. growth in the normal way. A series of assays was carried out for each element examined, and curves were drawn to illustrate the growth of yeast obtained with varying amounts of each element studied. It is realized that the influence exerted on growth by any added salt is the joint effect of the constituent radicles, but in view of the wide range of radicles investigated the picture presented should be of sufficient clarity.

Results obtained.—Table I summarizes the results obtained in the synthetic medium. Results are expressed in three ways:—

(*a*) As p.p.m. of the element required to reduce yeast growth to a half of the yield obtained in the control experiment—that is to 9·7 grm.

(*b*) As p.p.m. of the element required to inhibit yeast growth completely.

(*c*) As a direct "poisoning capacity" of the element to yeast, calculated on an arbitrary scale by regarding the poisoning capacity of the most toxic element—cadmium—as 10,000, and expressing the toxicity of the other elements in inverse proportion to this figure on the basis of the amounts required to reduce yeast growth to a half. Thus, it is necessary to add 55 p.p.m. of cobalt to the medium to obtain yeast half-growth and the poisoning capacity of cobalt is thus $\frac{0{\cdot}125 \times 10{,}000}{55} = 22{\cdot}8$.

From the table it may be seen that the

elements can be classified in four fairly well defined groups as follows:—

Group A (very poisonous).—Of all the elements examined only six fall in this category, *viz.*, cadmium, copper, silver, osmium, mercury and palladium. Of these, the two most likely to be met with in practice, cadmium and copper, are the most toxic of all elements.

Group B (moderately poisonous).—This group contains the common metals, nickel, chromium and tin, all of which may be encountered in the fabrication of modern brewery equipment. Some of the other elements in this group are likely to occur in the ash of brewing materials.

Group C (slightly poisonous).—The elements, selenium and thallium, have slight toxicity towards yeast.

Group D (very slightly poisonous or non-poisonous).—These elements show no toxic action in a concentration of 500 p.p.m. and thus have a poisoning capacity of less than 2·5.

TABLE I

INHIBITING EFFECT OF COMMON ELEMENTS ON YEAST GROWTH IN A SYNTHETIC MEDIUM

Designation.	Element.	P.p.m. reducing growth to one-half.	P.p.m. inhibiting growth completely.	*Poisoning Capacity.
Very poisonous	Cadmium ..	0·125	>10	10,000
	Copper ..	0·175	1·0	7,100
	Silver.. ..	0·22	0·4	5,700
	Osmium ..	0·72	1·0	1,730
	Mercury ..	1·55	1·8	810
	Palladium ..	2·7	10·0	460
Moderately poisonous	Cobalt ..	55	115	22·8
	Lithium ..	63	125	19·8
	Boron ..	66	200	19·0
	Nickel ..	83	approx. 185	15·0
	Fluorine ..	90	,, 160	13·9
	Vanadium ..	93	,, 200	13·4
	Chromium ..	142	,, 260	8·8
	Arsenic ..	157	,, 230	8·0
	Tellurium ..	223	,, 350	5·6
	Beryllium ..	240	,, 350	5·2
	Tin	285	>350	4·4
Slightly poisonous	Selenium Thallium	approx. 400	500–600	approx. 3
Very slightly poisonous or non-poisonous.	This group includes: (*a*) elements with a poisoning capacity of less than 2·5, *viz.*—lead, iron, zinc, aluminium, tungsten, molybdenum, manganese, and the halogen elements (iodine, bromine, and chlorine) as their potassium salts. (*b*) elements showing no toxic action in a concentration of 500 p.p.m., *viz.*—antimony, strontium, barium, uranium, thorium, lanthanum, rubidium, caesium, titanium, cerium, and zirconium. (*c*) in addition to the above, the following which are required in yeast nutrition and are non-toxic: magnesium, potassium, sodium, calcium, sulphur (as sulphate), and phosphorus (as phosphate).			

