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Incorporation of ^{59}Fe into Different Iron Compounds of Liver Tissue

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Techniques are described for isolating and radioassaying the iron of individual iron proteins from rat liver. These compounds include two ferritins, catalase, cytochrome b and cytochrome c.

After intraperitoneal injection of ^{59}Fe, radioactivity appears most rapidly in supernatant ferritin, followed by microsome ferritin, cytochrome b and catalase. After four hours, cytochrome b is the most radioactive.

It is suggested that the injected iron reaches the liver over a period of several hours. Because only a part of the iron of ferritin is labile, the average specific activity of ferritin iron does not rise as high as that of intracellular free iron. Cytochrome b which appears to have a turnover time of only a few hours more nearly reflects the free iron specific activity. It is possible that cytochrome b is a precursor of catalase.

There have been several reports of the use of radio-iron to study iron metabolism *in vivo*[1-4]. The techniques used for isolation of individual iron compounds and the rather low specific activity of the iron employed have limited the conclusions to be drawn. By restricting our investigation to a single rather homogeneous organ, rat or guinea pig liver; by utilizing the Hogeboom-Schneider [5] cell fractionation; by using high specific activity iron; and by developing new isolation methods for the iron containing proteins, we hoped to get a closer view of iron metabolism than has heretofore been possible. Following a single injection of radio-iron, ferritin has been isolated from the microsomes and from the 105 000×g supernatant fraction, cytochrome b from the microsomes, cytochrome c from the mitochondria, and catalase from the supernatant fraction and mitochondria. The radio-iron content of these substances has been followed from one hour to fourteen hours.

EXPERIMENTAL

Iron determination. Each substance to be examined was digested with sulfuric acid and hydrogen peroxide and the total iron was determined using the sulfosalicylic acid method [6,1]. In each case additional ferric sulfate was added to bring the total amount to

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500 μg and all the iron was then precipitated as iron sulfide by saturating the solution with hydrogen sulfide. The iron sulfide was filtered on hard paper and assayed with a shielded end-window counter. Under these conditions the counting efficiency for ^{59}Fe was about 3 %.

Table 1. Iron distribution in liver tissue.

	3 rat livers 16 g	3 rat livers 17 g	4 guinea pig livers 191 g	4 guinea pig livers 208 g
Nw. fraction	0.74 mg iron	0.92	2.4	2.4
Mtw. fraction	0.47	0.50	0.81	0.8
Microsomes	1.14	1.30	1.9	1.9
Microsome ferritin	1.10	1.17	0.9	0.8
Supernatant ferritin	2.05	2.80	1.6	1.0
Cytochrome b (hemin iron)	0.029	0.037	0.218	0.22

Fractionation. The rats used were between four and six months old and weighed between 150 and 200 g. They had been on a high iron diet for at least three months. 20 000—100 000 counts per min. of ^{59}Fe (1—5 μg of Fe as ferric ammonium citrate) was administered intraperitoneally to each animal. After the chosen time three to five animals were sacrificed, the livers excised and homogenized in 0.25 M sucrose solution. Fractionation according to the method of Hogeboom and Schneider [5] yielded a nuclear fraction (10 min. 700 × g sediment), mitochondrial fraction (10 min. 5 000 × g sediment), microsomal fraction (40 min. 100 000 × g sediment), and a soluble fraction (100 000 × g supernatant). Table 1 shows average values for the iron content of these fractions and for several iron-proteins.

Supernatant ferritin. To the 100 000 × g supernatant was added one half its volume of saturated ammonium sulfate. The supernatant was saved for isolation of catalase. The precipitate was dissolved in water, repeatedly fractionated with ammonium sulfate (25 to 35 %), and eventually dissolved in water. The solution was heated 5 min. at 70°, and centrifuged to yield a clear solution of ferritin containing between 17 and 20 % iron. When 2 % of cadmium sulfate was added to a very concentrated solution of this ferritin, part crystallized as shown in Fig. 1.

Microsome ferritin. The microsomes were suspended by homogenization in ten volumes of water and the pH was adjusted to 5 by addition of acetic acid. After 30 min., the

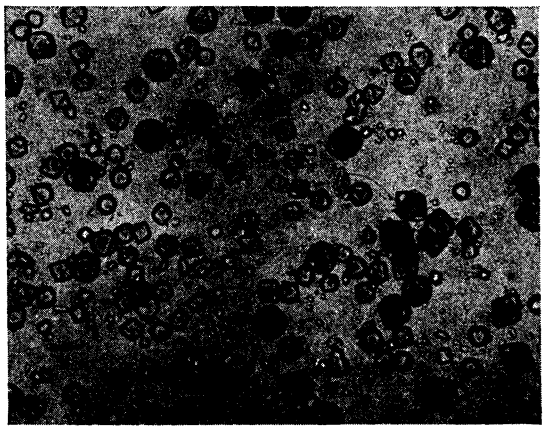

Fig. 1. Rat ferritin crystallized from cadmium sulfate (350 ×).

