THE EFFECTS OF IRON DEFICIENCY ON THE HEPATOCYTE: A BIOCHEMICAL AND ULTRASTRUCTURAL STUDY

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ABSTRACT

Effects of iron deficiency on the hepatocyte were studied quantitatively in the rat by combining ultrastructural and biochemical techniques. After 3–8 wk of an iron-deficient diet, the percentage of cytoplasm occupied by mitochondria increased progressively compared with complete diet values. The increment resulted primarily from an enlargement of individual mitochondria rather than from an increased mitochondrial number. Many mitochondria were completely divided by a double membrane, often at a point of constriction. After 2 days of iron administration, mitochondria were of heterogeneous size, shape, and electron opacity. After 5 days, essentially all mitochondria had become normal in configuration. The rate of reversal of the morphological abnormality was more rapid than would be anticipated if it coincided with known rates of renewal of mitochondrial DNA or protein.

The concentrations of mitochondrial cytochromes were more rapidly depressed as a result of iron deprivation than those of microsomal cytochromes. Cytochromes *c* and *a* were decreased after 3 and 8 wk of exposure to the deficient regimen. Cytochrome P 450 was not decreased after a 3 wk exposure to the deficient diet and responded normally to phenobarbital treatment with a fourfold increase in total hepatic content; its concentration was depressed only after 8 wk of exposure to the deficient diet. There was no reduction in cytochrome b_5 concentration.

A dietary deficiency of iron (1–4), copper (1, 5, 6), or riboflavin (7) in the mammal results in a selective depletion of mitochondrial proteins that require these constituents for their synthesis. A diet deficient in linoleate and arachidonate decreases the concentration of these essential fatty acids in mitochondrial lipids in an analogous manner (8). In addition to the changes in mitochondrial composition, all of these deficiency states also produce similar morphological abnormalities of the mitochondria (8–10). The ultrastructural abnormalities in the iron-deficient rat include enlarged, rounded, and electron-lucent mitochondria in the hepatocyte, and an enlargement and/ or an increase in number of mitochondria in bone marrow erythroblasts and heart muscle (10). The quantitative nature of the changes in morphology and composition, and their possible interrelationships, were studied by combining ultrastructural and biochemical techniques. The liver lends itself particularly well to a quantitative morphological and biochemical study of the consequences of iron deficiency. Its ultrastructural features are well defined, relatively pure cell fractions can be more easily isolated from liver than from other tissues,and the effects of the deficiency on the mitochondrial and microsomal cytochromes can be compared. The purposes of the present study are (*a*) to quantitate in terms of size and number the ultrastructural

changes of the mitochondria in liver, and their reversibility in the iron-deficient rat before and after iron administration; (*b*) to determine the relationship of changes in composition of the mitochondrial and microsomal fractions, particularly with respect to the cytochromes, to corresponding alterations in morphology; and (*c*) to evaluate the effect of iron depletion upon the response of the endoplasmic reticulum and its component cytochromes to phenobarbital stimulation.

METHODS

Experimental Animals

Male Wistar rats were given iron-deficient regimens based upon a diet of dried, partially skimmed milk (11). Iron-deficient rats II A and II B (Table I) were exposed to this diet starting at 10 days of age to avoid iron contamination from the maternal diet during the nursing period; they were weaned at 21 days, and were sacrificed at 30–38 days and at 59–66 days, respectively, (or after 3 and 8 wk on the low-iron diet alone). Iron-deficient group II C was placed on the regimen at 36 days of age and sacrificed at 64 days. Control rats (groups I A, I B, and I C—Table I) received the same regimen starting at 10 days of age, with the addition of 250 mg ferrous ammonium citrate added to each liter of drinking water. After weaning at 21 days, groups I A and I B were permitted free access to the diet, while rats in group I C were given weighed, daily quantities which restricted their rate of growth to approximate that of group II B. Phenobarbital in an amount of 8 mg/100 g body weight was administered intraperitoneally daily for 3 days; to certain of the animals in groups I A and II A that were utilized for studies of the microsomal cytochromes. Iron repletion was studied by treating certain of the iron-deficient rats in group II B with an initial intramuscular injection of 5 mg of elemental iron as iron dextran,[1] followed by iron supplementation in the drinking water as in the complete dietary regimen. All rats were killed after an overnight fast. The concentration of hemoglobin in tail blood was determined from assays after the hemoglobin was converted to cyanmethemoglobin (12). The significance of the difference between mean values for hemoglobin concentration was calculated by the *t* test.

Ultrastructural Studies

Samples of liver for electron microscopy were fixed in 1% buffered osmium tetroxide and/or 1% buffered glutaraldehyde, postfixed in 1% buffered osmium tetroxide, dehydrated in alcohol and propylene oxide, and embedded in Maraglas (The Marblette Co. Div. of Allied Products Corp., N. Y.). Sections were cut and stained with uranyl acetate and lead citrate. Mitochondrial size and number were evaluated on enlarged prints (20 × 24 cm), which covered an area of at least 4500 μ^2 per animal, at a magnification of 14,000–19,000. Areas occupied by mitochondria and nonmitochondrial cytoplasm were cut out and weighed; 1200–3000 mitochondrial cross-sections were included for each specimen. The two methods of fixation yielded similar results. Size distribution data were obtained by weighing the paper occupied by individual mitochondrial cross-sections.