*0·125 × 10,000/x, where x = p.p.m. required to reduce yeast growth to one-half the standard value.

It is of interest to see that such elements as iron, aluminium and zinc, commonly used for fabrication of components of brewery plant, fall into Group D. In addition, many of the elements in this group occur to varying extents in the ash of brewing materials. Further, a concentration of 1,000 p.p.m. of the halogen elements, iodine, bromine and chlorine (as their potassium salts) had no poisonous action,

whereas fluorine had a considerable toxic effect with a poisoning capacity of 13·9.

In addition to the four groups of elements discussed above, carbon, hydrogen, oxygen and nitrogen comprise the major portion of yeast dry substance, and the following are required in yeast nutrition: magnesium, potassium, sodium, calcium, sulphur and phosphorus.

It was necessary in certain cases to modify the composition of the basic medium to avoid precipitation of the added element by anions normally present. For example, when the effect of silver was being examined, all chlorides were kept out of the basic medium, the calcium normally added as chloride being present as nitrate. Similarly, the effects of barium, lead and strontium were examined in media free from sulphates. Phosphate was always used in the composition of the basic medium. The complete elimination of radicles such as chloride or sulphate from the medium may have had some effect on yeast growth, but such effect cannot as yet be ascertained.

Some of the elements were precipitated as sulphide upon the yeast by hydrogen sulphide evolved during normal growth. Addition of iron, copper, nickel or lead thus gave rise to black-coloured yeasts.

Results in non-synthetic media.—It can be seen from the above that growth of yeast in a synthetic medium can be inhibited completely by the presence of 1 p.p.m. of copper in the medium at the start of fermentation. The addition of molasses or malt worts at the start of the fermentation, however, gives very different results. For example, when 10% of the sucrose in the medium was replaced by beet molasses (1 grm. of sucrose replaced by 2 grm. of beet molasses containing 50% of sugars), an addition of 5 p.p.m. of copper gave virtually the same yield as the control (19·4 grm. of yeast) and additions of 10, 20 and 25 p.p.m. of copper gave yeast yields of 17·3, 10·8 and 5·0 grm. of yeast respectively. The addition of malt wort was found to have similar results. Thus, when 10% of the sucrose was replaced by equivalent fermentable matter as malt wort (1 grm. of sugar replaced by 10 ml. of malt wort of sp.gr. 1·059), additions of 5 and 10 p.p.m. of copper gave respective yields of 8·3 and 3·4 grm. of yeast. Indeed, no diminution of yeast yield was found by adding 30 p.p.m. of copper to the medium when all the sucrose of the medium was replaced by an equivalent quantity of malt wort; the only noticeable effect of the copper in this case was the darkening of the final yeast crop due to the formation of black copper sulphide which was deposited on the cells.

The protective action of molasses and malt wort was also found when cadmium, silver, mercury and osmium were added to the medium. No complete explanation of this protective action can yet be advanced. It does, however, appear likely that when molasses or malt wort are added, the metallic kations may be either partially precipitated by some of the constituents of the molasses or malt, or taken into a complex in which the metal is unionized and is, thus, virtually removed from effective poisonous contact with the yeast cells.

With respect to the poisoning of yeast by cadmium salts, it has been found that when a few p.p.m. of zinc are added to the medium the toxicity of cadmium is very greatly reduced. The proximity of zinc and cadmium in the periodic table may have something to do with this effect. If the metals are mutually antagonistic it is not difficult to believe that the poisonous effect of cadmium is due to its antagonizing the entry of essential zinc into the yeast cell. It is of great interest to note that complete inhibition of growth is obtained only when more than 10 p.p.m. of cadmium are present in the medium.

Discussion

Most practical manufacturing experience of toxicity of metals to yeast growth and fermentation has been gained with reference to poisoning by copper.

