de Boland, A. R., Garner, G. B., O'Dell, B. L., *J. Agric. Food Chem.* **23**, 1186 (1975).
Early, E. B., Deturk, E. E., *J. Am. Soc. Agron.* **36**, 803 (1944).
Gabriel, O., *Methods Enzymol.* **22**, 569 (1971).
Oberleas, D., Muhrer, M. E., O'Dell, B. L., *J. Nutr.* **90**, 56 (1966).
O'Dell, B. L., Boland, A. R., Koirtyohann, S. R., *J. Agric. Food Chem.* **20**, 718 (1972a).
O'Dell, B. L., Burpo, C. E., Savage, J. E., *J. Nutr.* **102**, 653 (1972b).
Saio, K., Koyama, E., Watanabe, T., *Agric. Biol. Chem.* **31**, 1195 (1967).
Smith, A. K., Rackis, J. J., *J. Am. Chem. Soc.*, **79**, 633 (1957).
Wolf, W. J., Briggs, D. R., *Arch. Biochem. Biophys.* **186**, 85 (1959).

Received for review September 24, 1975. Accepted December 15, 1975. A contribution of the Missouri Agricultural Experiment Station, Journal Series No. 7311. Supported in part by U.S. Department of Agriculture Grant No. 12-14-100-9186 administered by the Agriculture Research Service, Northern Regional Laboratory, Peoria, Ill.

Effect of Dietary Zinc and Copper Interrelationships on Blood Parameters of the Rat

Lalitha Murthy and Harold G. Petering*

The effects of varying both dietary zinc and copper on blood chemistry in male rats fed a semipurified diet were studied. Hematocrit and hemoglobin were directly related to serum zinc and serum copper levels. Serum glutamate oxaloacetate transaminase (SGOT) values were directly correlated with serum zinc values, and serum uric acid levels were directly related to serum copper levels. Serum cholesterol was inversely related to dietary copper as well as to serum copper values. Since there was no significant relationship of dietary zinc or serum zinc with serum cholesterol it was not surprising to find a direct relationship between serum cholesterol and the ratio of serum zinc/serum copper.

Because of our finding that cadmium, lead, and certain chemicals can affect zinc and copper metabolism in rats (Klauder and Petering, 1976; Klauder, 1975; Book et al., 1973; Murthy et al., 1972, 1976; Petering et al., 1967, 1971; Rice et al., 1974) we have been interested in establishing dose–response relationships to zinc and copper nutriture when these are varied within the suboptimal and optimal dietary ranges. This is important since an animal model for evaluating the toxicity of some environmental chemicals depends on controlling dietary intake of protective nutrients, in this case the essential trace metals. We have recently reported the nutritional interaction of varying zinc and copper as this is reflected in the serum and tissue levels of these two essential elements (Murthy et al., 1974), and we now wish to present evidence of interaction of these elements on several blood parameters which are frequently assessed in studying biological effects of environmental toxicants.

MATERIALS AND METHODS

The details pertaining to materials and design of the experiment were described in detail in a previous communication (Murthy et al., 1974). Twenty groups of three weanling Carworth strain male rats were fed a semipurified diet low in zinc and copper. Dietary zinc (zinc acetate) and copper (copper sulfate) were given in distilled deionized water at the following concentrations (μg/ml): 2.5, 5.0, 10.0, 20.0, and 40.0; and 0.25, 0.50, 1.00, and 2.00, respectively. A 5 × 4 factorial design for the 20 groups of animals was used. Food and water were available ad libitum. The experiment lasted for 60 days. Hematocrit, hemoglobin, white blood cell count, lymphocytes, and granulocytes were determined prior to kill or at necropsy by the routine clinical laboratory procedures. At necropsy the animals were anesthesized with phenobarbital sodium, blood was drawn by cardiac puncture, sera were separated, and the serum profile was obtained using an Auto Technicon sequential multi-analyzer (SMA 12) in which calcium, inorganic phosphorus, glucose, total protein, albumin, alkaline phosphatase, serum glutamate oxaloacetate transaminase (SGOT), uric acid, and cholesterol values were determined. For purposes of obtaining correlation between blood and/or serum profiles, serum copper and serum zinc values which were published earlier are referred to.