Certain principles of quantitative measurement of cell constituents from electron micrographs have been reviewed recently (13, 14). The fractional volume occupied by a cellular component is equal to its fractional area on a random cross-section. Thus, the percentage of total cytoplasmic volume occupied by the mitochondria is the same as the percentage expressed in terms of area. The volume of individual mitochondria is more difficult to estimate. Since mitochondria are not perfect spheres, their volume depends upon shape and axial ratio, both of which change in response to variations in dietary regimen (9, 10, 15, 16). In the present study, the calculation of cross-sectional area is used, since it relies on direct measurements and permits estimation of relative differences in mitochondrial size following several dietary regimens. The sections were taken within one to four cell layers of the portal triads, since average mitochondrial size and number vary with position in the lobule (13, 14).

Cell Fractionation

Mitochondrial and microsomal fractions were prepared by the methods of Sottocasa et al. (17), with the following modification: two centrifugations at 600 *g* for 8 min were employed to sediment the nuclear fraction, in order to reduce contamination of the mitochondrial supernatant by red blood cells. The yield of mitochondria was estimated by comparing cytochrome oxidase activity isolated in the fraction with that in the whole homogenate. The yield of microsomes was similarly determined, using nicotinamide adenosine dinucleotide phosphate (NADPH)–cytochrome *c* reductase as a marker enzyme. Cells other than hepatocytes, e.g. Kupffer cells, are ignored for the purposes of this calculation. Even though they are numerous, these cells account for only a small fraction of the liver volume and contain very little mitochondrial or microsomal material (14). Since recoveries of cell fractions were variable, total mitochondrial and microsomal phospholipid and protein per gram of liver were calculated from the estimates of yield obtained with the marker enzymes. Less

[1] Imferon, Lakeside Laboratories, Milwaukee, Wis.

than 1.2% of the total cytochrome oxidase activity in the whole homogenate contaminated the microsomal fraction, and less than 3.3% of the total NADPH–cytochrome *c* reductase appeared in the mitochondrial fraction. Recovery of microsomes from the total homogenate averaged about 50%. Cytochrome oxidase activity in the mitochondrial fraction approximated 25% of that present in the whole homogenate. Calculations were based upon yield, and it was assumed that the fraction isolated was representative. For example, mitochondrial constituents present in unfractionated material were estimated to be about four times the amount measured in the purified fraction. This calculation, although useful for comparing experimental groups, may lead to an overestimation of the cellular contribution of mitochondrial constituents. Total mitochondrial protein, for example, was sometimes calculated to represent as much as half of the protein present in whole homogenate, even though, from electron microscopic observations, it has been determined that mitochondria normally make up no more than a quarter of the cellular volume.

Chemical Analysis

Protein concentrations of cell fractions were determined according to the method of Lowry et al. (18), with bovine serum albumin as a standard. Phospholipid (PLP) was measured by determining the amount of phosphorus in washed chloroform extracts of cell fractions (19). The amount of phospholipid was calculated by multiplying the values for phosphorus by 25.

Enzyme Assays

NADPH–cytochrome *c* reductase activity was measured spectrophotometrically by following the reduction of cytochrome *c* at 550 mμ (17). Cytochrome oxidase activity was determined by measuring the oxidation of cytochrome *c* at 550 mμ (20).

Mitochondrial suspensions were clarified by incubation for at least 20 min at 4°C in 2% sodium deoxycholate and 50 mM phosphate buffer, pH 7.4. The difference spectrum of a sample reduced with a few milligrams of sodium dithionite was then measured against an oxidized sample. Cytochrome *c* ($c + c_1$) was determined from the wavelength pair 552–540 mμ, using 19.1 as the millimolar extinction coefficient (21) and cytochrome *a* ($a + a_3$) from the wavelength pair 605–630 mμ, using 12.0 as the extinction coefficient (22). Microsomal cytochromes b_5 and P 450 were determined spectrophotometrically (23). All difference spectra were performed in the Shimadzu split beam spectrophotometer model MPS-50L (Shimadzu Seisakusho Ltd., Kyoto, Japan).

RESULTS

Experimental Animals

Iron-restricted animals developed anemia promptly after initiation of the regimen. After 3 wk of iron restriction, the concentration of hemoglobin in group II A was 33% of the control group I A mean (Table I). The body weights of these animals averaged 74% of those of the corresponding control rats. After 8 wk of iron restriction, the animals in group II B were more markedly retarded in growth, but there was little further decrease in hemoglobin concentration. Rats fed a restricted amount of the complete diet (group I C) to keep their weights similar to those of the group II B animals had no depression of hemoglobin concentration but had a significant reduction in liver weight/body weight ($P < 0.02$). Liver weight/body weight in all other groups was essentially unaffected by diet.

