Studies on Copper Metabolism. V. Storage of Iron in Liver of Copper-Deficient Rats.* (19751)

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It has been demonstrated in previous studies in this laboratory(1,2) that swine depleted of copper develop an anemia which is morphologically indistinguishable from iron-deficiency anemia and which is accompanied by hypoferremia and an increase in the iron-binding capacity of the plasma. Evidence has been presented that in such animals there is 1) impaired ability to absorb iron from the gastro-intestinal tract; 2) incomplete mobilization of iron from the tissues; and 3) an inability to utilize parenterally administered iron for hemoglobin synthesis even when it is presented to the bone marrow in normal quantities. The fact that copper influences iron metabolism in such diverse sites as the mucosal cell, the liver, and the bone marrow, suggests that this element may, in some basic manner, be concerned wherever and whenever iron moves. Since ferritin has been shown to be present in the mucosal cell, the liver and the bone marrow(3) it seems plausible that copper may be concerned with the turnover of iron through ferritin.

In a previous study in swine(2) the iron in the livers and spleens of control, copper-deficient and iron-deficient pigs, and copper-deficient pigs given 200 mg of iron intravenously, was fractionated into the saline-soluble and the saline-insoluble portions. It has been shown by others(3-5) that the saline-soluble fraction contains most of the ferritin and that hemosiderin iron can be extracted with dilute hydrochloric acid from the residue remaining after saline extraction. The results of this preliminary study suggested that after the intravenous injection of iron into copper-deficient swine there was a tendency to store more iron as "hemosiderin" and less as "ferritin" as compared with control animals with approximately the same concentration of total iron in the livers. However, the results of this study were inconclusive.

The purpose of the present report is to present studies on the rate of uptake of parenterally administered radioactive iron into the saline-soluble (presumably ferritin) and saline-insoluble (presumably hemosiderin) fractions of the livers of control and of copper-deficient albino rats.

Methods. A total of 81 male weanling rats of the Sprague-Dawley strain were used. All of the rats were fed a diet of commercial canned milk diluted 1:1 with distilled water‡ supplemented with one mg of iron§ as ferrous chloride 3 times a week. A control group of 27 rats received, in addition, 0.1 mg of copper/rat as copper sulfate 3 times a week. These supplements were offered in a small quantity of milk in the morning to insure their complete ingestion. The amount of diet fed the control rats was restricted to the amount eaten by the deficient rats.

Two groups of copper-deficient rats were studied. When the mean hemoglobin level had decreased to 11.5 g %, the rats in the first copper-deficient group were given one ml of an iron solution buffered with 3% sodium citrate containing 0.04 μc of Fe^{59}‖ activity and 200 μg of total iron by the subcutaneous route. At the same time the control animals were given the same amount of radioiron. The animals in the second copper-deficient group were allowed to continue on the low-copper diet until the mean hemoglobin level reached 9.0 g %. At this time, the animals were given isotopic iron in the same amount as above. One-third of each group of rats was anes-

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‡ Containing 0.05 to 0.09 mg of copper/liter by analysis after dilution.

§ Carbonyl iron powder, grade RX, obtained from Antara Products, General Aniline and Film Corp.

‖ Obtained on allocation from the U. S. Atomic Energy Commission.

TABLE I. Partition of Subcutaneously Injected Radioiron Between Saline-Soluble (Ferritin) and Saline-Insoluble (Hemosiderin) Fractions of Liver from Control and Copper-Deficient Rats. Results expressed in μg of iron per total liver fraction $\pm$ the S.E. (standard error) of the mean.

Group	No. of rats	Fraction	Time after inj, hr 8	24	48
Control	27	Saline-soluble	50 $\pm$ 2.8	43 $\pm$ 2	42 $\pm$ 4.7
		Saline-insoluble	9 $\pm$ .6	10 $\pm$ .3	16 $\pm$ 1.3
Copper-deficient I	27	Saline-soluble	32 $\pm$ 2.1	32 $\pm$ 2.1	33 $\pm$ 2.7
		Saline-insoluble	8 $\pm$ .7	9 $\pm$ .7	11 $\pm$ .9
Copper-deficient II	27	Saline-soluble	52 $\pm$ 7.2	46 $\pm$ 6.1	49 $\pm$ 8.3
		Saline-insoluble	9 $\pm$ 1.4	7 $\pm$ 1.1	9 $\pm$ 1.3

thetized with ether and sacrificed by hemorrhaging at 8, 24 and 48 hours after the injection of iron. The livers were removed, blotted free of blood with gauze and weighed. The whole livers were then homogenized in a Waring blendor for 2½ minutes with sufficient 0.9% saline to make a total volume of 50 ml. A 15 ml aliquot was pipetted into 2 small (10 mm) test tubes and centrifuged at 2200 x g for one half hour. The supernatant fluids from both tubes were transferred to a 100 ml pyrex centrifuge tube. The insoluble residues were mixed with approximately 15 ml of 0.9% saline and transferred to one small test tube and centrifuged. The supernatant fluid was added to the first 2 supernates in the 100 ml centrifuge tube. The combined supernatant solutions were heated at 75°C for 15 minutes. The precipitated hemoglobin was removed by centrifugation and discarded. The supernatant solution was transferred to a second 100 ml centrifuge tube containing 10 to 15 ml of 0.9% saline and heated for 20 minutes in a boiling water bath. The precipitate, representing the saline-soluble or "ferritin" fraction was then separated by centrifugation, resuspended in saline, transferred to a 10 mm test tube, and recentrifuged.