Statistical Calculations. The statistical technique of analysis of variance was used to investigate the effects of alterations in dietary zinc and copper and their interaction on one another (Snedecor and Cochran, 1967). Subsequent comparisons testing individual effects (where appropriate) were carried out and then significance judged by Tukey's paired comparison test (Winer, 1962). Associations between the following measurements were investigated by calculating their correlation coefficients: hematocrit and hemoglobin; hematocrit and serum zinc; hematocrit and serum copper; hemoglobin and serum copper; hemoglobin and serum zinc; cholesterol and serum copper; cholesterol and serum zinc; cholesterol and the ratio of serum zinc to serum copper; cholesterol and the ratio of dietary zinc to dietary copper; SGOT and serum zinc; and uric acid and serum copper. (The values of serum zinc and copper were taken from the previously published paper; Murthy et al. (1974).)

RESULTS

Blood and Serum Parameters Showing No Variation Due to Dietary Zinc or Copper. Analyses of variance of the data for white blood cell count, lymphocytes, granulocytes, calcium, inorganic phosphorus, glucose, total protein, albumin, and serum alkaline phosphatase relating to the dietary levels of zinc and copper were done and found to be in the normal range for rats without any variation due to dietary zinc and copper.

Parameters Showing Variation Due to Alteration in the Levels of Dietary Zinc and/or Copper. *He-*

Kettering Laboratory, Department of Environmental Health, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267.

Table I. Mean Values of Hematocrit[a]

Cu concn, μg/ml of water	% volume of erythrocytes at Zn concn (μg/ml of water)					
	2.5	5.0	10.0	20.0	40.0	Means[b]
0.25	55.33	53.00	47.33	51.00	48.00	50.93[a]
0.50	55.67	50.33	50.67	48.33	46.00	50.20[a]
1.00	54.00	54.67	49.33	52.67	51.00	52.33[a]
2.00	58.00	51.00	50.00	53.00	51.00	52.60[a]
Means[b]	55.75[a]	52.25[ab]	49.33[b]	51.25[b]	49.00[b]	

[a] Analysis of variance showed that hematocrit values vs. zinc intake was significant ($p < 0.001$). [b] Different roman superscripts along a vertical column or horizontal line denote significant differences ($p < 0.05$).

Table II. Mean Values of Hemoglobin[a]

Cu concn, μg/ml of water	g/100 ml of blood at Zn concn (μg/ml of water)					
	2.5	5.0	10.0	20.0	40.0	Means[b]
0.25	17.50	16.77	13.93	16.07	15.27	15.91[a]
0.50	17.23	17.13	16.10	14.30	15.23	16.00[a]
1.00	17.97	16.90	15.93	16.07	16.53	16.68[a]
2.00	18.97	17.83	16.33	17.03	15.27	17.09[a]
Means[b]	17.92[a]	17.16[ab]	15.57[c]	15.87[bc]	15.57[c]	

[a] Analysis of variance showed that hemoglobin values vs. zinc intake was significant ($p < 0.01$). [b] Different roman superscripts along a vertical column or horizontal line denote significant differences ($p < 0.05$).

matocrit. Table I shows the effects of dietary zinc and copper on hematocrit. Analysis of variance of hematocrit data showed a significant variation in hematocrit due to dietary levels of zinc ($p < 0.001$). The decreasing linear trend between hematocrit and serum zinc was tested by means of the correlation coefficient and found to be $r = -0.556$ ($0.01 < p < 0.02$, $N = 20$).