When yeast is grown in either a molasses or malt-wort medium the operation can be carried out successfully in a copper fermenting vessel, provided that all the wort is present at the start of the operation. In this case, the yeast can tolerate quite high concentrations of copper (up to concentrations of the order of 30 or 40 p.p.m. on the medium) and yield will not be greatly reduced, although the yeast colour will be very dark. Under these conditions copper often accumulates in the final yeast—probably as an insoluble layer upon the outside of the cells, and the copper content of the final yeast can be as high as 1,000

p.p.m. on dry matter without much reduction in yield. When, however, yeast is to be grown in an exponentially-fed process, in which case the seed yeast is placed in a solution of inorganic salts and the sugar-containing wort is run into this over the course of the growth period, the situation is entirely changed. A concentration of only 1 or 2 p.p.m. of copper in the initial salt solution into which the yeast is seeded can substantially poison the seed yeast and cause reduced yield. Indeed, sufficiently bad poisoning leads to disappearance of the seed yeast after a few hours. A bare copper fermenting vessel cannot, in these circumstances, be used for this type of fermentation.

Copper poisoning is often accompanied by some interesting morphological effects. The budding daughter cells are often killed before the parent cell, which sometimes throws off a further bud at a later stage. Groups of three cells—mother cell and two attached buds—are quite commonly seen in yeast poisoned by copper.

From the results of the tests with synthetic wort it is clear that cadmium should never be employed at any stage in brewery operations. Nickel and tin could, in certain circumstances, give rise to poisoning, particularly in exponentially-fed operations. Other common metals have little or no toxicity and are not likely to lead to trouble.

Summary

1. The inhibiting effect of some 50 common elements towards yeast growth in synthetic media has been examined. Ten of the elements—carbon, hydrogen, oxygen, nitrogen, magnesium, potassium, sodium, calcium, sulphur and phosphorus—are normally required for full yeast growth and have no toxic properties. The other elements examined have been placed into four categories:—very poisonous; moderately toxic; slightly toxic; and very slightly toxic or non-toxic.

2. Cadmium is the most poisonous of the elements, but copper, silver, osmium, mercury and palladium are also extremely toxic. The toxicity of cadmium is reduced by adding to the medium a few p.p.m. of zinc.

3. Probably as a result of decreased ionization, the toxicity of these elements is reduced by the addition of either malt or molasses worts to the synthetic medium.

4. Copper poisoning can easily be encountered in practice when using copper plant in incrementally-fed yeast processes, and is often accompanied by interesting morphological changes in the yeast, budding in particular being abnormal.

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859

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THE INFLUENCE OF STORAGE, PASTEURIZATION, AND CONTAMINATION WITH METALS ON THE STABILITY OF VITAMIN C IN MILK

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In 1928 Hess (1) stated that " . . . it requires a minimum of about 16 ounces of cow's milk to protect an infant from scurvy or to cure it." Today, however, milk is almost entirely ignored as a source of vitamin C. This is probably due to the fact that a majority of the early studies indicated that milk was an uncertain source of the antiscorbutic factor. On the other hand, the fact should not be overlooked that before the discovery of vitamin C, milk was the only important source of this vitamin for infants. More recently Schwartze, Murphy and Hahn (2) state " . . . we would estimate that 200 cc. daily of our raw milk less than 12 hours old would probably suffice to protect an infant from scurvy."

Recently, while studying the influence of the cow's ration on the vitamin C content of milk, the authors were impressed with the appreciable amounts of this vitamin found in the samples tested (3). In 197 samples of fresh milk from individual cows the vitamin C content ranged from 17 to 37 milligrams per liter, averaging about 26.

While the vitamin C requirement of either adults or infants is not definitely known, 27 milligrams per day has been suggested as an adequate amount for an adult (4). Fresh milk, therefore, may be an important source of vitamin C. This vitamin C must be preserved until the milk is consumed if market milk is to be recognized as a reliable source.

