Effect of Zinc on Cytochrome Oxidase Activity. (20439)

G. D. DUNCAN, LOUISE F. GRAY AND LOUISE J. DANIEL. (Introduced by L. C. Norris)

From the U. S. Plant, Soil and Nutrition Laboratory, Bureau of Plant Industry, Soils and Agricultural Engineering, A.R.A. and the Department of Biochemistry and Nutrition, Cornell University, Ithaca, N. Y.

Excess dietary zinc causes a hypochromic, microcytic anemia in rats(1-3). The anemia is wholly or partially prevented by feeding additional copper(2,3). The need for copper in hemoglobin formation was discovered by Hart, Steenbock, Waddell and Elvehjem(4). Cohen and Elvehjem(5) and Schultze(6,7) reported that cytochrome oxidase activity was decreased in livers, hearts and bone marrow of rats fed a copper-deficient diet. Schultze and Kuiken(8) found that liver catalase activity was also decreased in copper deficiency. Thus, it is evident that copper is concerned with the formation of iron-porphyrin compounds.

Since zinc has been shown to induce a copper deficiency state in rats, this investigation was undertaken to determine the effect of dietary zinc on the activity of cytochrome oxidase in heart tissue.

Experimental. Animal studies. Rats originating from the Sprague-Dawley strain were started on experimental diets at the age of 21 to 25 days. Six rats (3 males and 3 females) were placed on each of 7 treatments. The basal diet was whole milk powder* to which mineral supplements of ferrous sulfate, cupric sulfate and manganous sulfate had been added. The rats on the basal diet received approximately 0.06 mg of copper, 0.38 mg of iron and 1 mg of manganese per day as a result of this supplementation. The following diets were fed *ad libitum* for a period of 5 weeks: 1) Basal, 2) Basal + 1.0% zinc, 3) Basal + 1.5% zinc, 4) Basal + 0.03% copper, 5) Basal + 1.0% zinc + 0.03% copper, 6) Basal + 1.0% zinc + 1 mg % folic acid, 7) Stock diet.† A-D Percomorph Liver Oil was administered weekly to each rat by dropper. Growth and hemoglobin were determined at the end of the experimental period. The animals were then sacrificed and cytochrome oxidase activity of the heart tissue was measured.

Enzyme studies. The cytochrome oxidase activity was determined as follows. The animal was decapitated and the heart removed at once. It was divided longitudinally and one half of it placed in 5.0 ml of ice cold 0.1 M potassium phosphate buffer, pH 7.2. The tissue was homogenized for 2 minutes in a Potter-Elvehjem glass homogenizer surrounded by an ice-water bath, and the sample was then diluted to the desired concentration with 0.1 M phosphate buffer. The diluted homogenate was allowed to stand exactly 5 minutes in an ice-water bath to permit large particles to settle out before pipetting into the reaction vessels. The enzyme activity was determined by the manometric method of Slater(9) with some minor modifications of reagent concentrations. Four concentrations of cytochrome *c* (Sigma Chemical Co.) were used at each of two levels of enzyme for a complete determination. The ferrocytochrome *c* was standardized spectrophotometrically using a molecular extinction coefficient at 550 mμ of 27,840(10). The test system consisted of 1.0 ml of 0.2 M potassium phosphate buffer (pH 7.2), 0.3 ml of 4 x 10^{-3} M aluminum chloride, 0.3 ml of 0.114 M sodium ascorbate, 1.0, 0.8, 0.7 or 0.6 ml of 1.94 x 10^{-4} M cytochrome *c*, and 0.2 or 0.3 ml of enzyme suspension containing approximately 1 mg of organic matter per ml. The volume of the reaction mixture was made to 3.0 ml by additions of heavy-metal-free water. The enzyme suspension was added last. Sodium hydroxide was found to be unnecessary in the center well of the Warburg flask. After a 10-minute equilibration period, oxygen consumption was measured at 10-minute intervals for a 40-minute period in a Warburg apparatus at 37°, with air as the gas phase. The activity was expressed as oxygen uptake in microliters

* Klim, The Borden Co., New York City.

† Big Red G.L.F. Dog Food Pellets, Cooperative G.L.F. Marketing Service, Inc., Canandaigua, N. Y.

TABLE I. Effect of Treatment on Growth, Hemoglobin Level and Cytochrome Oxidase Activity of Rats.*

Treatment	Wt change, g	Hemoglobin, g/100 ml	Cytochrome oxidase activity, Qo_2†
1. 0‡	149	14.1	1699
2. 1.0% zinc	101	5.5	500
3. 1.5% "	80	4.5	282
4. 0.03% Cu	146	13.8	1774
5. 1.0% zinc + 0.03% Cu	113	9.7	2304
6. 1.0% zinc + 1 mg % folic acid	111	5.0	391
7. Stock diet	161	14.2	1885

* Duration of animal experiment was 5 wk. All values are the mean for 6 animals, 3 males and 3 females.

† $Qo_2 = \mu l$ of oxygen uptake/hr/mg of organic matter calculated to infinite concentration of cytochrome *c*. The tissue used was heart.

‡ Mineralized whole milk powder.

