STUDIES ON COPPER METABOLISM

IV. THE INFLUENCE OF COPPER ON THE ABSORPTION OF IRON*

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In a previous paper in this series (1), evidence was presented that the absorption of iron from the gastrointestinal tract of swine deficient in copper is markedly reduced. This conclusion rests, in part, on the observation that in copper-deficient pigs fed the same quantity of either natural or radioiron for the same period of time as litter mate controls there was less iron absorbed, natural or isotopic, by the copper-deficient animals as indicated by the amount of iron in the blood, liver, kidney, spleen, and heart.

It is generally recognized that the extent of iron absorption can be determined most accurately and reliably by measuring the total body content of radioiron following the oral administration of the isotope (2). The observations in swine, referred to above, are subject to criticism because, for obvious technical reasons, the total body content of the isotope could not be measured.

The purpose of the present paper is to record the results of studies on the total body radioiron of male albino rats fed radioiron and different amounts of copper by mouth. A preliminary report of this work has been published (3).

Methods

Animals and Diet—A total of 165 male weanling albino rats of the Sprague-Dawley strain, weighing 35 to 50 gm., were used in this study. They were housed in individual galvanized iron cages. The basal diet consisted of canned evaporated milk mixed 1:1 with distilled water, which, after mixing, contained between 0.05 and 0.09 mg. of copper per liter. When an experiment involved copper-deficient animals, the daily milk intake of control groups receiving copper was restricted in order to keep the average body weight of all groups approximately equal.

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A solution of ferrous chloride was prepared from iron\textsuperscript{1} which was spectroskopically free of copper. All animals were given the daily equivalent of 0.5 mg. of iron. A solution of cupric chloride was prepared from analytical grade copper pellets. The animals were given a copper supplement as indicated below under "Results." The inert iron and copper supplements were administered on alternate days by adding them to a small portion of the diet.

A ferrous chloride solution containing Fe\textsuperscript{69} activity was prepared from an irradiation unit\textsuperscript{2} and the inert ferrous chloride solution described above. When the radioiron solution was substituted for inert iron, it was administered by stomach tube, as was the copper when this was given. The radioiron solution was given in such amounts that each animal received 0.5 mg. of iron daily.

\textit{Digestion of Whole Rat—}The carcass of each rat was placed in a 1000 ml. beaker, and 100 ml. of concentrated nitric acid and 25 to 30 ml. of distilled water were added. The beaker was allowed to stand in an oven at 37° overnight. The solution was then boiled gently with a few glass beads until clear and allowed to cool at room temperature until the fatty layer had solidified. The liquid portion was passed through iron-free glass wool in a 60 ml. funnel into a 200 ml. volumetric flask. By the use of a glass rod with a rubber policeman, the fat was washed 3 times with small portions of redistilled water and the washings added to the flask. The contents were then diluted to the mark. The fatty layer was discarded.

\textit{Determination of Radioactive Iron Content of Whole Rat—}The method previously reported from this laboratory was used (2). The samples were counted for a minimum of 4096 counts with a thin mica end window Geiger tube (counting efficiency 17 per cent). The geometry was kept constant and appropriate corrections made for background and coincidence loss. The standard, consisting of a small portion of the radioiron to be administered, was electroplated and counted in the same manner as the tissue samples.

\textit{Determination of Total Body Iron—}A 25 ml. aliquot of the nitric acid solution of the whole rat was pipetted into a 100 ml. Kjeldahl flask to which were added 10 ml. of concentrated nitric acid, 5 ml. of 70 per cent perchloric acid, and a few glass beads. The mixture was digested and evaporated by boiling until the residual perchloric acid solution was colorless. Occasionally, the addition of another 3 to 4 ml. of perchloric acid was neces-

\textsuperscript{1} Carbonyl iron powder, grade Rx (93 per cent iron), obtained from Antara Products, General Aniline and Film Corporation, 444 Madison Avenue, New York 22.

\textsuperscript{2} Irradiation unit containing 17 gm. of Fe in the form of iron carbonyl with 1 mc. of Fe\textsuperscript{69} and 0.9 mc. of Fe\textsuperscript{66} activity; obtained on allocation from the United States Atomic Energy Commission.
necessary to prevent drying of the residue. The flask was cooled and 15 ml. of redistilled water were added. This was followed by heating to dissolve any perchlorate precipitate formed. The contents were then transferred quantitatively to a 25 ml. volumetric flask and diluted to the mark with redistilled water (Solution A).