TABLE I
Experimental Animals

Group	Diet	Age	Weight	Liver weight	Hemoglobin
		days	*g*	*g/100 g body wt*	*g/100 ml*
I A	Complete—*ad libitum* (21)*	30–38	75.2 ± 3.2‡	3.6 ± 0.1§	14.1 ± 0.2
I B	Complete—*ad libitum* (11)	59–66	198.1 ± 7.0	3.8 ± 0.2	16.3 ± 0.3
I C	Complete—restricted (4)	64	94.5 ± 1.2	2.8 ± 0.03	17.1 ± 0.7
II A	Iron deficient (19)	30–38	55.8 ± 1.1	3.5 ± 0.1§	4.6 ± 0.2
II B	Iron deficient (11)	59–66	88.8 ± 7.8	3.5 ± 0.2	3.8 ± 0.2
II C	Iron deficient (6)	64	165.3 ± 4.2	—	8.2 ± 0.6

* Number of animals.
‡ Mean ± SE of mean.
§ Excludes phenobarbital-treated animals.

Ultrastructural Studies

Table II shows the changes in mitochondrial size and number in hepatocytes after 3 and 8 wk of the iron-deficient diet. In group II C, after the 3 wk exposure to the deficient diet, the percentage of the cytoplasm occupied by mitochondria was increased ($P < 0.01$) as compared to that in control groups I B ($P < 0.01$) and I C ($P < 0.05$). The percentage increased further after prolonged exposure to the deficient regimen (group II B). The increased contribution of the mitochondria to total cytoplasm was attributable primarily to enlargement of individual mitochondria rather than to an increase in mitochondrial number. Rats in group I C, whose growth rate was matched with group II B by restriction in quantity of diet, had a slight enlargement of mitochondria compared with *ad libitum*–fed group I B controls, but the enlargement was not as marked as the enlargement in iron-deficient animals of comparable age and weight. Enlargement of the mitochondria in group II B was reversed after 5 days of iron administration. The number of mitochondria per 100 μ^2 of cytoplasm was not increased in the iron-deficient animals.

The size distribution curves of mitochondrial cross-section areas from three representative animals are depicted in Fig. 1. In the animal given a complete diet *ad libitum* (I B), most of the mitochondrial cross-sections were below 0.5 μ^2 in area, while in the iron-deficient rat (II B), most areas were larger. Mitochondria in the group II C rats exposed to the iron-deficient diet for a briefer period were intermediate in size.

A large number of mitochondria in the iron-deficient animals was completely traversed by a double membrane. Figs. 2–5 illustrate several examples of this in the group II C animals. The membrane partitions often occurred at a point of narrowing or constriction and were contiguous with the inner limiting membrane (Figs. 2–5). Many mitochondria had invaginated areas limited by a single membrane, often in adjoining portion of two organelles (Figs. 3 and 4). Partitioned mitochondria were also observed occasionally in the more severely depleted group II B rats, but were rare in animals after treatment with iron, or in those on the complete dietary regimens.

Following administration of iron, the mitochondria rapidly reverted to normal size and configuration (Table I). After 2 days there appeared to be at least two populations of mitochondria (Fig. 6). Large, rounded, relatively electron-lucent mitochondria characteristic of the iron-deficient animal were still present, but there were also the normal, smaller, elongated, and electron-opaque mitochondria in the same cell resembling those of group I B animals. Other mitochondria were intermediate in size and electron opacity. After 5 days of iron administration, essentially all mitochondria reverted to normal size and appearance. There was a decline in mitochondrial area (per cent of total cytoplasm) to less than that found in either control group during this period of rapid "catch-up" growth.

Mitochondrial Composition

In spite of the marked increase in total mitochondrial area determined by electron microscopy, no significant increase in mitochondrial protein or PLP was detected per gram of liver

TABLE II

Liver Ultrastructure: Mitochondrial Number and Size in Iron Deficiency and after Iron Repletion

Group	Diet	Mitochondrial area	Mitochondrial no.	Mitochondrial cross-section area—mean
		% of cytoplasm	*per 100 μ^2 cytoplasm*	μ^2
I B	Complete—*ad libitum* (5)	21.4 ± 1.5	58.3 ± 4.6	0.37 ± 0.03
I C	Complete—restricted (4)	24.5 ± 1.4	46.4 ± 1.9	0.53 ± 0.03
II C	Iron deficient (3)	29.7 ± 1.0	51.6 ± 5.1	0.57 ± 0.04
II B	Iron deficient (4)	35.0 ± 3.6	40.0 ± 3.3	0.89 ± 0.04
	Repletion—2 days	23.2	33.3	0.70
		21.6	49.0	0.44
	Repletion—5 days	14.2	41.6	0.34
		16.4	47.5	0.35