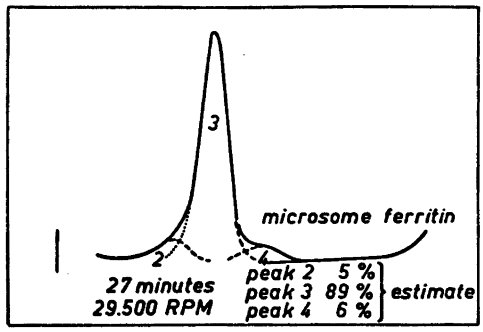

Fig. 2. Ultracentrifugation of microsome ferritin.

suspension was centrifuged and the supernatant was decanted. The sediment was extracted once more with water. The combined supernatants were treated as the supernatant above to isolate the microsome ferritin. This contained 18 to 22 % iron.

Cytochrome b. The sediment remaining after extraction of the ferritin from the microsomes could be solubilized with desoxycholic acid. In agreement with Strittmatter and Ball [7] the only photometrically detectable cytochrome was cytochrome b. Pyridine hemochromogen determinations confirmed this observation. Routinely the extracted microsomes were treated with 1:1 000 HCl-acetone which releases the hemin. The acetone solution was filtered, concentrated and partitioned between water and *peroxide-free* ether. The ether phase was evaporated and ^{59}Fe was determined.

Catalase. To the 100 000 × g supernatant from which ferritin had been isolated more ammonium sulfate was added to 60 % saturation. The precipitate was dissolved in 0.1 M phosphate buffer, pH 7.0, and dialyzed against the same buffer. Four volumes of the dialyzed solution were shaken with one volume of chloroform ethanol (1:3). This denatures all of the hemoglobin present. After centrifugation, the supernatant solution was heated to precipitate the catalase. The precipitate was treated with HCl-acetone and worked up like the cytochrome b. Catalase could also be isolated in the same fashion from the mitochondria.

Cytochrome c. A suspension of mitochondria was adjusted to pH 4.2 with 1.0 N H_2SO_4 and allowed to stand at 4° for one hour. After centrifugation the solution was adjusted to pH 7.0 with 1.0 N NH_4OH and again centrifuged. The clear solution was passed through

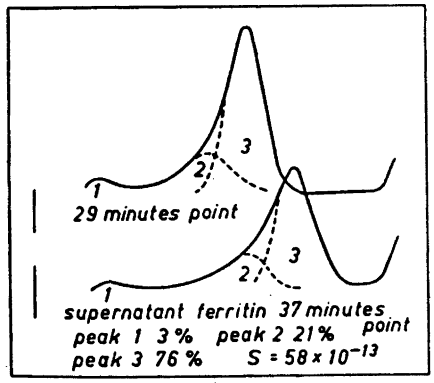

Fig. 3. Ultracentrifugation of supernatant ferritin.

a 4 × 1 cm column of Amberlite XE-69 in the ammonium form. (We are indebted to Rohm and Haas, Inc., and to their associates Chas. Lemming and Co., Ltd., for gifts of this material). All of the cytochrome c is retained by the upper part of the column while hemoglobin and other pigments pass through. After washing with about 20 ml of water, the cytochrome c is eluted with about 4 ml of 1 N sodium sulfate. After reduction with dithionite, cytochrome c was determined by its absorption at 550 mμ. Radio-iron was determined as described above.

RESULTS AND DISCUSSION

Although the isolation of ferritin from rat liver has not been described previously, it was not surprising that ferritin could be obtained. Interestingly, ferritin prepared in this manner from both the microsomes and from the supernatant fraction, gives very sharp peaks in the ultracentrifuge in contrast to previous observations on other ferritins [8] (Figs. 2 and 3). Nonetheless, both of our ferritins give sedimentation constants of 58 and 60×10^{-13} in essential agreement with other workers. Occasionally, we observe in the "supernatant ferritin" a small amount of a colorless protein with a sedimentation constant about 7.5 or 10×10^{-13}. This may be due to apoferritin. The electrophoretic mobilities of our two ferritins are within about 10 % of each other at pH 6.0 namely $-2.2\ u \times 10^{-5}$ for microsome and $-1.95\ u \times 10^{-6}$ for supernatant ferritin. (Figs. 4 and 5).

The radioactivity data is summarized in Table 2 and Fig. 6. The ratios of specific activities at each time interval are quite significant. It appears that ^{59}Fe reaches the liver rather slowly over a period of as much as ten hours. The activity reaching the liver is first incorporated into the "supernatant ferritin" more than twice as rapidly as into the microsome ferritin. This may be compared with earlier observation [9] that ^{14}C-leucine is incorporated into supernatant ferritin ten times as rapidly as into microsome ferritin in a 20 min. period and supports our belief that these two ferritins are biochemically distinct. With the passage of time, iron atoms or perhaps entire ferritin molecules move from the supernatant ferritin pool to the microsome ferritin pool and from ten hours onward the specific activities in each fraction are essentially the same.

