EFFECT OF ASCORBIC ACID ON COPPER DEFICIENCY IN RATS

by

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>vi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>METHODS AND MATERIALS</td>
<td>9</td>
</tr>
<tr>
<td>Determination of Hemoglobin Concentration</td>
<td>13</td>
</tr>
<tr>
<td>Determination of Serum Alkaline Phosphatase</td>
<td>13</td>
</tr>
<tr>
<td>Determination of Serum Copper</td>
<td>14</td>
</tr>
<tr>
<td>Determination of Liver Copper</td>
<td>15</td>
</tr>
<tr>
<td>RESULTS</td>
<td>16</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>24</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>34</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>35</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Composition of Basal Ration</td>
<td>10</td>
</tr>
<tr>
<td>2. Vitamin Mixture Ingredients</td>
<td>11</td>
</tr>
<tr>
<td>3. Mineral Mixture Ingredients</td>
<td>12</td>
</tr>
<tr>
<td>4. Effect of Ascorbic Acid on Body Weight Gain, Serum Alkaline Phosphatase Activity and Hemoglobin Concentration in Rats Fed Copper-deficient and Copper-supplemented Rations</td>
<td>17</td>
</tr>
</tbody>
</table>
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Effect of Ascorbic Acid on Average Body Weight Gain in Rats Fed Copper-deficient and Copper-supplemented Rations.</td>
<td>18</td>
</tr>
<tr>
<td>2.</td>
<td>Effect of Ascorbic Acid on Hemoglobin Concentration in Rats Fed Copper-Deficient and Copper-supplemented Rations.</td>
<td>20</td>
</tr>
<tr>
<td>3.</td>
<td>Effect of Ascorbic Acid on Serum Alkaline Phosphatase Activity in Rats Fed Copper-deficient and Copper-supplemented Rations.</td>
<td>23</td>
</tr>
<tr>
<td>4.</td>
<td>Effect of Ascorbic Acid on Serum and Liver Copper Concentration in Rats Fed Copper-deficient and Copper-supplemented Rations.</td>
<td>30</td>
</tr>
</tbody>
</table>
ABSTRACT

In this experiment, the effect of 3% ascorbic acid on copper deficiency in albino rats was studied. Copper-deficient rats with or without ascorbic acid weighted about 25% less than the copper-supplemented rats. Ascorbic acid had no significant effect on weight gain in either copper-deficient or copper-supplemented rats.

The hemoglobin concentration in the blood of the copper-deficient rats was very low. There was a difference of about 10.16 gm/\% at the end of the experiment between the copper-supplemented and the copper-deficient rats. Ascorbic acid had no significant effect on hemoglobin concentration in all the rats of our experiments.

Serum alkaline phosphatase activity was less than normal in the copper-deficient rats during the early stage of growth after weaning. The decrease in alkaline phosphatase activity was mainly due to old age and copper deficiency.

Ascorbic acid had no specific effect on serum concentration of copper. Liver copper level in the copper-supplemented rats was at least twelve times higher than in rats fed copper-supplemented rations with 3% ascorbic acid. The 3% ascorbic acid was very effective in reducing copper concentration of the liver.
INTRODUCTION

More than 45 years ago, laboratory animals fed a milk ration developed nutritional anemia. Researchers later showed that the nutritional anemia was due to the copper-deficient ration composed of milk.

Over the last four decades, the advances in copper deficiency research was indeed remarkable. In 1931, Elvehjem and Kemmerer (1) developed an improved technique for the production of nutritional anemia in rats. Dreosti and Quicke in 1966 introduced a method for the rapid induction of copper deficiency in adult rats (2).

It had been speculated that copper could be an important substance in metabolism. Elvehjem and Hart (3), in 1929, suggested that copper was essential for hemoglobin biosynthesis in chicks. Hill and Matrone (4) showed that young chicks fed a copper-deficient ration containing adequate amounts of iron developed the nutritional anemia. It is possible that copper had an important physiological role. Other factors such as cadmium, molybdenum, sulfate and zinc also influenced the function of copper. In some animals, the amount of copper intake also significantly influenced the level of copper in the blood and liver. This happened in animals fed either toxic or copper-deficient rations (5, 6). However liver copper levels in rats were rather constant if the copper intake was in the range of 10 to 100 ppm per day.
Approximately 200 ppm dietary copper per day was needed to elevate liver copper level from about 20 ppm to 68 ppm dry weight (7).