Soon after the discovery of vitamin C, it was found that quick boiling of milk destroyed less of the vitamin than pasteurization at 143° F. for 30 minutes (5), and that contamination of the milk with copper greatly increased the loss (6). These findings were confirmed in later studies and some important differences were demonstrated with certain types of commercial pasteurizers (7, 8, 9, 10). It has not been determined whether the lower vitamin C content usually found in pasteurized milk is due to loss during the process of pasteurization, or to an increased rate of progressive loss after this treatment, or whether both may be contributing factors.

It is known that, during pasteurization, milk may be somewhat contaminated with the metals of which the pasteurizer is made (11, 12). The effect

[1] Contribution No. 292, Department of Chemistry; Contribution No. 106, Department of Dairy Husbandry.

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of contact with milk pail and storage can, and the effects of contact with metal and air in the process of cooling and straining are less certain. The relation of such slight contamination to either immediate loss of vitamin C or to increased progressive loss is unknown.

The present study was undertaken to determine more clearly the relative effect of several factors on the vitamin C in milk. The loss during storage of both raw and pasteurized milk was first studied. Loss during the process of pasteurization, and after contamination with metals either by direct addition to the milk, or by addition to the ration, was also studied.

I. INFLUENCE OF STORAGE AND PASTEURIZATION

Procedure

To obtain milk as free as possible from any kind of contamination, samples were taken directly from aluminum milk pails at the college dairy barn and cooled in glass bottles. Parts of some of these samples were pasteurized in 50 ml. pyrex glass flasks. Samples of milk were also obtained at several milk plants using different types of commercial pasteurizers.[2] Since some of these plants were as far as 125 miles from the laboratory, approximately 9 hours elapsed between the collection and testing of samples from such plants.

Samples were taken just before pasteurization and again after the milk had been pasteurized, cooled and bottled. They were packed in ice, brought to the college laboratory, and tested after several intervals for vitamin C with 2-6-dichlorophenolindophenol (Dubey and King 13). The samples were stored at approximately 40° F. during the intervals between tests. The types of pasteurizers studied, the intervals after pasteurization at which the milk was tested, the Vitamin C content and the losses due to storage, to pasteurization, and to both pasteurization and storage are shown in table 1.

Results

In the study of table 1, first consideration should probably be given to the greater stability of vitamin C in fresh raw milk than in any of either the raw or pasteurized samples secured at commercial pasteurizers. Even the usual procedure of straining and cooling over a well-tinned, copper, tubular cooler produced an appreciate loss during storage.

No immediate loss resulted from pasteurization by the short time high temperature method (Types B and D). This agrees with the results of other workers where pasteurization involved similar temperatures and holding periods (8, 9, 10). The progressive loss due to storage also was

[2] The help of Mr. O. L. Fretz of Kansas City, Mr. L. Brandon of Lawrence, and Mr. J. B. Jervis of Topeka in securing and collecting these samples is very much appreciated.

TABLE 1

Influence of storage and pasteurization on vitamin C in milk

	Type				Samples: Number plants represented	Samples: No. samples	Samples: Raw or Past.	Average values for vitamin C: Hours after sampling (Vit. C in Mg./liters): 2	9	24	48	Loss in per cent due to storage only, and to storage plus pasteurization. (Hours stored): 2–24 Storage only	2–24 Past.+ Storage	9–24 Storage only	9–24 Past.+ Storage	9–48 Storage only	9–48 Past.+ Storage
	FRESH RAW MILK (not pasteurized)																
A	Direct from milk pail				1	1275	Raw	25.7				7					
	" " " "				1	89	Raw	25.0		23.3							
	Strained, and cooled over well tinned copper				1	68	Raw	21.6		15.4							
	LABORATORY FLASK PASTEURIZATION (Using raw milk aged 9 hours in type B and aged 3 hours in type C)																
	Pasteurizer used	Material	Temp. °F.	Holding time min.													
B	Conical flasks heated in water bath	Pyrex glass	160	1	1	13	Raw	21.0		19.9		5.2					
							Past.	21.3		20.7		2.8	1.4				
							% loss	−1.4		−4.0							
C	As in type B	Pyrex glass	143	30	1	8	Raw	20.5	17.5	12.5		39		29			
							Past.	15.8	13.3	10.2		35	50	23	42		
							% loss	23	24	18							