It is extremely interesting that the specific activity of the cytochrome b is lower than either ferritin at first and then higher than either. Obviously, the

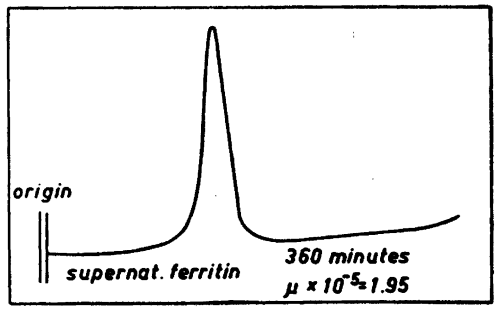

Fig. 4. Electrophoresis of supernatant ferritin.

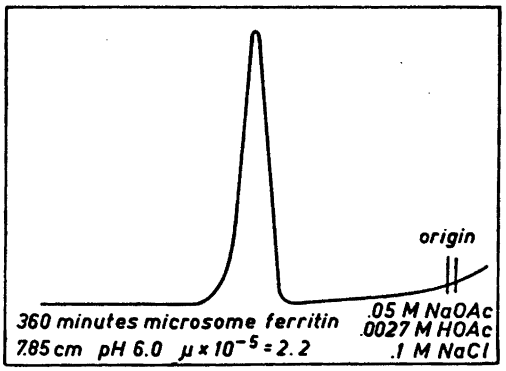

Fig. 5. Electrophoresis of microsome ferritin.

specific activities of all iron precursor substances must have been as high or higher than that of cytochrome b at its highest point. Ultimately, this requirement includes free iron. It would appear that free iron entering the liver is very rapidly picked up or equilibrated with a small amount of the ferritin iron. Accordingly, in the early phase there is a nearly linear rise in the average specific activity of the ferritins and in the total activity of the ferritins, which include about half of the iron of the liver. Because the superficial or most labile ferritin iron (which is in equilibrium with the free iron) is not rapidly equilibrated with the more deeply "buried" iron of the ferritin, the specific activity of the free iron is at all early times higher than the average activity of ferritin iron atoms. Substances synthesized more or less directly from free iron might be expected to reflect the rapid rise in free iron activity to levels much higher than the ferritin average. Cytochrome b appears to be such a substance.

The very high incorporation rate of cytochrome b is made doubly interesting because this substance is found in the microsomes where the bulk of the cell's protein synthetic activity is located. Cytochrome b has as yet no well defined cell function. Perhaps it is a precursor of one or several hemoproteins. If cytochrome b were the precursor of catalase, one might expect the peak of the catalase curve to come at the point that the two curves intersect. This is obviously not the case, but possibilities such as the presence of comparatively

Table 2. Specific activity of the isolated iron compounds in counts per min. per μg Fe from guinea pig livers.

	Minutes						
	80	195	240	320	675	1 170	1 440
Microsome ferritin	0.115	0.251	0.364	0.497	0.335	0.237	0.271
Supernatant ferritin	0.255	0.386	0.350	0.554	0.440	0.374	0.338
Cytochrome b hemin iron	0.055	0.305	0.396	0.983	0.700	0.621	0.528
Catalase	0.029	0.061	0.089	—	0.297	0.268	0.281

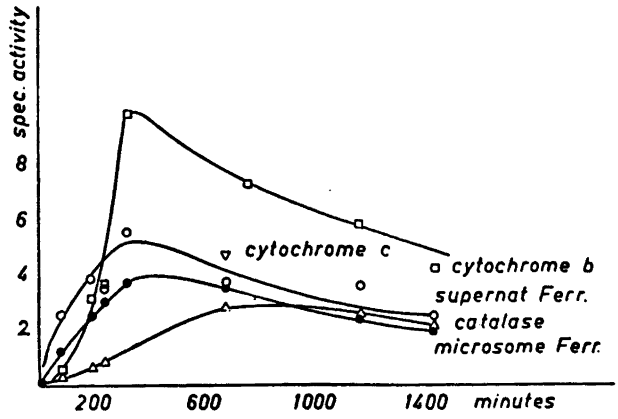

Fig. 6. Specific activity (counts per minutes per μg Fe) of the different iron compounds of liver. The animals were sacrificed 30—1 400 minutes after the injection.

radioactivity inert blood catalase [1] or inert catalase-like substances in the cell extract tend to reduce the observed specific activity of liver catalase by dilution. Hence it is still presumptuous to exclude cytochrome b as a precursor of catalase.

A final noteworthy observation concerns the rather short time required to reach maximum specific activity in each substance in comparison to previous observations [1]. This may very well be due to the much smaller doses of iron and hence the much shorter time required to bring free iron in the liver to its maximum specific activity. From our data we believe that the turnover time of cytochrome b is sufficiently short to permit its being an important precursor of hemoproteins. In turn it is possible that the turnover time of catalase may be short enough to account for the rapid disappearance of catalase on tumor implantation [10]. Liver catalase may also be released to provide part of the blood catalase. The single cytochrome c point serves only to show that liver cytochrome c must also have a rather high turnover rate.

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