The severity of copper deficiency could be increased by certain chemicals. The addition of small amount of zinc, iron and cadmium to a copper-deficient ration would induce a more severe copper deficiency in chicks (8). Gray and Daniel (9) showed that the addition of 10 to 50 ppm molybdenum significantly increased the severity of copper deficiency in the copper depleted rats. The symptoms were further intensified when sulfate was added with molybdenum at the same time. However, molybdenum had no effect on the control rats (9). In general, the addition of copper to a copper-deficient ration would correct the copper-deficient anemia in rats, chicks, swine and turkey poults. On the contrary, in certain animals copper-deficient anemia could be developed without restriction of dietary copper intake. According to Lewis and Allcroft (10), lambs and cattle consuming apparently normal amounts of copper, molybdenum and sulfate in their rations eventually developed the copper-deficient anemia. Copper-deficient rats usually had a significantly lower erythrocyte copper level. Rats fed 100 to 200 ppm dietary copper showed a very high level of copper in the erythrocytes (7). Ruminants did not show direct correlation between blood copper and liver copper levels. Underwood confirmed in 1962 that the liver copper levels in cattle ranged from 30 to 1000 ppm while the blood copper remained normal (11). Also, the liver copper in
Sheep varied from 50 to 4000 ppm with little change in blood copper level (12). This apparently suggested that the storage and excretory mechanism in copper metabolism was not the same in different kinds of animals.

The development of copper deficiency in some species increased mortality and weight loss. Starcher (in 13) and Miller (in 14) reported that copper deficiency in chicks and turkey poults was characterized by aortic rupture and a decrease in the intramolecular cross-linking of the elastin. Starcher et al. (13) showed that the increase of elastin content of the aortas of young chicks was directly related to dietary intake of copper in the ration. In the copper-deficient chicks, the lysine concentration of the elastin was 3 times that of the control elastin. The collagen from the copper-deficient chicks was more soluble than the collagen from normal chicks. In copper-deficient pig aortas there was a decrease in elastin and an increase in hexosamine (15). In the normal elastin there was a relatively high concentration of valine (16). Starcher and others (13) suggested that during the periods of rapid elastin biosynthesis, copper was a necessary co-factor.

A dietary deficiency of copper could also cause connective tissue defects and mortality from internal hemorrhages. Simpson and Harms (17) in 1964 reported that about 50% of the young chicks fed a copper-deficient ration died of hemorrhages. Usually, the principal pathologic alteration found in the copper-deficient animals was elastic
fiber degeneration in the aortas. However, the exact cause of this fiber
degeneration was not clearly established. Chicks seemed to be more
susceptible than other animals to copper deficiency and mortality from
aortic rupture. O'Dell et al. (18) showed that the high mortality in
copper-deficient chicks resulted from rupture of major blood vessels.
The aortas of the copper-deficient chicks were larger in proportion
to body weight because they had a thicker than normal aortic wall. The
copper-deficient aortas were significantly higher in water and lipid con-
tents. The concentration of hydroxy-proline, which made up 13% of the
collagen, was nearly 4 times higher in copper-deficient aortic tissue.
The normal chick aortas contained about 47% elastin; the copper-defi-
cient chick aortas had only about 26% elastin. The copper-deficient
aortas accumulated a large fraction of non-elastic, non-collagen protein,
and had an increased concentration of soluble collagen. The percentage
of nitrogen extracted by saline was nearly twice as high in the copper-
deficient chick aortas as in the normal chick aorta. However, these
nitrogenous residues were not collagen protein. The elastin of the cop-
per-deficient aortas also contained a very high concentration of lysine
and significantly less desmosine and isodesmosine (10). Starcher et al.
(13) also showed that the elastin of the aortas of the copper-deficient
chicks solubilized much more rapidly in formic acid than the elastin of
normal aortas.
Carlton and Henderson (19) clearly demonstrated that in copper-deficient chicks the principal gross lesion was massive internal hemorrhage, often accompanied by large clots of blood over the liver and also in the loops of the intestine. Usually the site of fatal hemorrhage was the aorta at the base of the heart or at the level of the adrenal glands. Carlton and Henderson (19) showed that the cause of aortic rupture often appeared to result from weakening of the blood vessel wall following severe and widespread degeneration.

It had been suggested that copper is necessary for the catalysis of elastin and collagen cross-linkage, especially during the active periods of elastin biosynthesis. At the site of collagen biosynthesis, ascorbic acid was also required for effective collagen formation (20). However, Simpson et al. (21) reported that in the copper-deficient and copper-supplemented turkey pouls, the ratio and quantities of amino acid residues in isolated elastin were not significantly different.