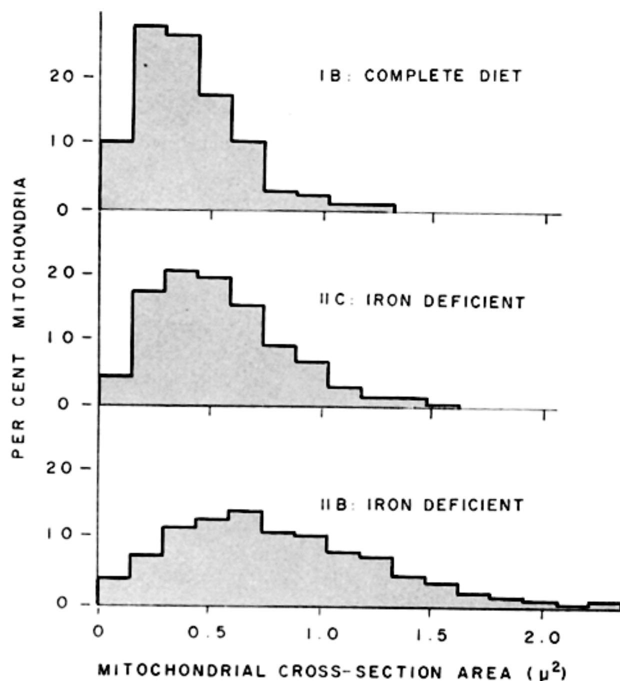


FIGURE 1 Size distribution of mitochondrial cross-sections in hepatocytes from three representative animals. In control rat I B most mitochondrial cross-sections are less than 0.5 μ^2. In rat II B, exposed to the iron-deficient diet for 8 wk, most mitochondrial cross-sections are greater than 0.5 μ^2 in area. Mitochondria in rat II C, exposed to the iron-deficient diet for 3 wk, are intermediate in size.

(Table III). The ratio of PLP to protein was higher in the older animals (I B, I C, II B), but at each age the ratio of PLP to protein in rats on the deficient regimens to those on the complete regimens was similar. Since PLP is restricted to the membrane, this indicates that there is no major alteration in the ratio of membrane to matrix. The cytochromes of the electron transport chain are located within the membranes of the mitochondrial cristae (16), and their concentration within the membranes may be best expressed per milligram of PLP, rather than on a protein basis. Cytochrome *c* per milligram of PLP was slightly, but significantly, decreased ($P < 0.02$) after 3 wk of the low-iron regimen. After 8 wk, the depression of the value ($P < 0.01$) below that of the control groups was somewhat greater. Changes in cytochrome *a* concentration were similar. Cytochrome *c* concentration expressed per gram of liver, or as hepatic cytochrome *c* per 100 g body weight was also decreased below the levels in the *ad libitum*-fed, control animals. Weight-restricted animals, I C, had similar concentrations of cytochrome *c* but lower concentrations of cytochrome *a* than the *ad libitum*-fed, control animals, I B. Cytochrome concentrations per gram of liver, or per 100 g body weight in group I C, were reduced largely on the basis of decreased total mitochondrial PLP and diminished liver weight per 100 g body weight (Table I).

Composition of the Microsomal Fraction

Iron-deficiency resulted in little change in the composition of the microsomal fraction. After 3 wk of the deficient regimen (group II A) the iron-deficient animals showed no significant difference in microsomal phospholipid or protein compared to group I A control animals (Table IV). Cytochromes P 450 and b_5 per mg PLP were also essentially unchanged. Treatment with phenobarbital for 3 days produced almost identical responses in the deficient and control animals. Liver weight and phospholipid and protein per gram of liver were all increased. The rise in the PLP/protein ratio is similar to that described by others (24), and is consistent with an increase in phospholipid-rich, smooth-surfaced membranes.

Cytochrome P 450 was similarly increased in both groups, while cytochrome b_5 was not significantly affected.

After 8 wk of the deficient regimen (group II B, Table IV) cytochrome P 450 per milligram of PLP was decreased in the iron-deficient animals compared to group I B, animals given complete diet *ad libitum* ($P<0.01$). The values for microsomal PLP, protein, and cytochrome b_5 were similar in the two groups. Animals given restricted quantities of the complete diet (group I C) had increased microsomal protein and a correspondingly decreased PLP/protein ratio. Cytochrome P 450 per milligram of PLP was similar to that of control group I B. Cytochrome b_5 per milligram of PLP was greater than in the other two groups, but because of the small liver size the amount of cytochrome b_5 per 100 g body weight was similar in all groups.

Group II B and I B animals had similar concentrations of microsomal PLP and protein (Table IV). The weight-restricted rats (I C) had a higher microsomal protein, which contributed to a decreased ($P<0.001$) PLP/protein ratio. Chronic iron depletion appeared to reduce cytochrome P 450 per milligram of PLP below the levels in groups I C ($P<0.01$) and I B ($P<0.1$). On the basis of liver weight and body weight there appeared to be a similar depression. Cytochrome b_5 content did not differ strikingly among the three groups, except for some elevation per milligram of PLP in group I C compared with the other two groups. Chronically iron-depleted animals (II B), therefore, appeared to have a significant depression only of cytochrome P 450 concentration in the microsomal fraction.