(μl) per hour per mg of organic matter. The *organic matter* was determined by the method of Johnson(11), using a 0.1% solution of sucrose as the standard. Duplicate 0.4 ml aliquots of the suspension were taken at the time of pipetting the homogenate into the Warburg vessels, and placed in pyrex test tubes. The color developed was read in a filter colorimeter using Corning filters 3389, 3389, 5113 (½ std. thick) with a transmission peak at 440 mμ with a spread of approximately 75 mμ at the 1% level. The Qo_2 (organic matter) was determined at each level of cytochrome *c*. The reciprocals of the four Qo_2 values were plotted against the reciprocals of the corresponding cytochrome *c* concentrations on rectangular coordinate paper and extrapolated to zero [infinite cytochrome concentration](9).

Results and discussion. The adverse effect of excess dietary zinc on growth and hemoglobin formation in the young rat has been confirmed in this study. These results are presented in Table I. A marked growth retardation was observed at both the 1.0 and 1.5% levels of zinc ($P < 0.01$). The addition of copper to the diet containing 1.0% zinc improved growth significantly ($P < 0.05$), although this improved growth was still considerably below that obtained for the animals on the basal diet. Since Albert(12) has shown folic acid to be a good chelating agent for heavy metals, this compound was included in one of the treatments to learn if it would reduce the toxic effect of zinc. The results presented indicate that it was without effect.

A level of 1.0% zinc in the diet resulted in a reduction in hemoglobin concentration of about 40% of normal ($P < 0.01$) and the addition of 1.5 zinc caused a further significant decrease ($P < 0.05$). The addition of 0.03% copper to the diet containing 1.0% zinc was effective in partially preventing the anemia ($P < 0.01$). Folic acid had no effect on the hemoglobin level, as might be expected in a microcytic anemia.

The effect of excess dietary zinc on cytochrome oxidase activity is presented in Table I. The results show the enzyme activity was decidedly lower in the presence of excess dietary zinc ($P < 0.01$). When copper alone was added to the basal diet, no change in enzyme activity occurred; however, the addition of copper to the 1.0% zinc diet actually caused an increase in cytochrome oxidase activity above that of the basal animals ($P < 0.01$). The following is a possible explanation of this increase. An anemic animal with a low concentration of hemoglobin must circulate the blood more frequently in an attempt to supply the tissues with the oxygen needed for metabolism. This necessitates a marked increase in heart action. The higher-than-normal enzyme activity may have been the result of the increased need on the part of the heart tissue itself for those enzymes involved in energy production. In this group of rats (copper and zinc) a moderate anemia existed, although the copper supply was adequate for high cytochrome oxidase activity. It is evident from these results that the synthesis of cytochrome oxidase takes precedence over that of hemoglobin. This was also an earlier observation of Schultze(7).

Folic acid did not alter the effect of zinc on cytochrome oxidase activity, and hence these values serve as additional data confirming the deleterious effect of zinc on the activity of this enzyme.

The cytochrome oxidase activity observed in the rats on the stock diet was greater than that of the rats on the basal diet. It is improbable that this difference was due to a higher copper content in the stock diet, since

the enzyme activity was not changed when 0.03% copper was added to the basal diet.

It was believed important to eliminate the possibility that the presence of ionic zinc in the homogenates was the cause for the low cytochrome oxidase activity. The concentration of zinc in the heart tissue of the animals on the 1.0% zinc diet was determined by the method of Parks, *et al.*(13). This level of zinc, 80 μg per g of heart tissue (dry weight), was added *in vitro* to heart homogenates of normal rats. No reduction in cytochrome oxidase activity was observed, indicating that the effect of zinc was not occurring in this manner. It is of interest to note that the copper content (13) of the hearts of animals receiving 1.0% zinc was 40% of the normal value. [Normal, 27 μg per g; 1.0% zinc, 10 μg per g (dry weight).]

The coefficient of correlation was determined for the relation between the hemoglobin values and cytochrome oxidase activities. There was a highly significant correlation between these sets of values, $r = 0.79$.

The exact nature of the action of zinc on hemoglobin and cytochrome oxidase formation is not known, although sufficient evidence exists to show that excess dietary zinc induces a copper deficiency state. Since many metals form chelates with protoporphyrin, it may be that zinc is competing with copper in some manner in the formation of iron porphyrins.

Addendum. While this paper was in preparation an abstract was published in the *Fed. Proc., 12,* 283 (1953), describing similar studies by Van Reen and Pearson. Their findings, although on rat liver instead of heart tissue, are in agreement with the results of the studies presented here.

Summary. 1. Excess dietary zinc resulted in lowered growth, hemoglobin concentration and cytochrome oxidase activity in the rat. 2. The addition of copper to the zinc diet increased growth and hemoglobin, but to suboptimum levels, whereas the cytochrome oxidase activity was raised to a greater than normal level. 3. A highly significant correlation was found between hemoglobin level and cytochrome oxidase activity. 4. The inhibitory effect of zinc was not due to the presence in the homogenate of free zinc ions, since the *in vitro* addition of inorganic zinc to normal rat hearts in amounts equivalent to that found in the zinc-toxic hearts had no effect on the activity of cytochrome oxidase.

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Received June 22, 1953. P.S.E.B.M., 1953, v83.