Although adequate copper was required for normal growth and metabolism, high levels of copper in rations would cause copper toxicity. DeGoey and Wahlstrom (22) showed that swine fed 500 ppm copper developed large gastric ulcers with profuse bleeding and the liver copper level increased 80-fold. Hematocrit and hemoglobin value were decreased. Gray and Daniel (9) reported similar effect of molybdenum on copper metabolism. Molybdenum had no effect in decreasing the amount of copper stored in the liver of the swine fed high copper
levels. Molybdenum had little effect on weight gain and did not affect copper toxicity. Gipp and Walker (23) demonstrated that young pigs fed a ration containing 80 ppm iron and 250 ppm copper had a significantly lower level of plasma copper, iron and hemoglobin than those fed the 80 ppm iron and 15 ppm copper. They suggested that the damaging effect of high copper level, 250 ppm, fed to young pigs was possibly due to the interference of copper with iron metabolism. Underwood (11) showed that rats fed a ration containing 200 ppm copper developed symptoms of hemolytic jaundice. McMaster (24) showed that the growth of guinea pigs was reduced by the addition of 10 times normal copper or no copper to the rations.

Copper deficiency in animals also affected the activity of certain bone enzymes. It had been demonstrated that there was interrelationship of dietary copper and amine oxidase activity in the formation of vascular and connective tissue (21, 25). Rucker et al (25) reported that in copper-deficient chick bone, amine oxidase activity was reduced 30 to 40% as compared with the normal chicks. Cytochrome oxidase activity was reduced significantly in the mitochondrial fraction of the copper-deficient chick bones. However, bone catalase activity was not affected in the copper-deficient chicks (25). According to Wohlrab (26), copper-deficient rat mitochondria contained a normal concentration of cytochrome b and cytochrome C1. Cytochrome C concentration was
significantly lower than the controls. Cytochrome a and a₃ concentrations were also very much lower than the controls.

Hill and Starcher (27) showed that the addition of 0.3% ascorbic acid to a copper-deficient ration of chicks increased the severity of copper deficiency. Ascorbic acid had decreased the growth rate of copper-deficient chicks as well as aortic elastin formation, and increased mortality. However, ascorbic acid had no effect on the severity of iron and zinc deficiency. Voelker and Carlton (28) also demonstrated that miniature swine fed a copper-deficient ration with 2.5% ascorbic acid developed a severe copper deficiency. The copper deficient miniature swine with added dietary ascorbic acid died about 16 days sooner than did those fed copper-deficient ration without ascorbic acid. Ascorbic acid also increased the severity of copper deficiency as was shown by Hill and Starcher (27). However, Van Campen and Gross (29) demonstrated that the addition of 1% ascorbic acid to a copper-deficient ration did not increase severity of copper deficiency in adult rats. He claimed that liver copper level, mortality and weight gain were not significantly affected by adding 1% ascorbic acid to the rations of adult rats. Dietary ascorbic acid had little or no effect on the excretion of copper in rats. Also, if the copper deficiency was not severe, the addition of ascorbic acid had no effect on mortality.

Simpson et al. (21) showed that turkey poults fed a copper-deficient ration supplemented with 0.3% ascorbic acid had high mortality
from angiorrhexis. The addition of ascorbic acid also reduced the arginine content of the elastin, but increased the other elastin-amino acids.

In the present experiments with albino weaning rats, 3% ascorbic acid was added to copper-deficient and copper-supplemented rations to study the effect of high ascorbic acid on growth rate, hemoglobin concentration, hepatic and serum copper level and serum alkaline phosphatase activity.
METHODS AND MATERIALS

Holtzman male weaning rats, weighing 50-60 gm, were used in the experiments. The rats were housed in individual cages with screen bottoms. The room temperature was maintained at 24.5° C. Drinking water supplied all rats was deionized through an ion-exchange resin after distillation. All rats were fed the basal ration (Tab. 1) for 3 days. Then the rats were divided at random into four groups of six rats. Each group was fed a specific ration as follows:

- Group A - Basal (Tab. 1) + 3% ascorbic acid.
- Group B - Basal ration only.
- Group C - Basal + 20 ppm CuSO₄ + 3% ascorbic acid.
- Group D - Basal + 20 ppm CuSO₄.

All rations were stored in a cold room at -1° C. The ascorbic acid was added to the ration every day during the feeding period. Deionized distill water was mixed with the dry ration at the time of feeding to form a gruel. The rats were fed these rations ad libitum for ten weeks. All rats were weighed at weekly intervals. The air conditioned room was automatically timed to cut off all light from 7 p.m. to 7 a.m. to allow normal night time activity for the rats. The hemoglobin concentration and the serum alkaline phosphatase activity were measured at weekly intervals. Blood was collected from the tail
Table 1. Composition of Basal Ration$^a$

<table>
<thead>
<tr>
<th>Components</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried skim milk</td>
<td>77.6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.0</td>
</tr>
<tr>
<td>Corn oil$^b$</td>
<td>10.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.4</td>
</tr>
<tr>
<td>Vitamin mixture$^c$</td>
<td>1.0</td>
</tr>
<tr>
<td>Mineral mixture$^d$</td>
<td>1.0</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100.0</strong></td>
</tr>
</tbody>
</table>

$^a$ The basal ration contained no CuSO$_4$ and ascorbic acid. These two ingredients are added to the ration as needed.