TABLE 1—(*Continued*)

	Type				Samples			Average values for vitamin C									
								Hours after sampling				Loss in per cent due to storage only, and to storage plus pasteurization. (Hours stored)					
					Number plants represented	No. samples	Raw vs. Past	2	9	24	48	2–24		9–24		9–48	
								Vit. C in Mg/liters				Storage only	Past + Storage	Storage only	Past + Storage	Storage only	Past + Storage
	Pasteurizer used	Material	Temp. °F.	Holding time min.													
	COMMERCIAL PASTEURIZATION (Using mixed raw milk from night and morning, aged 17 hours and 5 hours respectively, when sampled)																
D	Internal tubular continuous flow (short time-high temp.)	Stainless steel	160	0.3	2	3	Raw		17.1	15.0	12.5			12		27	
							Past.		16.9	16.1	12.0			4.8	6	29	30
							% loss		1.7	−7.3	4.0						
E	Tubular preheater, glass lined holding vat	Tinned copper, glass	143	30	1	2	Raw		15.4	12.0	9.1			22		41	
							Past.		11.1	9.8	5.9			12	37	47	62
							% loss		28	18	35						
F	Spray vat	Stainless steel	143	30	4	11	Raw		16.4	14.4	12.1			12		36	
							Past.		10.8	7.6	4.8			30	54	55	71
							% loss		34	47	60						
G	Coil vat	Tinned copper	143	30	5	11	Raw	23.4	18.8	16.5	11.9	29		12		37	
							Past.	16.4	11.6	7.6	4.9	53	68	34	60	58	74
							% loss	29	38	54	59						
H	Internal tubular continuous flow (30 min.-holding	Tinned copper	143	30	2	5	Raw		13.4	11.1	8.7			17		35	
							Past.		6.0	3.3	2.7			45	75	55	80
							% loss		55	70	69						

[a] Precision of measurement is about 1 mg. per liter. Variations of less than about 5 per cent have doubtful significance.

very much less than with any other method of pasteurization studied. In fact, practically no loss resulted from storage for 24 hours after pasteurization. This was true for both the laboratory pasteurization in glass flasks (Type B) and for commercial flash pasteurization in stainless steel equipment (Type D).

It was to be expected that pasteurization by holding at 143° F. for 30 minutes would reduce the vitamin C either immediately or after storage of the pasteurized milk. An immediate loss was found even when the milk was pasteurized in glass flasks where there was very little agitation and very little contact with outside air (Type C). The raw milk used in Type C pasteurizers did not show as good storage stability of vitamin C as the milk used in Type B pasteurizers. It was more nearly comparable to the raw milk used in the commercial pasteurizers. The first observed loss after pasteurization in commercial types E and G was only slightly greater than the immediate loss under laboratory conditions.

The loss of vitamin C on storage of pasteurized milk is shown in table 1 both as percentage of the first value after pasteurization, and also as percentage of the raw milk of equal age. By either comparison the progressive loss after type C pasteurization was no more than for the raw milk. Among all the commercial types using the 30 minute holding period, only the glass lined vat (Type E) produced pasteurized milk where loss of vitamin C on storage was as small as in the raw milk.

It was anticipated that the stainless steel spray vats (Type F) would be superior to the tinned copper coil vats (Type G) in conserving the vitamin C content of milk. The agitation of the milk was less vigorous in the spray vat and the stainless steel construction should minimize contamination with copper. The results indicated some superiority for type F compared to type G. That the difference was not as marked as was expected can be attributed to at least two possible factors. In the first place there was probably contamination of the milk with copper in equipment through which the milk was handled before and after pasteurization. This may account for the greater storage loss with type F than with type E. In the second place, as previously indicated, prolonged holding (143° F., for 30 min.) even without contact with any metal (Type C) resulted in serious loss of vitamin C.