The radioactivity in the saline-soluble fraction and in the original saline-insoluble residue was measured in a vial sample scintillation counter as described previously(6). A standard containing 10% of the injected amount of radioiron was counted along with each group of samples. By comparison of the radioactivity in the liver fractions with the standard, the iron represented by the total counts was calculated.

Results and discussion. The results are presented in Table I. Although the same total amount of iron did not reach the liver in all 3 groups, in all groups 80 to 85% of the radioiron taken up by the liver was found in the saline-soluble (ferritin) fraction within 8 hours. Fifteen to 20% of the iron reaching the liver was present in the saline-insoluble (hemosiderin) fraction. No significant difference, either in the partition of the iron after 2 days or in the rate of uptake of the iron into the 2 fractions was observed between the control and the copper-deficient animals. Likewise no significant differences were observed between the more severely (Group II) copper-deficient and the less severely (Group I) copper-deficient rats.

Since no studies were made on the efficiency of the method used in separating ferritin from hemosiderin and since these compounds were not identified other than on the basis of solubility, it cannot be stated conclusively that the rate of ferritin synthesis was unaltered. However, the results suggest that the derangement in iron metabolism in copper-deficient rats is not a consequence of an abnormality in the manner of storage of iron in the liver.

Summary. No alteration from the normal in the uptake of parenterally administered radioiron into the saline-soluble and saline-insoluble fractions of liver iron was observed in copper-deficient albino rats.

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Serum Lipids Studied by Electrophoresis on Paper. (19752)

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Several lines of investigation have indicated the lipids of human serum and plasma to be associated with proteins. Analysis of electrophoretically separated protein fractions of serum has revealed the presence of lipid in all(1), the highest concentration being in the β-globulin fraction(1-4). Nearly all the plasma cholesterol and phospholipid may be recovered in Cohn fractions III_0, IV, V, and VI(5) and in pooled plasma an α and a β lipoprotein have been described, the latter containing 75% of the total plasma cholesterol(6). A large number of lipoprotein classes has been disclosed by ultracentrifugation of plasma(7).

Microelectrophoresis of proteins on filter paper(8) has proved to be a useful method for the separation and study of serum proteins(8-11). In the present study histochemical and other procedures applicable to the detection and characterization of lipids in tissue sections have been applied to paper strips on which electrophoresis of human serum samples had been performed and the lipid and protein patterns were compared. A brief communication describing a similar approach to the study of blood lipoproteins has recently been published(12).

Methods. The procedure used for electrophoresis was, with minor modifications, that described by Durrum(8). The sera studied were obtained from 74 individuals and included both normal subjects and hospitalized patients suffering from various disorders. The serum sample (0.1-0.2 cc) was applied transversely as a band approximately 1 cm wide across a 42 x 11 cm piece of Whatman 3 MM paper and the paper adjacent to the band was then quickly moistened with sodium barbital buffer (0.025 M, pH 8.6). The paper was then placed in a lucite-covered glass aquarium so that the middle of the paper rested on a silicone-coated glass rod traversing the aquarium 20 cm above its floor and the ends (6 cm apart) dipped into buffer-containing electrode vessels in which were also immersed platinum electrodes connected, via mercury-filled glass tubes, to lead wires from the power supply. The paper on either side of the serum line was then drenched thoroughly with buffer and voltage applied. The line of application of the serum was generally 4.5 cm from the apex on the cathode side; in this position the γ globulins moved very little, their migration toward the anode being very nearly balanced by the electro-osmotic flow of liquid toward the cathode. If the line of origin was more toward the cathode there was a greater rate of movement of all bands toward the anode but without greater resolution, while if the placement were more toward the anode the components of least mobility moved toward the cathode. Three hundred volts, providing a current of 0.4 milliampere per cm of width, applied for 5 hours moved the front of the fastest band approximately 15 cm from the origin. The paper was then air-dried in a hood, heated for 10 minutes in an oven at 100°C, and, after removal of the outer 0.5 cm on each side, was cut into parallel longitudinal strips 1-2 cm wide to be stained separately. Proteins were stained according to Durrum's method(8) by immersion of a strip in alcoholic bromphenol blue solution saturated with mercuric chloride and subsequent washing in water. For demonstrating lipids another paper strip was immersed in a saturated solution of Sudan IV in 40% ethanol, 50% glycerol