$^b$ "Mazola" brand corn oil. A product of Best Foods; A division of CPC International Inc., Englewood Cliffs, N. J.

$^c$ Vitamin mixture supply per 100 g diet (see Table 2).

$^d$ Mineral mixture supply per 100 g ration (see Table 3).
<table>
<thead>
<tr>
<th>Components</th>
<th>mg/100 g diet</th>
<th>Components</th>
<th>mg/100 g diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A (^a)</td>
<td>0.35 mg</td>
<td>Riboflavin</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Vitamin D (^b)</td>
<td>1.00 mg</td>
<td>Pyridoxine HCL</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Vitamin E (^c)</td>
<td>20.00 mg</td>
<td>Niacin</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>Vitamin B(_{12})</td>
<td>0.01 mg</td>
<td>Ca-pathothenate</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>Menadione</td>
<td>0.15 mg</td>
<td>Biotin</td>
<td>(0.05) mg</td>
</tr>
<tr>
<td>Thiamine</td>
<td>1.00 mg</td>
<td>Folic acid</td>
<td>0.2 mg</td>
</tr>
</tbody>
</table>

a. Vitamin A crystalline acetate. 0.344 mcg = 1 U.S.P. Product of Nutritional Biochemicals Corporation, Ohio.

b. Vitamin D\(_2\), crystalline form. Equivalent to 500,000 D\(_3\) per gram. Nutritional Biochemicals Corporation, Ohio.

c. \(\alpha\)-Tocopherol. A product of Nutritional Biochemicals Corporation, Cleveland, Ohio.
Table 3. Mineral Mixture Ingredients

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>mg/100 g diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO₄ . H₂O</td>
<td>10 mg</td>
</tr>
<tr>
<td>MnSO₄ . H₂O</td>
<td>5 mg</td>
</tr>
<tr>
<td>KI</td>
<td>5 mg</td>
</tr>
<tr>
<td>ZnSO₄ . 7H₂O</td>
<td>2 mg</td>
</tr>
<tr>
<td>MgSO₄ . 7H₂O</td>
<td>100 mg</td>
</tr>
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vein. Hemoglobin concentration was determined by the cyanmethemoglobin method (30), and serum alkaline phosphatase by the sigma method (31). All rats were sacrificed at the end of the 10th week.

Blood was collected by puncturing the aorta. Heparin was added to the portion of the blood used for hemoglobin determination. Serum was obtained by the regular method of coagulating the blood at 37° C for 30 minutes and then kept in the refrigerator at 5° C for 24 hours. Whole livers were collected for copper determination.

**Determination of Hemoglobin Concentration**

Add 0.02 ml blood to 5.0 ml Hycel cyanmethemoglobin reagent. Mix well and read the optical density at 540 mu against the reagent blank. Record the hemoglobin concentration in gm % by comparing with a standard curve. This method measures the total hemoglobin including the oxyhemoglobin, carboxy-hemoglobin, and metamoglobin. The normal erythrocytes are completely lysed by the Hycel reagent.

**Determination of Serum Alkaline Phosphatase**

Pipette exactly 0.1 ml serum into a test tube containing 0.5 ml sigma 104 phosphatase substrate solution and 0.5 ml sigma alkaline

2. Product of Sigma Chemical Co., St. Louis, Missouri.
buffer solution$^3$ at pH 9.4. Place the mixture in a water bath of 38°C for exactly 30 minutes after adding the serum. Then add 10 ml 0.02 N NaOH to stop the reaction. Measure the optical density at 410 μm. Obtain the alkaline phosphatase concentration from a standard curve.

The function of the sigma 104 phosphatase substrate in the serum is indicated in the following reaction (31, 32):

$$\text{p-nitrophenyl} + \text{H}_2\text{O} \xrightarrow{\text{phosphatase}} \text{p-nitrophenol} + \text{H}_3\text{PO}_4$$

The p-nitrophenyl phosphate compound is colorless in acid or alkali. Upon hydrolysis of the phosphate group, the salt of p-nitrophenyl is yellow in alkali and colorless in acid. One sigma unit of phosphatase will liberate 1 um of p-nitrophenol per hour under the specified condition.

**Determination of Serum Copper**

Mix 1 ml of serum with 5 ml of TCA and set aside for one hour. Then centrifuge for 10 minutes at 2000 r.p.m. and decant the supernatant into a 60 ml cylindrical separatory funnel. Wash the precipitate with an additional 5 ml TCA and centrifuge again at 2000 r.p.m.

for 10 minutes. Add this supernatant to the same separatory funnel along with 1 ml 1% sodium diethyldithio carbamate, 1 ml 1% sodium EDTA and 3 ml methy isobuty ketone (MIBK). Shake the solution for 10 minutes. Discard the lower layer and centrifuge the remaining layer at 2000 r.p.m. for 10 minutes. Run the clear solution through a Perkin-Elmer model 303 atomic absorption spectrophotometer at 3257° A wave length (33, 34).