In the commercial method using coil vats (Type G) it was possible to observe the rate of loss of vitamin C for the 2 to 24 hour storage period. The initial loss for these samples was not greatly different from the similar loss in samples similarly pasteurized in glass flasks (Type C). The progressive loss on storage was distinctly greater in type G than in types C or E, and slightly greater than in type F.

The internal tubular pasteurizers (Type H) should be as effective as the laboratory flasks (Type C) in avoiding contact of the hot milk with outside air. All the vat pasteurizers (Types E, F, G) permit much greater contact of the milk with outside air. On the other hand, the amount of metallic surface in contact with the milk and the velocity of the milk at this surface is greater in type H.

It was impossible to evaluate exactly the amount of exposed copper in various parts of the equipment in the two plants using type H pasteurizers, or to distribute the cause for the loss of vitamin C between the type of pasteurizer and the condition of the pasteurizer and necessary equipment. In one plant the vitamin C in the raw milk was improved by avoiding a poorly tinned cooler at the receiving vat. In the other plant the final cooler appeared to be the part containing most serious exposure of copper. When this cooler was used with a new short time high temperature pasteurizer, no serious loss of vitamin C was caused. In both plants the pasteurizers themselves appeared to be in fair condition. In these two plants both the initial loss and the progressive loss on storage were greater than for any other type of pasteurizer studied.

The results with laboratory type C and with commercial types E, F, G and H, indicate that the 30 minute holding process of pasteurization is not well adapted to the conservation of vitamin C in milk.

II. INFLUENCE OF CONTAMINATION WITH METALS

Procedure

The results just reported might suggest that contamination of milk with copper is indicated when the loss of vitamin C on storage after pasteurization is excessive. If addition of the metals contained in stainless steel should not increase the loss of vitamin C in milk, the unexpected loss in stainless steel vats could be assumed to be due to other causes.

The effect of added metals was studied by adding sulfates of several metals to the milk. Sulfates were used because they are readily soluble salts. The metals studied were copper, iron, nickel, and chromium. Although aluminum is corroded by milk, it was not included because it has repeatedly been found equal to glass in avoiding loss of vitamin C. Tin was not included because it is not readily corroded by milk and also because any solution in which it could be added would be very strongly acid.

The first tests were conducted with copper and iron solutions because these metals are known to catalyze the oxidation of vitamin C. The first object was to obtain an estimate of the minimum concentration of added metal needed to produce a detectable effect on the vitamin C in the milk. Preliminary tests with copper and iron were followed by a series of tests using about the minimum detectable addition of metal. In each test the

sample of milk was divided into four parts. One was used as a standard or check and one of the metals was added to each of the other three parts. Copper in the concentration of 5 p.p.b. was used in each of these 8 tests. Ten p.p.b. of copper and 100 p.p.b. of iron were also used in 4 of the tests while in the other 4 tests these were replaced by 1000 p.p.b. of chromium and 1000 p.p.b. of nickel.

The amounts and kinds of metal added, the times at which vitamin C was tested, and the amounts found, are shown in tables 2, 3, and 4.

TABLE 2

Vitamin C in milk to which copper was added as sulfate[a]

ADDED COPPER[b] P.P.M.	RAW VS. PAST.	PER CENT OF INITIAL CONTENT	PER CENT LOSS 2–24 HRS. STORAGE	HOURS AFTER PASTEURIZING 2	9	24	48
				Vit. C in mg./liter			
0	R	100	23	23.4	21.5	17.9	11.1
	P	84	18	19.7	17.9	16.0	11.1
			% loss	16	17	11	0
0.1	R	92	45	21.5	18.5	11.7	6.2
	P	68	54	16.0	13.5	7.4	2.5
			% loss	25	27	37	75
0.3	R	80	79	18.5	13.5	3.1	3.7
	P	39	73	9.2	4.9	2.5	1.8
			% loss	50	64	19	51
0.6	R	68	84	16.0	7.4	2.5	2.5
	P	13	42	3.1	1.8	1.8	1.8
			% loss	80	76	28	28

[a] These samples were prepared from a single composite of morning's milk taken from a can at the college creamery about 7:30 a.m. The copper was added about 8:00 a.m. The pasteurization at 143° F. for 30 min. in glass flasks was completed about 9:00 a.m.