Although analysis of variance of the hematocrit data did not show an effect due to variation in dietary copper, a positive correlation was found between hematocrit and serum copper, $r = 0.495$ ($p < 0.05$, $N = 20$). This indicates that there is evidence of the influence of both zinc and copper on hematocrit as we have reported earlier, namely, that serum copper level is influenced by dietary zinc levels despite the fact that statistical interactions are not significant (Murthy et al., 1974).

Hemoglobin. The data in Table II show the relationship between hemoglobin and dietary zinc and copper. Analysis of variance of the results showed that hemoglobin values like hematocrit were inversely related to dietary zinc ($p < 0.01$), but the negative correlation coefficient between hemoglobin and serum zinc was significant only at the 10% level ($r = -0.399$, $N = 20$). Although analysis of variance showed no significant relationship between dietary copper and hemoglobin, a significant positive correlation $r = 0.564$ ($p < 0.01$, $N = 20$) between serum copper and hemoglobin was obtained. So again, there is evidence of hemoglobin being influenced by both dietary zinc and copper just as in the case of hematocrit.

When hemoglobin and hematocrit values were correlated a high positive correlation $r = 0.817$ ($p < 0.001$, $N = 20$) was obtained. Thus, it is obvious that zinc and copper are antagonistically related with the hematopoietic process in the physiological dietary range of intakes.

Serum GOT. Analysis of variance of the relationships among dietary zinc and copper intakes and SGOT levels (Table III) showed a significant variation with zinc ($p = 0.05$). In addition, a significant correlation coefficient $r = 0.624$ ($p < 0.01$, $N = 20$) was obtained between SGOT and serum zinc. Physiologically, these relationships are important, because they suggest that zinc may be involved in the transamination reactions.

Serum Uric Acid. The results showing variations in serum uric acid in relation to dietary levels of zinc and copper are presented in Table IV. Analysis of variance

Table III. Mean Values of SGOT[a]

Cu concn, μg/ml of water	mU/ml of serum at Zn concn (μg/ml of water)					
	2.5	5.0	10.0	20.0	40.0	Means
0.25	165.3	167.3	198.0	135.3	150.0	163.2
0.50	190.3	182.0	170.7	153.3	174.7	174.2
1.00	184.0	167.3	204.0	171.3	151.3	175.6
2.00	140.0	217.3	243.0	178.7	159.3	187.1
Means	169.9	183.5	203.9	159.7	158.8	

[a] Analysis of variance showed that SGOT varied significantly with dietary zinc levels ($p = 0.05$).

Table IV. Mean Values of Serum Uric Acid[a]

Cu concn, μg/ml of water	mg/100 ml of serum at Zn concn (μg/ml of water)					
	2.5	5.0	10.0	20.0	40.0	Means[b]
0.25	3.60	2.73	3.07	2.33	3.00	2.95[a]
0.50	3.50	2.35	2.77	2.80	3.13	2.91[ab]
1.00	3.17	2.27	2.33	2.17	2.27	2.44[b]
2.00	1.70	2.33	3.10	2.00	2.23	2.27[b]
Means[b]	2.99[a]	2.42[a]	2.82[a]	2.32[a]	2.66[a]	

[a] Analysis of variance showed that serum uric acid varied significantly with dietary copper levels ($p < 0.05$). [b] Different superscripts along a vertical column or horizontal line denote significant differences ($p < 0.05$).

of individual data showed that serum uric acid varied significantly with increased dietary copper ($p < 0.05$) but not with dietary zinc. When group mean values of serum uric acid were correlated with serum copper a significant negative correlation coefficient $r = -0.526$ ($p = 0.02$, $N = 20$) was obtained.