**Determination of Liver Copper**

Dry the whole liver to constant weight. Wet ash the dry liver with 4 ml of sulfuric acid and sufficient nitric acid to digest the liver (33, 34). Then transfer the digested pale yellow solution to a 25 ml volumetric flask and make to volume with de-ionized water. Chelate and extract 5 ml of this diluted solution as described previously in the preparation of serum copper for atomic absorption spectrophotometry determination (33, 34). Express the concentration of copper in the liver in ppm dry weight basis.
RESULTS

In our experiments, mortality did not occur in rats fed the basal ration supplemented with 20 ppm copper whether ascorbic acid was added to the ration or not. Only one rat in group A (Cu-deficient with ascorbic acid) and one in group B (Cu-deficient without ascorbic acid) died at the seventh week after feeding the copper deficient rations. The exact cause of death was not known. Both rats showed sign of internal hemorrhage and blood clotting around the heart area.

There was an apparent weight loss in those rats fed the copper-deficient rations with or without addition of ascorbic acid (Fig. 1). Weight gain was rather steady in all rats for the first three weeks after weaning. At the end of the fourth week, weight loss appeared in the copper-deficient rats. However, there was no abrupt change of weight as was reported on chicks and miniature swine (27, 28). On the average, the 20 ppm copper-supplemented rats (group C & D) were about 100 gm heavier than the copper-deficient rats (group A & B) at the end of the experiment. The difference in weight gain between group C and D (copper-supplemented) was not obvious. However, in the copper-deficient rats, the average weight of group A (with ascorbic acid) was about 10 gm heavier than group B (without ascorbic acid).
Table 1. Effect of Ascorbic Acid on Body Weight Gain, Serum Alkaline Phosphatase Activity and Hemoglobin Concentration in Rats Fed Copper-deficient and Copper-supplemented Rations.

<table>
<thead>
<tr>
<th>ITEM</th>
<th>UNITS</th>
<th>GROUP&lt;sup&gt;d&lt;/sup&gt;</th>
<th>WEEKS AFTER WEANING&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Body Weight</strong></td>
<td>Gms</td>
<td>A</td>
<td>73.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>75.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>70.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>68.3</td>
</tr>
<tr>
<td><strong>Hemoglobin Conc.</strong></td>
<td>Gm%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>7.1</td>
</tr>
<tr>
<td><strong>Alkaline Phosphatase</strong></td>
<td>Sigma&lt;sup&gt;b&lt;/sup&gt;</td>
<td>A</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>11.81</td>
</tr>
</tbody>
</table>

a. Grams of hemoglobin in 100 ml blood.
b. One Sigma unit equals to 1 um of p-nitrophenol liberated per hour by the alkaline phosphatase.
c. Average values as determined at the end of each week.
d. Group A - Copper-deficient + ascorbic acid.
   Group B - Copper-deficient.
   Group C - Copper + ascorbic acid.
   Group D - Copper.
Figure 1. Effect of Ascorbic Acid on Average Body Weight Gain in Rats Fed Copper-deficient and Copper-supplemented Rations.
Hemoglobin concentration in all groups increased steadily in the first three weeks after weaning (Table 4). In the beginning of the fourth week hemoglobin concentration in the copper-deficient rats (group A & B) began to decrease steadily (Fig. 2). At the end of the experiment, the average value of the hemoglobin level in the 20 ppm copper-supplemented rats (group C & D) was about 10.16 gm % heavier than the average of the copper-deficient rats (group A & B). There was only a slight difference in hemoglobin concentration between the copper-supplemented rats (group C & D).

Serum copper levels in the copper-supplemented rats (group C & D) was much higher than in the copper-deficient rats (group A & B). The serum copper in the copper-supplemented rats without ascorbic acid (group D) was slightly higher than in the copper-supplemented rats with ascorbic acid (group C). However, the difference was not obvious (Table 4).

The liver copper level showed a different picture of the copper status of the rats. Only the control rats (group D) had a high level of copper 17.92 ppm (dry wt.). The rest of the rats (group A, B & C) had liver copper levels of less than 1.6 ppm (Fig. 4). It is interesting to note that the liver copper level in group A rats was slightly higher than in group B & C rats. The copper-supplemented rats with 3% ascorbic acid added to the ration were unable to store copper in their livers as well as did the control rats. The liver copper level in these rats (group
Figure 2. Effect of Ascorbic Acid on Hemoglobin Concentration in Rats Fed Copper-deficient and Copper-supplemented Rations.
C) was as low as in the copper-deficient rats. However, group C did not develop nutritional anemia. Also weight gain and alkaline phosphatase activity were not obviously different from the control rats. It was apparent that the low liver copper level was not a prime factor in inducing copper-deficient anemia. On the other hand, low serum copper level had a direct connection with copper-deficient anemia.