A 1.0 ml. aliquot of Solution A was diluted to 25 ml. with redistilled water, and 2 ml. of the latter solution were used for the iron analysis according to the method of Gubler et al. (1).

**Determination of Total Body Copper**—A modification of the method of Gubler et al. (4) was used for the determination of copper. A 5 ml. aliquot of Solution A above was pipetted into a 50 ml. test-tube and 8.0 ml. of a saturated sodium citrate solution, 5.0 ml. of a saturated sodium pyrophosphate solution, and 1.0 ml. of concentrated ammonium hydroxide were added, with mixing after each addition. The optical density of the final solution was then read in an Evelyn photoelectric colorimeter with a 440 m\(\mu\) glass filter. 1 ml. of a 0.1 per cent solution of sodium diethyl dithiocarbamate was added and the solution read again immediately after mixing. The optical density due to the copper was taken as the difference between these two readings. Occasionally after the addition of the carbamate solution, a cloudiness appeared in the colorimeter tube which interfered with the measurement. In such instances, all the reagents were added to the 5.0 ml. aliquot in a 50 ml. separatory funnel and 10 ml. of amyl alcohol were added, followed by the addition of 1.0 ml. of sodium diethyl dithiocarbamate. The amyl alcohol layer, after the extraction of the “copper carbamate,” was taken off, centrifuged for 10 minutes at 2000 r.p.m. to remove the unseparated droplets of the aqueous fraction, and read in the photoelectric colorimeter. The \(K\) value (concentration versus density) was previously determined for both the aqueous and amyl alcohol solutions by the use of a standard copper solution prepared from analytical grade copper pellets. Blanks for the reagents were prepared in the same manner as the samples.

**Results**

**Influence of Dietary Copper on Absorption of Iron**—A total of 74 rats was divided into five groups and fed the milk diet supplemented with iron (0.5 mg. per day). The animals in Group I received no copper supplement; Group II, 0.05 mg. of copper per rat per day; Group III, 0.25 mg.; Group IV, 0.50 mg.; and Group V, 1.0 mg. After a period of 28 days, radioiron was substituted for inert iron. 14 days later, at a time when all of the animals had received a total of 0.02\(\mu\)c. of Fe\(^{69}\), the mineral supplements were discontinued. The animals were sacrificed by ether anesthesia
4 days later, and total body radioiron, inert iron, and copper were determined. The results are presented in Table I.

Groups I through IV showed significant differences\(^2\) in the percentage of the total amount of radioiron absorbed. Group V did not differ significantly from Group II, although the former received 20 times as much copper as the latter. With respect to hemoglobin concentration, only Group I was significantly different from the remaining groups. The differences in total body iron were significant between Groups I and II and between Groups III and IV, but the differences between Groups II, III, and V were not significant. In contrast, the mean total body copper concentration tended to be higher in each successive group.

**Table I**

<table>
<thead>
<tr>
<th>Group No.</th>
<th>No. of rats</th>
<th>Copper supplement (mg. per day)</th>
<th>Radioiron absorbed (per cent of oral dose)</th>
<th>Total body iron (mg.)</th>
<th>Hb*</th>
<th>Total body copper† (g. per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>17</td>
<td>None</td>
<td>8.8 ± 0.7</td>
<td>3.75 ± 0.15</td>
<td>12.3 ± 1.0</td>
<td>0.125 ± 0.004</td>
</tr>
<tr>
<td>II</td>
<td>21</td>
<td>0.05</td>
<td>11.9 ± 0.5</td>
<td>5.09 ± 0.17</td>
<td>16.8 ± 0.3</td>
<td>0.214 ± 0.008</td>
</tr>
<tr>
<td>III</td>
<td>12</td>
<td>0.25</td>
<td>16.7 ± 0.8</td>
<td>4.87 ± 0.15</td>
<td>17.4 ± 0.3</td>
<td>0.239 ± 0.010</td>
</tr>
<tr>
<td>IV</td>
<td>12</td>
<td>0.50</td>
<td>19.7 ± 2.2</td>
<td>5.91 ± 0.26</td>
<td>16.4 ± 0.4</td>
<td>1.211 ± 0.181</td>
</tr>
<tr>
<td>V</td>
<td>12</td>
<td>1.00</td>
<td>11.1 ± 0.9</td>
<td>5.36 ± 0.15</td>
<td>19.0 ± 0.4</td>
<td>1.737 ± 0.216</td>
</tr>
</tbody>
</table>

Mean ± the standard error.