DISCUSSION

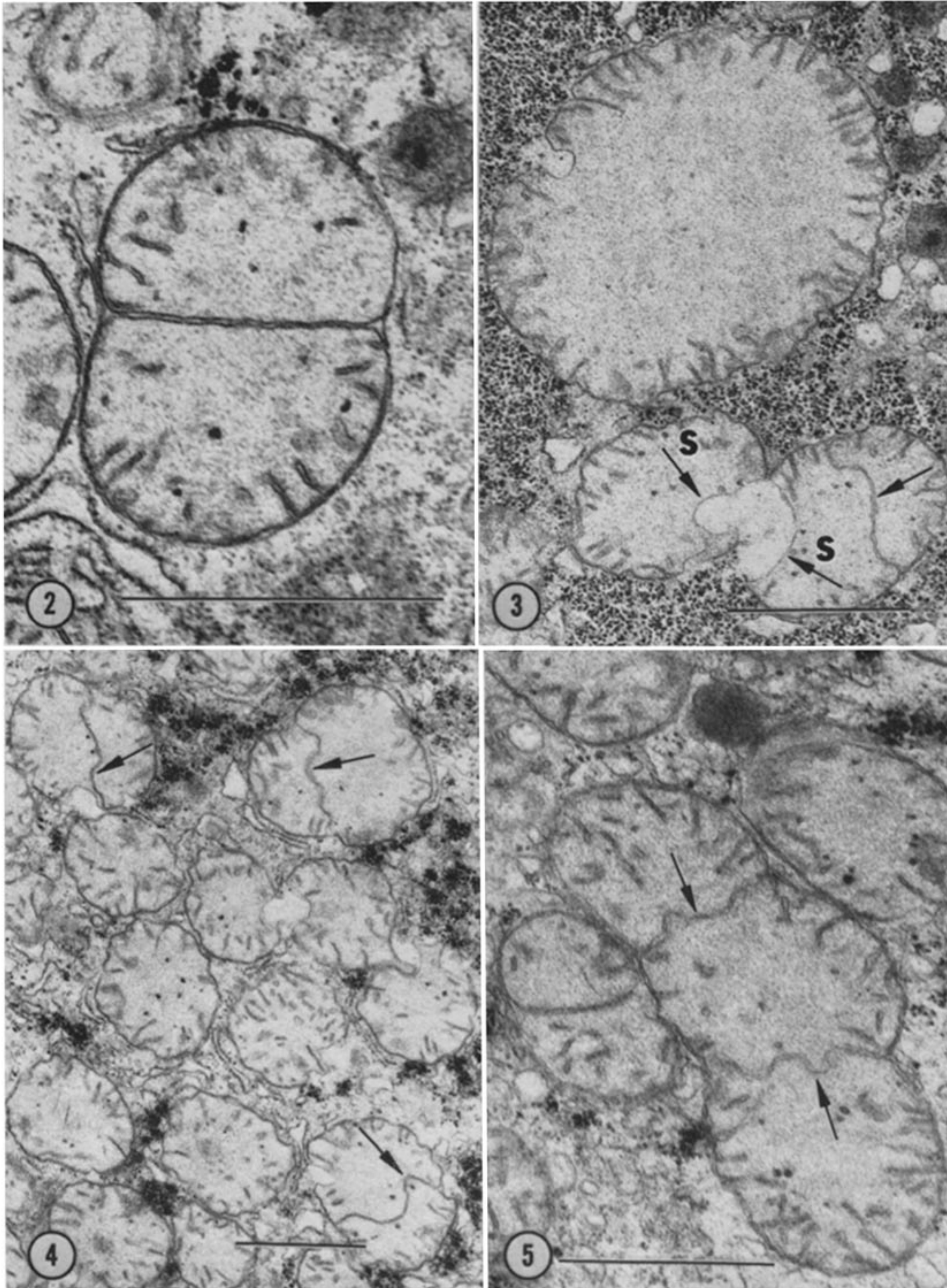
The ultrastructural morphology of the hepatocyte in the iron-deficient rat is dominated by enlarged, rounded, and electron-lucent mitochondria which occupy an increased proportion of the total cytoplasm. The size of individual mitochondria is increased while the number of mitochondria per given volume remains fairly constant. These morphological changes in the mitochondria might be related to either altered composition or to function of the individual organelles, or to a response of the organelles to changes in their cellular environment. The similarities in appearance of the liver mitochondria in animals with deficiencies of iron, copper (10), riboflavin (9), or essential fatty acid (10), each of which results in depletion of certain components of the mitochondrial membrane, suggest the former. The arrangement of proteins and lipids within membranes is believed to favor specific groupings of enzyme molecules (25). It is possible that certain small deviations from the normal stoichiometry could alter the properties of the membrane. An alternate possibility, that the abnormal mitochondrial morphology represents a response to the cellular environment, is suggested by, perhaps, analogous changes in morphology in vitro (26) and in vivo (27, 28) that can be produced by altering the availability of substrates, adenosine diphosphate, and cations. The rounding and/or enlargement of mitochondria observed in extreme or chronic hypoxia (29–31) and in short-term fasting (15) also seem likely to represent a morphological change in response to factors external to the organelle, such as substrate availability. However, the heterogeneous appearances of the mitochondria after 2 days of iron administration—some normal, some retaining

FIGURE 2 A mitochondrion completely traversed by a double membrane at a point of narrowing. The membrane partition is contiguous with the inner limiting membrane. 3 wk iron-deficient diet. × 47,000. Scale marker 1 μ.