Alkaline phosphatase activity of all rats increased steadily during the first two weeks after weaning (Table 4). Alkaline phosphatase activity of the copper-supplemented rats (group C & D) increased much faster than that of the copper-deficient rats (group A & B). At the end of the fourth week after weaning, the average alkaline phosphatase activity in the copper-deficient rats (group C & D) was 12.7 sigma units; in the copper-deficient rats (group A & B) was only 9.3 sigma units. At the beginning of the fifth week (Fig. 3), the serum alkaline phosphatase of all rats began to decrease rapidly in activity as reported for miniature swine (28). At the end of the experiment, the serum alkaline phosphatase activity of the rats fed the copper-supplemented ration with ascorbic acid (group C) was 8.26 sigma units. Rats fed the copper-supplemented ration without ascorbic acid (group D) was 8.78 sigma units. Copper-deficient rats with ascorbic acid (group A) showed an alkaline phosphatase activity of 6.82 sigma units. The alkaline phosphatase activity of the copper-deficient rats without ascorbic acid (group B) was 7.40 sigma units (Table 4). The alkaline phosphatase
activity between the copper-deficient and the copper-supplemented rats was not as large as that reported for miniature swine (28). It appeared that the decrease of alkaline phosphatase activity during the last week of the experiment was due to age of the animals rather than due to the copper deficiency. However, during the first four weeks, alkaline phosphatase activity in the copper-supplemented rats was about 25% higher than that in the copper-deficient rats whether ascorbic acid was added or not.
Figure 3. Effect of Ascorbic Acid on Serum Alkaline Phosphatase Activity in Rats Fed Copper-deficient and Copper-supplemented Rations.
DISCUSSION

Van Campen and Gross (29) reported that when adult rats were fed either a copper-deficient or a copper-supplemented ration the growth weight was not affected by adding 1% ascorbic acid to the ration. Also 1% ascorbic acid had no effect on liver copper. Mortality seemed to increase only when the rats developed severe copper deficiency. Van Campen and Gross suggested that rats were less susceptible to high levels of ascorbic acid than were chicks, pigs or turkey poult. However, the addition of 1% ascorbic acid to the copper-deficient ration in Van Campen's experiment may have been too low to exert an effect; or perhaps the copper deficiency induced was not severe enough to be detected.

In the experiments reported here, the addition of 3% ascorbic acid to the rations of weaning rats had only a slight effect on the weight gain, serum copper level and hemoglobin concentration. Yet, 3% ascorbic acid was highly effective in depressing liver copper storage in the copper-supplemented rats. There was a 100 gm difference in weight gain between the copper-supplemented and the copper-deficient rats (Table 4). In general, ascorbic acid appeared to have at the most only a slight effect on serum copper and hemoglobin concentration.
Hill and Starcher (27) showed that the addition of 0.1% ascorbic acid to copper-deficient ration fed to chicks induced low weight gains, low aortic elastin and high mortality. Voelker and Carlton (35) reported that the addition of 2.5% ascorbic acid to a copper-deficient and copper-supplemented rations of the miniature swine resulted in decreased weight gain. The swine that were fed a copper-deficient ration with ascorbic acid showed a large drop in weight gain. Carlton and Henderson (36) reported that ascorbic acid decreased growth rate, lowered hemoglobin and hematocrit levels in chicks. Simpson et al. (21) successfully produced a high mortality from vascular ruptures in turkey poults by feeding a copper-deficient ration with added ascorbic acid.

In our experiments, ascorbic acid did not lower weight gain as reported by Voelker and Carlton (35). They showed that addition of ascorbic acid to copper-deficient rations reduced weight gain by about 40 to 50%. In our experiment, copper-deficient rats with 3% ascorbic acid in their rations (group A) weighed about 10 gm more than the copper-deficient rats receiving no ascorbic acid (group B). Also the rats receiving ascorbic acid (group A) appeared to be more active and healthier than those not receiving ascorbic acid (group B). In the copper-supplemented rats, ascorbic acid had no effect in reducing weight gain (Fig. 1). Van Campen and Gross (29) reported similar results for rats.
Although ascorbic acid had no effect on animals fed normal levels of copper, it had damaging effect on the animals fed high levels of dietary copper. McMaster (24) reported that an intake of copper 10 times the normal dietary level (55 mg/kg diet) by the guinea pigs would increase the severity of both liver damage and copper storage mechanism. Livers of these guinea pigs contained approximately 50% less ascorbic acid in their livers than the controls. Guinea pigs fed high copper and 100 times normal ascorbic acid (2 gm/kg diet) suffered severe loss of weight. All guinea pigs received excessive copper and varied levels of ascorbic acid showed outward signs of copper intoxication within one week. Livers of guinea pigs fed excessive dietary copper and ascorbic acid showed large inflammatory areas and fat or glycogen infiltration (24).