* Tail blood.
† Wet tissue.

**Influence of Copper Administered Simultaneously with Single Dose of Radioiron on Iron Absorption**—In order to determine whether copper influences the absorption of the iron with which it is administered, a total of forty-eight rats was divided into five groups and these were fed the milk diet supplemented with iron (0.5 mg. per rat per day) and copper (0.05 mg. per rat per day) for a period of 22 days. A single oral dose of 0.5 mg. of iron (0.02 µc. of Fe\(^{69}\) activity) was given by stomach tube together with the amounts of copper indicated in Table II. 4 days later, the animals were sacrificed by ether anesthesia, and the total body radioiron and total body copper determined. No significant difference in the amount of radioiron absorbed was noted among the five groups (Table II).

**Influence of Amount of Copper in Tissues on Absorption of Iron**—Since the amount of copper given simultaneously with a single dose of radioiron failed to influence the amount of iron absorbed, the following experiment was designed to determine whether the concentration of copper in the tissues influences iron absorption.

\(^2\) Significant difference at the 5 per cent level as determined by the Fisher \(t\) test (5).
A total of forty-three rats was divided into five groups. All animals were fed the milk diet supplemented with iron (0.5 mg. per rat per day). The animals in Group I were not given a copper supplement. The animals in Group II received 0.05 mg. of copper per rat per day; Group III, 0.25 mg.; Group IV, 0.50 mg.; and Group V, 1.0 mg. After 38 days on the above diet, the copper supplements were omitted from the diet for 48 hours in order to clear the copper from the gastrointestinal tract. All the animals were then given 0.5 mg. of radioiron (0.02 μc.) without copper by stomach tube. 4 days later they were sacrificed by ether anesthesia and total body radioiron and copper were determined. The results are presented in Table III. Significant differences in iron absorption were found between Groups I, II, and III, receiving 0, 0.05, and 0.25 mg. of copper daily, respectively. The absorption of iron was maximal in Group III.

### Table II

*Influence of Copper Administered Simultaneously with Single Dose of Radioiron on Iron Absorption*

<table>
<thead>
<tr>
<th>Group No.</th>
<th>No. of rats</th>
<th>Copper supplement with radioiron</th>
<th>Radioiron absorbed</th>
<th>Total body copper*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg. per cent of oral dose</td>
<td>mg. per 100 gm.</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>10</td>
<td>None</td>
<td>27.9 ± 2.9</td>
<td>0.141 ± 0.015</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>0.05</td>
<td>22.9 ± 2.2</td>
<td>0.140 ± 0.015</td>
</tr>
<tr>
<td>III</td>
<td>9</td>
<td>0.25</td>
<td>26.0 ± 1.8</td>
<td>0.159 ± 0.018</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>0.50</td>
<td>28.4 ± 1.8</td>
<td>0.272 ± 0.024</td>
</tr>
<tr>
<td>V</td>
<td>9</td>
<td>1.00</td>
<td>27.3 ± 2.0</td>
<td>0.507 ± 0.037</td>
</tr>
</tbody>
</table>

Mean ± the standard error.

* Wet tissue.

### Table III

*Influence of Amount of Copper in Tissues on Absorption of Iron*

<table>
<thead>
<tr>
<th>Group No.</th>
<th>No. of rats</th>
<th>Copper supplement prior to radioiron</th>
<th>Radioiron absorbed</th>
<th>Total body copper*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg. per day</td>
<td>mg. per cent of oral dose</td>
<td>mg. per 100 gm.</td>
</tr>
<tr>
<td>I</td>
<td>9</td>
<td>None</td>
<td>7.5 ± 0.9</td>
<td>0.112 ± 0.007</td>
</tr>
<tr>
<td>II</td>
<td>9</td>
<td>0.05</td>
<td>11.6 ± 1.0</td>
<td>0.201 ± 0.008</td>
</tr>
<tr>
<td>III</td>
<td>9</td>
<td>0.25</td>
<td>15.0 ± 1.3</td>
<td>0.352 ± 0.021</td>
</tr>
<tr>
<td>IV</td>
<td>8</td>
<td>0.50</td>
<td>10.7 ± 0.9</td>
<td>0.418 ± 0.031</td>
</tr>
<tr>
<td>V</td>
<td>8</td>
<td>1.00</td>
<td>10.6 ± 1.6</td>
<td>0.760 ± 0.106</td>
</tr>
</tbody>
</table>

Mean ± the standard error.