FIGURE 3 Adjacent to a giant mitochondrion are two smaller organelles, the adjoining portions of which are invaginated and bound by single membranes (*s*). One of the mitochondria is completely traversed by a double membrane (arrow). 3 wk iron-deficient diet. × 28,000. Scale marker 1 μ.

FIGURE 4 Three mitochondria (arrows) are completely partitioned by a double membrane. Several invaginated portions of the mitochondria appear to be limited by a single membrane. 3 wk iron-deficient diet. × 20,000. Scale marker 1 μ.

FIGURE 5 An elongated mitochondrion is divided by two double membrane partitions (arrows) both at points of narrowing of the organelle. 3 wk iron-deficient diet. × 33,000. Scale marker 1 μ.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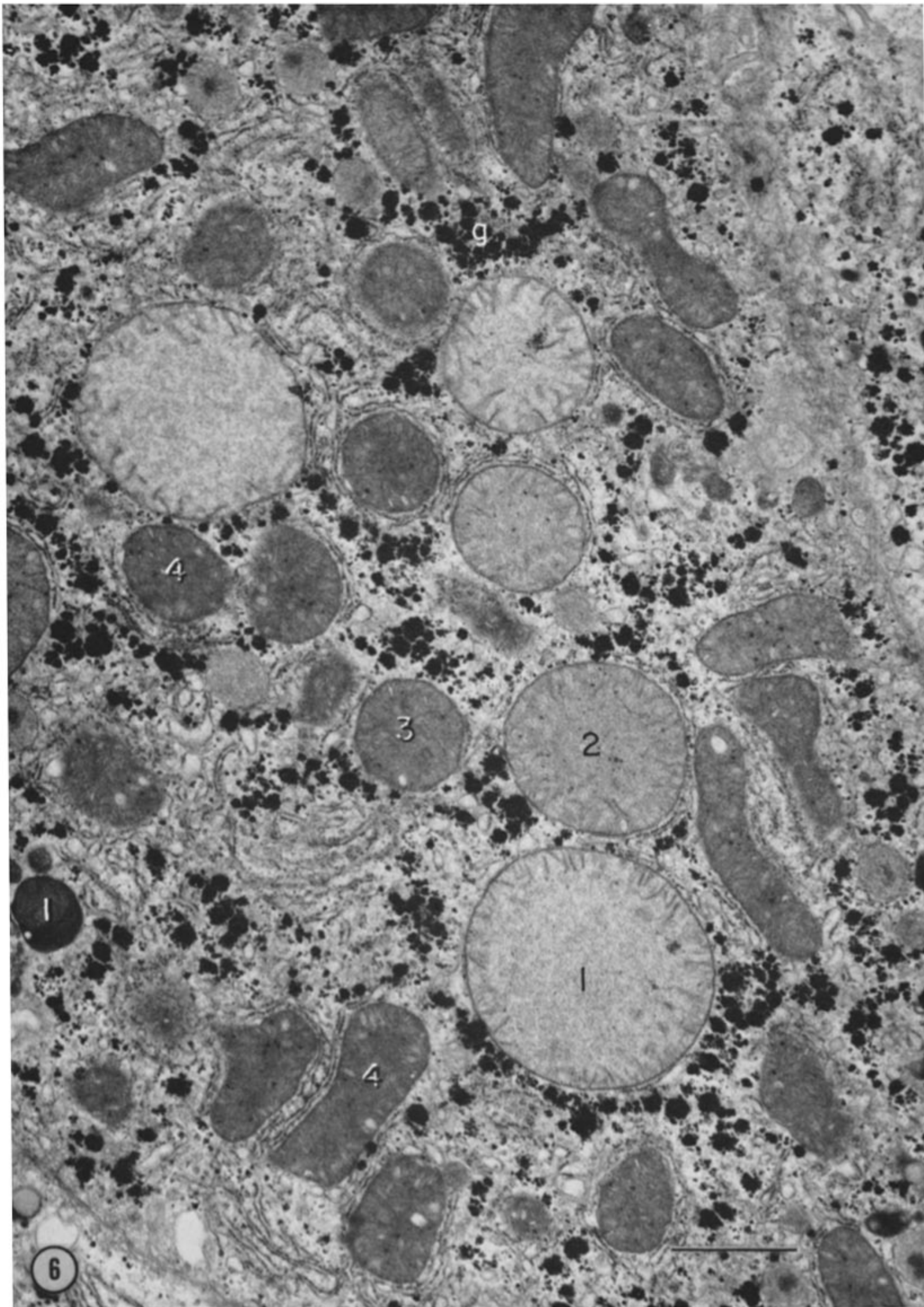

FIGURE 6 Hepatocytes from an iron-deficient rat (II B) 2 days after a single intramuscular injection of 5 mg of elemental iron as iron dextran, followed by the complete dietary regimen. Some mitochondria (*1*) are rounded and electron-lucent as in the untreated iron-deficient animal. Others (*4*) in the same cell are elongated and electron opaque as in the animal on a complete diet. Some mitochondria are intermediate in appearance (*2* and *3*). Glycogen granules (*g*) and a lipid droplet (*l*) are visible. × 20,000. Scale marker 1 μ.