Serum Cholesterol. We had previously reported that serum cholesterol is inversely related to dietary copper and that dietary zinc enhances this relationship. Therefore, it was of interest to examine this relationship under the present experimental conditions. The group means of serum cholesterol are presented in Table V. Analysis of variance of all individual values ($N = 56$) showed no significant variation due to dietary zinc; there was, however, a significant decrease in serum cholesterol as dietary copper was increased ($p < 0.01$). None of the interaction terms were significant. When cholesterol group

Table V. Mean Values of Serum Cholesterol[a]

Cu concn, μg/ml of water	mg/100 ml at Zn concn (μg/ml of water)					
	2.5	5.0	10.0	20.0	40.0	Means[b]
0.25	124.0	133.3	146.7	151.3	135.3	138.1[a]
0.50	120.7	131.0	122.7	121.7	108.0	120.8[ab]
1.00	98.7	100.7	100.0	109.3	111.3	106.0[b]
2.00	74.0	100.7	127.5	102.7	114.0	103.8[b]
Means[b]	104.3[a]	116.4[a]	126.7[a]	121.2[a]	117.2[a]	

[a] Analysis of variance showed that serum cholesterol vs. copper intake was significant ($p < 0.01$). [b] Different superscripts along a vertical column or horizontal line denote significant differences ($p < 0.05$).

mean values were correlated with serum copper values a correlation coefficient $r = -0.782$ was obtained and found to be significant ($p < 0.01$, $N = 20$). No such significant relationships, however, were observed between serum cholesterol and serum zinc ($r = 0.276$, $p < 0.1$, $N = 20$). In view of the reports (Klevay, 1973, 1975) that serum cholesterol is positively related to the dietary zinc/dietary copper ratio, we decided to examine the relationship between dietary zinc/dietary copper and serum zinc/serum copper with cholesterol. Indeed, our results show a positive correlation $r = 0.462$ ($p < 0.05$, $N = 20$) for dietary zinc/dietary copper and a positive correlation $r = 0.484$ ($p < 0.05$, $N = 20$) for serum zinc/serum copper. These results are to be expected if one remembers that there was no variation in cholesterol with changes in dietary zinc or serum zinc (cf. Table V) while a highly significant correlation between serum copper and serum cholesterol was found. Therefore, when ratios are formulated between the pairs of either serum zinc and serum copper or dietary zinc/dietary copper they obviously must show a positive correlation with serum cholesterol.

DISCUSSION

By carefully controlling the intake of copper and zinc of male Carworth rats, it has been possible to show that in the suboptimal to optimal levels there are changes in hemoglobin, hematocrit, SGOT, serum uric acid, and cholesterol. These changes are due to variations in the dietary intake of zinc and copper and which in turn are associated with serum levels of these essential microelements. In the case of hemoglobin and hematocrit there was definite evidence of interaction between these parameters and dietary zinc and copper intake. Similar results were reported previously from our laboratory (Petering et al., 1971). The effect of zinc on hemoglobin may be due to a suppressive effect of zinc on the function, utilization, or absorption of copper as has previously been noted. Thus, the stimulatory effect of copper on hemoglobin is to be expected in the light of the known action of ceruloplasmin in the mobilization of iron for porphyrin synthesis (Osaki et al., 1966). The inhibitory influence of zinc on hematocrit is not so readily explained unless it has an effect on the size of the red blood cells, which was not determined, since it would seem that zinc intake should be directly related to red blood cell production, i.e., protein synthesis and cell proliferation.

These data do show, however, that the effect of high levels of zinc to produce anemia is an extension of a phenomenon which can be identified in the physiological range of zinc nutriture, namely, the fact that increasing dietary zinc inversely affects hemoglobin and hematocrit. It can be inferred from these observations that supplementation of iron alone to prevent anemia is not sufficient unless there are optimal levels of dietary zinc and copper (Klauder, 1975; Klauder and Petering, 1976).

The reduction of serum uric acid with increased dietary copper and serum copper could be understood on the basis of increased levels of either tissue or serum uricase which is a copper metalloenzyme (Mahler et al., 1955; Baum et al., 1956). Thus, it would be of interest to investigate whether the administration of superoptimal levels of copper (10–100 μg/ml of water) would further reduce serum uric acid levels and in fact affect serum uricase levels.