* Wet tissue.
Groups IV and V, receiving 0.5 and 1.0 mg. of copper daily, did not show a significant difference compared with Group II, even though the mean total body copper concentration had increased with the increasing amounts of copper in the diet.

**DISCUSSION**

Under the conditions of these experiments, it is apparent that copper influences the absorption of iron in the rat. This observation is in agreement with our studies in swine (1, 6) and also finds support in the literature in the limited data presented by Houk et al. (7). However, it is at variance with the work of others (8–11).

Elvehjem and Sherman (8) observed that, when rats deficient in both iron and copper were fed iron, the iron content of the liver and spleen increased. From this they concluded that copper does not influence iron "assimilation." Neither Cunningham (9) nor Josephs (10) observed a significant difference between the total content of body iron in weanling rats fed a milk diet plus iron and weanling rats fed a milk diet plus iron and copper. These studies led Schultze in 1940 (11), in his review of the rôle of copper in blood formation, to conclude that copper is not necessary for the absorption and storage of iron in tissues.

It is not surprising that no effect of copper on iron absorption was observed in these earlier studies. The observations of Elvehjem and Sherman took into account only the iron content of the liver and spleen and no consideration was given to the fact that the hemoglobin iron compartment was not increasing. The failure of Cunningham and Josephs to detect a difference between the total amount of iron in the bodies of rats fed diets with and without copper can be explained in several ways. Cunningham fed 0.2 mg. of iron per rat per day, which is a suboptimal amount (12). Josephs observed as great an increase in hemoglobin in the animals fed iron as in the animals fed both iron and copper. This would suggest that the "copper-deficient" animals were receiving copper from an undetermined source, since it has been repeatedly demonstrated by many observers that no significant degree of hemoglobin regeneration occurs when only iron is administered to copper-deficient animals (13). Finally, as indicated by our own studies reported here, the determination of total body iron is not as sensitive a method of measuring the rate of iron absorption as is the measurement of total body radioiron. This is understandable, because with the isotope method the measurements of iron absorption can be confined to the period when differences in the concentration of body copper have been produced.

It is interesting that increasing the amount of copper in the diet beyond 0.25 to 0.50 mg. per rat per day did not favor greater iron absorption. The explanation for this is not evident. It would seem unlikely that this
was the result of administering toxic amounts of copper, since Cunningham (9) failed to observe any detrimental effect of 7.5 mg. of copper daily on the growth of rats. The larger amount of copper seemed, if anything, to depress the absorption of iron. Thus it is unlikely that exposure to high concentrations of dietary copper would in itself result in the production of hemochromatosis, as postulated by Mallory (14).

It should not be concluded from our studies in the rat that the only function of copper in mammalian tissues is in relation to the absorption of iron from the gastrointestinal tract. Also it does not follow that the anemia associated with a deficiency of copper is the result only of failure to absorb adequate amounts of iron. The various aspects of the relation of copper to iron metabolism have been discussed in the two preceding papers in this series (1, 6).

**SUMMARY**

Evidence is presented which indicates that, under the conditions of these experiments, there is less absorption of iron from the gastrointestinal tract of rats deficient in copper than in rats supplied with copper. The amount of radioiron (Fe\textsuperscript{59}) absorbed was favored by amounts of dietary copper up to levels of 0.25 to 0.50 mg. per rat per day. Above this amount there was no greater iron absorption; in fact, there appeared to be somewhat reduced absorption.

The influence of copper on iron absorption is not due to the simultaneous administration of copper with the iron but appears to be correlated with the level of copper in the tissues.

**BIBLIOGRAPHY**

STUDIES ON COPPER METABOLISM: IV. THE INFLUENCE OF COPPER ON THE ABSORPTION OF IRON
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