[b] Addition to another sample of milk of 20 times the maximum amount of the water used in making these solutions caused no loss of vitamin C.

TABLE 3

Vitamin C in milk to which ferrous sulfate was added

ADDED IRON P.P.M.	PER CENT LOSS ON STORAGE 2–7 hrs. raw	past.	7–24 hrs. raw	past.	HOURS AFTER PASTEURIZATION 2 raw	past.	7 raw	past.	24 raw	past.
					Vit. C in mg. per liter					
0	16	13	46	54	19.2	17.6	16.1	15.3	8.7	7.0
1.0	18	17	36	45	15.2	14.0	13.1	11.7	8.4	6.4
			% loss		21	21	19	23	6	9

TABLE 4

Vitamin C in pasteurized milk to which various metals were added.

METAL ADDED P.P.B.	AVERAGE		TEST NUMBER							
	Extra losses	Mg./1	1	2	3	4	5	6	7	8
			Vit. C 24 hrs. after past. (mg./1)							
0	0	10.3	12.0	12.9	11.1	6.8	7.5	8.0	11.4	11.4
5 copper	28	7.4	7.4	8.0	8.0	3.7	6.1	6.1	10.1	10.1
10 "	60	4.2	3.1	3.7	6.8	8.1				
100 iron	8	10.1	12.3	12.3	9.9	6.1				
1000 nickel	19	7.9					4.7	6.1	10.1	10.8
1000 chromium	11	8.6					5.7	6.7	10.8	11.4

[a] The losses were calculated as average percentages of the individual mg./1 of vitamin C in the samples to which no metal was added. Losses of less than 20 per cent have doubtful significance.

Results

It is evident from table 2 that addition of copper produced an extra progressive loss of vitamin C in both raw and pasteurized milk. Copper did not produce a serious immediate loss in the raw milk. This finding is based on the estimated initial vitamin C contents obtained by extrapolating the values in table 2 to the time the copper was added. The losses 2 hours after pasteurization or 3 hours after addition of copper are small relative to the losses on longer storage and indicate the same finding. There probably was an immediate loss on pasteurization after the addition of 0.1 or more p.p.m. of copper. The data are, however, inadequate to distinguish accurately between an immediate loss and a progressive loss whose rate slowed as the vitamin content of the milk diminished. When only 5 p.p.b. of copper were used, the extra loss of vitamin C was evident only after pasteurization and storage for 24 hours.

The tests in table 4 indicate that 5 p.p.b. of copper added to milk produced a decrease of vitamin C in the pasteurized stored milk. This result is confirmed by comparison with the progressively shorter intervals needed to detect the influence of the larger amounts of added copper.

In table 3 the loss due to 1.0 p.p.m. added iron appears to be somewhat more than the minimum significant loss. It is also noteworthy that the extra loss was as evident 2 hours after addition of iron as at any other time and was about equally evident in the raw and the pasteurized milk.

When 100 p.p.b. of iron was added (Table 4), the observed loss of 8% although rather consistent, was hardly more than the experimental error.

Addition of 1.0 p.p.m. of chromium did not produce a significant loss of vitamin C either in the stored pasteurized milk as shown in table 4 or in the raw milk. Tests at intervals of 2 hours and 9 hours after pasteurization did not show greater effects.