Mortality of the rats in these experiments was not apparent. All rats in the copper-supplemented rations of groups C and D survived to the end of the experiment. In the copper-deficient rats two died at the seventh week after weaning. Anyway, the rat population used in our experiment was a little too small for mortality evaluation with statistical reliability. However, Van Campen and Gross (29) showed that 39% of the rats fed a copper-deficient ration died 8 weeks after weaning. The addition of 1% ascorbic acid to the copper-deficient ration increased the mortality to 52%.
Several reports showed that copper deficiency caused a decrease in aortic elastin (13, 17, 37, 38). However, Simpson et al. (21) believed that the low level of aortic elastin in the turkey aorta did not induce high mortality from aortic rupture in the turkey poults. Only when ascorbic acid was added to the copper-deficient ration was there a highly significant increase in mortality from aortic rupture (21). In our experiments, the addition of 3% ascorbic acid to the copper-deficient ration did not seem to effect mortality in the rats. Van Campen and Gross (29) reported that, with severe copper deficiency, all rats died regardless of whether ascorbic acid was added to the ration or not.

In our experiments, hemoglobin concentration in the copper-supplemented groups C and D were much higher than in the copper-deficient groups A and B at the end of the experiment. Ascorbic acid appeared to have a slight effect in maintaining a higher hemoglobin concentration in copper-deficient rats. Although the hemoglobin concentration of groups A and B was not apparently different, the slightly higher hemoglobin concentration in group A indicated a reason why rats in group A weighed more and appeared more active than rats in group B. The results reported here were similar to those reported by Voelker and Carlton (35).

The serum and liver copper levels of this experiment are shown in Table 4. Serum concentration of copper was very much higher in the copper-supplemented rats (group C and D) than in the
copper-deficient rats (group A and B). Serum copper in the copper-deficient rats with ascorbic acid was slightly higher than it was in the copper-deficient rats without ascorbic acid. On the contrary, the serum concentration of the copper-supplemented rats with ascorbic acid (group C) was slightly lower than the copper-supplemented rats without ascorbic acid (group D). However, the difference was not great. In this experiment, the results were similar to those reported by Voelker and Carlton (35). But in the copper-deficient experiment there was some discrepancy. In our experiments, the addition of ascorbic acid to the copper-deficient ration gave slightly higher serum concentration of copper. In Voelker and Carlton's experiment, the addition of ascorbic acid to the ration of copper-deficient swine gave a lower serum concentration of copper. Except during the 12th week to the 14th week in their experiment, the serum concentration of copper rose to a level slightly higher than those copper-deficient swine without ascorbic acid (35).

The liver copper level of copper-supplemented rats without ascorbic acid (group D) was the highest with an average of 17.92 ppm dry weight (Fig. 4). Hepatic copper level of rats in groups A, B and C was less than 1.6 ppm dry weight. The copper-deficient rats without ascorbic acid (group B) had the lowest hepatic copper content with an average of 1.32 ppm. However, it was rather interesting that there was a difference of 16.5 ppm, in the hepatic copper level between the
copper-supplemented rats receiving ascorbic acid and those that did not (group D). Voelker and Carlton (35) reported that hepatic copper level in swine fed 10 ppm copper-supplemented ration with 2.5% ascorbic acid was about one-third lower than in copper-supplemented swine without ascorbic acid. The difference in his and our results could be due to the lower dietary copper in the rations or perhaps a different mechanism in copper storage between the swine and the rats. However, the results reported by Van Campen and Gross (29) did not show significant difference in hepatic copper concentration when ascorbic acid was added to the copper-supplemented rations. The reason was probably because that the 1% ascorbic acid was not high enough to be effective. Also the copper deficiency induced in 8 weeks was perhaps not a long enough period for adult rats of 250 to 350 gm to become copper deficient. Adult rats on a copper deficient ration usually require 2 to 6 months before their copper stores are depleted (39, 40).

In our experiments, 3% ascorbic acid in the rations definitely had some effect on liver copper in young rats. It perhaps even delayed the depletion of serum copper in the copper deficient rats. The hepatic copper levels in the copper-supplemented rats with added ascorbic acid (group C) was apparently as low as in the copper-deficient rats (groups A & B). But the serum copper level of the copper-supplemented rats with ascorbic acid (group C) was almost equal to the control rats (group D). But there was no sign of copper deficiency or development of anemia in the copper-supplemented rats with added ascorbic acid (group C).
Figure 4. Effect of Ascorbic Acid on Serum and Liver Copper Concentration in Rats Fed Copper-deficient and Copper-supplemented Rations.