The relationship which was found in this experiment between copper intake or serum copper and serum cholesterol levels is in agreement with our earlier report (Murthy et al., 1972). The fact that serum cholesterol levels are inversely related to serum copper concentration and greatly influenced by copper nutrition is of importance in both the design and the interpretation of experiments relating serum cholesterol to atherosclerosis and cardiovascular diseases, both in animals and man. These data raise questions about the meaning of nutritional work on serum cholesterol where the copper nutriture was not controlled, or where copper antagonistic agents may have been present.

In addition, our data indicate that when serum copper was low due to low intake of copper then an excessive intake of zinc tended to elevate serum cholesterol levels due to zinc–copper interrelationships. As pointed out earlier, it is very important to emphasize that neither dietary zinc nor serum zinc was directly related to serum cholesterol even though serum cholesterol was correlated with serum zinc/serum copper ratios. Therefore, we suggest that dietary copper governs the metabolic fate of cholesterol more than the zinc/copper ratio does. This is not in agreement with other reports (Klevay, 1973, 1975) and in fact may be an explanation of the results published therein. Furthermore, it would be of interest to determine whether the zinc/copper relationship in the diet will hold true at very high dietary levels of zinc and copper since Klevay also has only used diets relatively low in copper.

ACKNOWLEDGMENT

We wish to acknowledge the advice of Linda Levin in the statistical treatment of the data, and the assistance of Patricia Coburn in carrying out some of the experiments, and K. L. Stemmer for SMA-12 analytical results. We thank L. M. Klevay for suggestions relating to nutritional design of this experiment.

LITERATURE CITED

Baum, H., Mahler, H. R., Hubscher, G., *Biochim. Biophys. Acta* **22**, 528 (1956).

Book, R., Murthy, L., Shirley, T., Srivastava, L., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **Abst. No. 1381, 32**, 468 (1973).

Klauder, D. S., Ph.D. Thesis, submitted to University of Cincinnati, Department of Environmental Health, Cincinnati, Ohio, 1975.

Klauder, D. S., Petering, H. G., *Environ. Health Perspect.*, in press (1976).

Klevay, L. M., *Am. J. Clin. Nutr.* **26**, 1060 (1973).

Klevay, L. M., *Am. J. Clin. Nutr.* **28**, 764 (1975).

Mahler, H. R., Hubscher, G., Baum, H., *J. Biol. Chem.* **216**, 625 (1955).

Murthy, L., Highhouse, S., Levin, L., Petering, H. G., *Trace Subst. Environ. Health, 9, Proc. Univ. Mo. Annu. Conf.*, 9th, in press (1976).
Murthy, L., Klevay, L. M., Petering, H. G., *J. Nutr.* **104**, 1458 (1974).
Murthy, L., O'Flaherty, E., Petering, H. G., presented at the 9th International Congress of Nutrition, Mexico City, Mexico, Sept 1972, p 136.
Murthy, L., Sorenson, J. R. J., Petering, H. G., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **Abst. No. 31**, 2726 (1972).
Osaki, S., Johnson, D. A., Friedman, E., *J. Biol. Chem.* **241**, 2746 (1966).
Petering, H. G., Johnson, M. A., Stemmer, K. L., *Arch. Environ. Health* **23**, 93 (1971).
Petering, H. G., et al., *Cancer Res.* **27**, 1115 (1967).
Rice, D. P., Murthy, L., Shirley, T., Menden, E., *Trace Subst. Environ. Health, 7, Proc. Univ. Mo. Annu. Conf., 7th*, 305 (1974).
Snedecor, G. W., Cochran, G. W., "Statistical Methods", 6th ed, Iowa State College Press, Ames, Iowa, 1967, p 163.
Winer, B. J., "Principles in Experimental Designs", McGraw-Hill, New York, N.Y., 1962.