Table III

Mitochondrial Protein, Phospholipid (PLP) and Cytochromes

Group	Diet	Mitochondrial yield	Total mitochondrial		PLP/Protein	Cytochrome *c*	Cytochrome *a*
			PLP	Protein			
		%	*mg/g liver*	*mg/g liver*		*mμ moles/mg PLP*	*mμ moles/mg PLP*
Diet for 3 wk:							
I A	Complete—*ad libitum* (8)	25 ± 2	10.8 ± 0.6	88 ± 7	0.125 ± 0.004	2.63 ± 0.04	2.22 ± 0.05
II A	Iron deficient (8)	20 ± 1	10.8 ± 0.5	94 ± 3	0.113 ± 0.005	2.35* ± 0.09	1.97† ± 0.11
Diet for 8 wk:							
I B	Complete—*ad libitum* (12)	23 ± 2	13.2 ± 0.9	76 ± 6	0.177 ± 0.005	2.68 ± 0.11	2.56 ± 0.22
I C	Complete—restricted (4)	25 ± 1	10.1 ± 0.9	63 ± 6	0.161 ± 0.006	2.75 ± 0.12	1.98‡ ± 0.03
II B	Iron deficient (11)	24 ± 2	12.0 ± 0.9	72 ± 6	0.169 ± 0.009	2.11‡ ± 0.08	1.88‡ ± 0.08

* $P < 0.02$.
‡ $P < 0.01$ comparison with group I B.
† $P \cong 0.05$.

Table IV

Microsomal Protein, PLP and Cytochromes after Iron Deprivation: Response to Phenobarbital Treatment

Group	Diet	Liver weight	Microsomal yield	Total microsomal		PLP/Protein	Cytochrome P 450	Cytochrome *b*
				PLP	Protein			
		per 100 body wt	%	*mg/g liver*	*mg/g liver*		*△ OD 450–490/mg PLP*	*△ OD 424–410/mg PLP*
Diet for 3 wk:								
I A	Complete—*ad libitum* (6)	3.9 ± 0.1	41 ± 2	12.9 ± 0.6	34.9 ± 2.3	0.366 ± 0.018	0.230 ± 0.014	0.303 ± 0.023
II A	Iron deficient (5)	3.7 ± 0.1	50 ± 3	11.9 ± 0.8	30.7 ± 1.5	0.387 ± 0.017	0.216 ± 0.011	0.374 ± 0.033
Phenobarbital treated:								
I A	Complete—*ad libitum* (6)	5.4 ± 0.2	59 ± 5	16.8 ± 0.6	38.0 ± 1.4	0.443 ± 0.007	0.487 ± 0.022	0.243 ± 0.055
II A	Iron deficient (5)	4.9 ± 0.3	56 ± 4	18.0 ± 0.8	39.3 ± 2.0	0.462 ± 0.014	0.445 ± 0.044	0.300 ± 0.022
Diet for 8 wk:								
I B	Complete—*ad libitum* (6)		36 ± 2	18.7 ± 1.7	31.4 ± 4.0	0.605 ± 0.026	0.263 ± 0.009	0.243 ± 0.011
I C	Complete—restricted (4)		50 ± 1	15.6 ± 0.4	41.3 ± 1.4	0.366 ± 0.012	0.255 ± 0.030	0.343 ± 0.003
II B	Iron deficient (4)		44 ± 2	16.0 ± 0.5	29.1 ± 2.8	0.566 ± 0.068	0.185* ± 0.016	0.247 ± 0.026

* $P < 0.01$ comparison with group I B.

the abnormal configuration, and others intermediate in appearance within the same cell (Fig. 6)—particularly favors the hypothesis that the variations in morphology of the individual organelles are related to alterations in their composition. Presumably the mitochondria of normal configuration were also the first to approach normal composition.

It is noteworthy that the rate of reversal of the morphological abnormality in the present study and in the riboflavin-depleted rats (9) was more rapid than would be anticipated if the rate of repair coincided with the normal rates of mitochondrial renewal (32, 33). The heterogeneity of appearance of the mitochondria after 2 days of iron treatment suggests a gradual but not a uniform reversal of the mitochondrial abnormality in contrast to what might be anticipated from the reports of Tandler et al. (9) and of Luck (34). Tandler et al. attribute the disappearance of abnormally enlarged mitochondria after 3 days of riboflavin treatment to division of the organelle, which would be expected to result in an abrupt restoration of individual, enlarged organelles to normal size. However, Luck found that the composition and buoyant density of mitochondria in a choline-requiring strain of *Neurospora crassa*, after a change in the choline content of the medium, were altered gradually and uniformly. It was postulated that the composition of mitochondrial membranes also changed in a uniform fashion and was not restricted to an individual, identifiable population of "new" mitochondria. We attempted to pursue this question by separating small from large mitochondria on sucrose gradients for analysis of cytochrome concentrations, but were unable to obtain adequate resolution between the two.