Group A - Cu-deficient + Ascorbic Acid
Group B - Cu-deficient
Group C - Cu-supplemented + Ascorbic Acid
Group D - Cu-supplemented
Van Campen and Gross (29) demonstrated that 1% ascorbic acid significantly depressed intestinal absorption of copper in rats if both ingredients were administered intraduodenally or orally. There was no effect if the ascorbic acid was given intraperitoneally. Hunt and Carlton (41) also showed that the administration of ascorbic acid by intra-muscular injection did not increase the severity of copper deficiency in chicks. Dietary ascorbic acid intake also had little or no effect on the excretion of copper (29). So, the low hepatic copper content in the copper-supplemented rats with added ascorbic acid (group C) in our experiments was possibly due to ascorbic acid causing poor intestinal absorption of copper. In such a case, the poor copper absorption would indirectly cause liver copper to drain into the serum to maintain a normal serum concentration of copper.

In our experiments, the addition of 3% ascorbic acid to the copper-deficient ration did not increase the severity of copper deficiency in the rats. This experiment supported the suggestion of Van Campen and Gross (29) that rats are less susceptible to high level ascorbic acid and copper deficiency than chicks or turkey poults. Besides, critically low hepatic copper level in the rats did not necessarily indicate symptom of copper deficiency unless the serum concentration of copper was also very low.

Serum alkaline phosphatase activity in all rats increased progressively during the first five weeks. But the alkaline phosphatase
activity of the copper-deficient rats with or without ascorbic acid (groups A & B) increased rather slowly (Fig. 3). From the sixth week on, alkaline phosphatase activity began to decrease. McClellan and others (42) showed that the decrease in activity of alkaline phosphatase in pigs fed copper-supplemented rations was inversely related to age change. In our experiments, serum alkaline phosphatase activity change was also inversely related to age change no matter whether ascorbic acid was added to the ration or not. In the copper-deficient rats with ascorbic acid (group A), the alkaline phosphatase activity was slightly lower than in the copper-deficient rats without ascorbic acid (group B). Serum alkaline phosphatase activity was much higher in copper-supplemented rats with or without ascorbic acid during the early stage of growth. Ascorbic acid was not effective in lowering serum alkaline phosphatase activity. Voelker and Carlton (35) reported that a close correlation existed between alkaline phosphatase activity and hemoglobin concentration. Briton and Hill (43) also observed that there was a decrease in alkaline phosphatase activity in chicks fed copper-deficient rations. They suggested that there was a stimulating effect of copper on the activity of the enzyme. McClellan and others (42) believed that the significantly low level of alkaline phosphatase activity in copper deficiency could be due to decreased osteoblast activity. In our experiments, it was not possible to evaluate the exact cause of decrease in alkaline phosphatase activity.
Csopak and Falk (44) showed that two Cu$^{2+}$ ions were tightly bound to each alkaline phosphatase molecule. They suggested the formation of the copper-alkaline-phosphatase complex involved the coordination of the Cu$^{2+}$ to two specific sites of the enzyme (44). Riley and Spearman (45) strongly suggested that vitamin A specifically induced the DNA-directed synthesis of alkaline phosphatase. Jeghers and Marravo (46) also confirmed that alkaline phosphatase was increased by hyper-vitaminosis A in human plasma. It is apparent that there is more than one factor which can cause the decrease of alkaline phosphatase activity. Further investigation is needed to locate the exact cause of the decrease of alkaline phosphatase activity in copper-deficient rats.
CONCLUSIONS

In this experiment, the addition of 3% ascorbic acid to the copper-deficient and copper-supplemented rations for weaning rats gave the following results:

1. The addition of 3% ascorbic acid did not affect weight loss significantly. The actual cause of weight loss was mainly due to copper deficiency.

2. Ascorbic acid had no significant effect in lowering the hemoglobin concentration in copper-deficient rats. But it slightly lowered the hemoglobin level of the copper-supplemented rats.

3. Ascorbic acid did not increase the severity of low serum copper level. The low serum concentration of copper was mainly due to copper deficiency.

4. Ascorbic acid had a significant effect, possibly an indirect effect, in depleting liver copper in the copper-supplemented rats. However, there was no severe effect in depleting the hepatic copper of the copper-deficient rats.

5. Possibly, ascorbic acid did not decrease serum alkaline phosphatase activity. The decreasing activity of alkaline phosphatase was mainly due to copper deficiency and old age.
REFERENCES


31. Sigma Technical Bulletin No. 104 (4-60), "The Calorimetric Determination of Phosphatase in Serum or Other Fluid."