Received for review November 24, 1975. Accepted April 20, 1976. This work was supported by Grant ES-00159. This is the third of a series of papers dealing with zinc metabolism; see Murthy et al. (1974).

Determination of Chromium in Several Proposed Standard Samples and of Zinc and Chromium in Wheat Milling and Beet Sugar Refining Samples

James J. Christensen,* Patrick A. Hearty, and Reed M. Izatt

The following chromium concentrations have been determined for the indicated proposed biological standards (values in nanograms per gram are given in parentheses): NBS bovine liver (50.1), NBS/NIOSH freeze-dried urine (2.42), Doisy's serum (2.55), Bowen's standard kale (344), and Tascosa wheat coarse ground (22.8) and fine ground (45.1). The refining processes for flour and sugar beets were examined to determine the steps at which zinc and chromium were removed. Values of zinc and chromium were determined to be 116 and 2.15 ng/g in refined sugar, and 5180 and 33 ng/g in refined flour.

A growing awareness of the importance of trace elements in biochemical processes and in nutrition has stimulated interest in the determination of their concentration in biological materials. The biologically important trace elements include zinc, which is known to be an essential nutrient and a cofactor in numerous enzyme systems (Parisi and Vallee, 1969), and chromium, which has been suggested to be essential for proper carbohydrate metabolism (Mertz, 1975). Zinc is found in sufficient quantities, in most biological materials, that its determination poses no special problems (Christian and Feldman, 1970). On the other hand, while numerous methods are available for chromium analysis on a macro scale (Bachman and Banks, 1969), its determination in biological samples has been difficult and unreliable (Parr, 1974). Much of this difficulty is due to the fact that this element occurs in extremely low levels in most biological materials. The controversy over the nutritional essentiality of chromium and the questions concerning its mechanism of action are not likely to be resolved until accurate measurement of its concentration in such materials can be made. Since chromium occurs in biological materials in the range of nanograms per gram, accurate determinations were not possible until recent years. Even with the application of modern analytical methods, such as atomic absorption, gas chromatography, and neutron activation, very little agreement among researchers has been produced. The variation among reported values for chromium in human blood and its constituents has been discussed by Underwood (1971), and similar discrepancies have been noted in values obtained from the analysis of other biological and environmental samples (McClendon, 1974). One is, therefore, led to question much of the published data concerning chromium in biological materials.

The removal of trace elements during food processing may affect adversely the nutrition of much of the population of the industrialized nations (Mertz, 1972). Refined sugar and flour are two products from which trace elements, including zinc and chromium, are largely removed during processing (Schroeder, 1971). The implications of this removal may be rather far reaching, as these food products are consumed in considerable amounts by a large segment of the population.

In the present study, procedures for sample preparation and analysis by the method of flameless atomic absorption are reported, along with chromium values for several proposed reference materials of biological origin. In addition, the refining processes for flour and sugar beets were examined to determine the steps at which zinc and chromium were removed.

EXPERIMENTAL SECTION

Materials. The following reagent grade chemicals were used: HNO_3 (Mallinckrodt), $HClO_4$ (Baker and Adamson), HCl (J. T. Baker), 2 M tetramethylammonium hydroxide (TMAH) in methanol (Southwestern Analytical Chemicals), $ZnSO_4$, and K_2CrO_4. All water used was doubly distilled and deionized. The following proposed biological reference standards were obtained from the sources indicated: bovine liver (National Bureau of Standards, SRM 1577), freeze-dried urine (NBS/NIOSH, supplied by J. O. Pierce, University of Missouri, Columbia, Mo., 1974), Doisy's serum (J. R. Doisy, State University of New York at Syracuse, 1974), Bowen's standard kale (H. J. M. Bowen, Reading University, Reading, Berks., U. K., 1974), and

Departments of Chemical Engineering (J.J.C.) and Chemistry and Contribution No. 70 from the Center for Thermochemical Studies, Brigham Young University, Provo, Utah 84602.