The large number of mitochondria that are completely traversed by a double membrane in the iron-deficient rats are of particular interest. Similar mitochondria were described by Fawcett (15) after short-term fasting and in fasted animals after refeeding, and by Tandler et al. (9) in riboflavin-deficient mice, especially within the first few days after initiation of a complete diet. Both studies suggested that the mitochondria might be in the process of dividing. In iron-deficient animals "dividing" mitochondria were most abundant prior to treatment with a complete diet, in contrast to the riboflavin-deficient animals. However, there was no evidence of an accumulation of larger numbers of organelles per given area (Table II). Indeed, the total number of hepatocyte mitochondria in the iron-deficient animals would appear to be decreased roughly in proportion to the decrease in body weight and liver weight (Tables I and II). Therefore, if the numerous, partitioned mitochondria in iron-deficient animals represent an increased rate of proliferation, an increased rate of degradation of the organelles would also have to be postulated. This hypothesis would be consistent with the prominence of autophagic vacuoles containing mitochondria as well as other cellular components that have been observed in other forms of malnutrition (35), but this was not a striking feature in the present study.

The biochemical abnormalities in the mitochondrial fraction were quantitatively less impressive than the morphological changes. The protein and PLP content of the mitochondrial fraction in the iron-deficient animals was not increased as might be anticipated from the increased contribution of the mitochondria to the total cytoplasmic volume indicated by the ultrastructural studies. An enlargement of the organelle without a proportionate increase in protein and PLP, a situation which might result from a shift of fluid from other intracellular compartments, is suggested by the relative electron lucency of the mitochondria.

Cytochrome *c* concentrations withiin the mitochondrial membranes were consistently depressed in iron-deficient animals. The effect of iron deficiency upon cytochrome *a* was generally proportional to that upon cytochrome *c*. The unusually low cytochrome *a* values in the weight-restricted group are unexplained. Previous studies of iron-deficient animals have indicated a moderate depression of cytochrome *c* concentration in the liver (2–4); in other tissues cytochrome *c* concentration is more profoundly depressed (3, 4).

No close relationship among mitochondrial respiratory function, changes in mitochondrial morphology, and a decreased cytochrome content is apparent. Morphological alterations can be most striking in tissues whose cytochrome content is close to normal (heart muscle in iron deficiency [4]), and less profound in tissues with marked cytochrome depletion (liver in copper deficiency [36]). There is no evidence of abnormal respiratory function of liver mitochondria in iron-deficient animals (37).

The rate of reversal of the morphological

abnormality in the iron-deficient animals after administration of a complete regimen is probably more rapid than the rate of reversal of the cytochrome deficiency. This can be inferred from previous experiments in the copper-deficient rat (36). Reversal of cytochrome *a* and *c* deficiencies after treatment of copper and iron deficiency, respectively, occurs at a roughly similar, rapid rate in intestinal mucosa and at a much slower rate in skeletal muscle (11, 34), tissues in which the depression is profound enough to allow an accurate estimation of the rate of repair. In the copper-deficient rat, complete reversal of severe cytochrome *a* depletion in the liver required about 10 days (36), roughly consistent with the normal rate of renewal of mitochondrial protein (32) and DNA (33). Presumably, the rates of reversal of cytochrome *c* depletion in iron deficiency and of cytochrome *a* depletion in copper deficiency are similar in liver as in the other two tissues. The differences in cytochrome concentration between control and deficient animals in the present study were too small to allow accurate estimation of the rate of reversal.

There were no striking changes in either the ultrastructure of the endoplasmic reticulum or in the composition of the microsomal fraction. The microsomal cytochromes appeared to be more resistant than mitochondrial cytochromes to iron depletion. The iron-deficient animals were severely anemic and had a significant depression of cytochrome *c* before any effect upon the microsomal cytochrome, cytochrome P 450, was detectable. In response to phenobarbital treatment, there was a fourfold absolute increase in cytochrome P 450 in the iron-deficient rats above that found in the animals on a complete diet. This normal response to phenobarbital treatment implies an unusually high priority for the trace amounts of iron from the deficient diet and/or from the catabolism of hemoglobin and other heme proteins. A possible source of iron is hemoglobin, through the destruction of newly produced, misshapen red blood cells. The production of bilirubin per gram of circulating hemoglobin in iron-deficient rats is twice that in normal animals (38). It has been postulated that some of this bilirubin also originates from a very rapidly catabolized hepatic pool of microsomal heme protein, a labile pool of iron in an iron-poor animal, to which the endoplasmic reticulum of the hepatocyte, by its proximity, might have preferential access.

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