The effects of 1.0 p.p.m. added nickel were more pronounced in the pasteurized than in the raw milk. The average effect was as great after 2 hours' storage as after 24 hours' storage. The average effect at both ages appeared to be more than the error of measurement but the effect in different samples of milk varied greatly.

It may be estimated conservatively that compared to copper, at least 20 times as much iron or 200 times as much chromium or nickel are required to produce comparable destruction of vitamin C. The significance of these ratios in choosing materials for pasteurizing equipment is further emphasized by the fact that copper or brass is probably 100 fold more soluble in milk than stainless steel (12). Of the four metals studied copper was the only one for which both pasteurization and storage increased the destructive effect of vitamin C in milk. This fact further substantiates the earlier inference that excessive loss of vitamin C after pasteurization and storage indicates contamination with copper.

III. INFLUENCE OF ADDED COPPER IN THE RATION

Procedure

Although Elvehjem, Steenbock and Hart (14) reported that addition of copper to the ration produced no significant increase of copper in the milk, it seemed worth while to test the effect of the added copper in the ration on the rate of loss of vitamin C in pasteurized milk. Accordingly eight cows were each fed 0.3 gram of copper as copper sulfate solution sprinkled on the ration each evening for four days. Samples of milk from each of these cows and from eight other cows giving milk of approximately equal vitamin C content were collected for 3 milkings following the first copper supplement and again after the fourth supplemental feeding.

Each sample of milk was divided into two parts, one of which was pasteurized at 160° F. for 1 minute. Both parts were analyzed after 2 hours and 24 hours storage at 80° F.

Results

The copper supplement did not at any time produce a detectable difference in the vitamin C content of fresh raw milk or of freshly pasteurized milk. The loss after pasteurizing and storing averaged 30 per cent for the control milk and 49 per cent for the milk produced on the copper supplemented ration. The difference of 19 per cent ± a standard error of 5.6 per cent indicates a critical ratio of 3.4. In other words, there is less

than 1 chance in 1000 that there is not a real difference between the two groups. The loss is the same order of magnitude as that produced by 5 p.p.b. of copper to the milk. The simplest interpretation of this result is that the increased copper in the ration did slightly increase the amount of copper in the milk.

The amount of added copper in milk needed to produce this effect on vitamin C stability is less than could be detected by chemical methods (11, 14). The deduction, that copper added to the ration produced additional copper in the milk, extends rather than conflicts with the test made by Elvehjem, Steenbock, and Hart. The large increase of copper in the ration needed to produce a barely detectable decrease of vitamin C stability in the milk indicates that normal variations of copper in the ration, probably would not produce any significant effect on the vitamin C content of milk.

CONCLUSIONS

Milk, either raw or pasteurized by the short time, high temperature process, but uncontaminated with copper was held 35 hours without serious loss of vitamin C. Such milk may be an important source of vitamin C in human nutrition.

Stainless steel tubular short time high temperature pasteurizers were found to be well suited to the preservation of vitamin C in milk. There was no significant destruction of vitamin C during the pasteurizing process, and very little loss when the milk had been stored 24 hours after pasteurization.

The 30 minute holding process of pasteurization as investigated in five different types of pasteurizers was found not to be well adapted to the preservation of vitamin C in milk. Excessive losses were found even when contamination with copper was reduced to a minimum. The losses were frequently serious during the storage period after pasteurization as well as during the pasteurizing process.

Pasteurized milk to which 5 p.p.b. of copper had been added contained less vitamin C 24 hours after pasteurization than check samples without added copper. The addition of 0.1 p.p.m. of iron or 1.0 p.p.m. of either chromium or nickel to the milk produced less effect than 5 p.p.b. of copper. Only in the case of copper did both pasteurization and storage increase the destructive effect of the added metal.

Addition of 0.3 gram of copper daily to the ration produced a significant decrease in the stability of vitamin C in pasteurized milk and probably produced a corresponding increase in the copper content of the milk. Normal variations of copper in the ration would probably not produce any significant effect on the vitamin C content of milk.

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