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THE EFFECTS OF IRON DEFICIENCY ON RAT LIVER ENZYMES

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Summary.—The effect of iron deficiency on a number of iron containing enzymes in rat liver has been examined. In addition, 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase have been assayed. Of the mitochondrial electron transport reactions only succinate-cytochrome C reductase activity was decreased in iron deficient animals. Microsomal reductase enzymes associated with the NADPH-oxidase system were also markedly decreased although cytochrome P450 concentrations were unaffected. Both 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase were reduced in young iron deficient rats but the former had returned to control levels at the age of 14 weeks.

IRON CONTAINING enzymes, or those requiring the presence of iron for optimal activity, are involved in a wide variety of metabolic functions and are found in most parts of the cell. Despite the small proportion of the total body iron associated with these activities, there is evidence that impaired function may result from iron deficiency (Jacobs, 1969; Dallman, 1974).

Earlier studies have suggested abnormalities in the electron transport pathway (Beutler, 1957; Richmond, Worwood and Jacobs, 1972) and the present investigation has been carried out to define this in more detail with regard to the liver. In addition, the microsomal reductase system, which includes cytochrome P450 and cytochrome b_5, and the dehydrogenases of the pentose phosphate pathway, 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase have been examined. Normal and iron deficient rats have been studied at the ages of 7 and 14 weeks.

MATERIALS AND METHODS

Animals.—All experiments were performed using male rats of the Wistar strain (Tucks Laboratories, Rayleigh, Essex). The rats were placed on their respective diets (iron deficient and iron supplemented) *ad libitum* immediately on weaning at 21 days old. The iron deficient diet was that described by McCall *et al.* (1962) and contained 1–2 mg iron per kg body weight. The supplemented diet was identical except for the addition of 180 mg of iron per kg (as $FeSO_4 . 7H_2O$). All animals were kept in plastic cages and given deionized water to drink.

A total of 48 animals was used. Twelve animals from both the control and the iron deficient group were killed at the age of 7 weeks and further animals from each group were killed at 14 weeks.

Preparation of subcellular fractions.—The animals were starved for 20 h before killing but were allowed water. They were killed by a blow on the back of the neck, the livers were then immediately excised and placed in ice-cold 0·25 mol/l sucrose containing 3·4 mmol/l tris adjusted to pH 7·4 with HCl (hereafter referred to as "sucrose solution"). All subsequent operations were performed in vessels kept in ice and in centrifuges maintained at 4°C. After rinsing and blotting, the weighed livers were diced and homogenized in a Teflon-glass Potter–Elvehjem type homogenizer, the pestle being passed through the suspension 3 times. The suspension was made up to 10% (w/v) with further sucrose solution. Fractionation was performed on 25 ml of suspension made up from 12·5 ml volumes from 2 separate homogenates. Thus, each determination represents the average for the 2 animals.

The nuclear fraction was obtained by centrifuging 25 ml of whole suspension at 365 *g* for 10 min using an MSE 2L Mistral centrifuge.

The supernatant was decanted and the pellet resuspended by homogenization in a further 20 ml volume of sucrose solution before repeating the centrifugation under the same conditions. The pellet obtained was suspended in 10 ml of sucrose solution by means of the Dounce homogenizer (Kontes Glass Co.) before assay.

The pooled supernatants from the above procedure were then centrifuged at 8800 *g* for 7 min in a MSE Superspeed " 50 " centrifuge. The supernatant, including the microsomal " fluffy layer ", was removed for subsequent preparation of a microsomal fraction and the resuspended pellet centrifuged again. The resulting pellet was made up to 10 ml to form a mitochondrial fraction and the supernatant (again including the fluffy layer) pooled with the first " microsomal " supernatant which were then equally distributed between 2 centrifuge tubes and centrifuged at 80,000 *g* for 40 min. The supernatant fractions obtained were retained for assay and one of the microsomal pellets resuspended and made up to a volume of 5 ml with sucrose solution. The second pellet was suspended in 20 ml of isotonic KCl solution (11·4 g/l) before centrifuging as above. This fraction was used for assay of cytochrome P450, and the treatment with KCl is essential to remove contaminating haemoglobin. The sedimented pellet was made up to 5 ml with isotonic KCl.

The percentage distribution of protein obtained by this procedure was: nuclear fraction 14·3 ± 0·6%; mitochrondrial fraction 25·3 ± 1·0%; microsomal fraction 20·0 ± 0·7% soluble fraction 41·0 ± 1·5%. Studies using marker enzymes indicated that cross contamination of any of the fractions was always less than 10%. Attempts to isolate a lysosomal fraction led to much greater cross contamination and this procedure was abandoned. Only 3 particulate fractions and the supernatant were routinely separated.

Assay of protein, enzyme activities and cytochrome P450.—Protein was determined by the method of Gornall, Bardawill and David (1949) after solubilizing a 100 µl sample of the protein with 100 µl of 10% potassium deoxycholate. A standard reference solution of bovine serum albumin (Sigma Chemicals Co.) was used.

All enzyme assays were performed on the same day as the cell fractionation, the fractions being diluted 50-fold with ice-cold water before assay, in order to disrupt the external mitochrondrial membranes. All determinations were carried out in incubation volumes of 1·0 ml and the volume of diluted enzyme preparation (generally 40 µl or 100 µl) selected to give a convenient rate of enzyme reaction. Changes in optical density were measured continuously on the Pye Unicam SP1800 spectrophotometer fitted with an AR25 recorder. Cuvettes were thermostatically maintained at 25°.

NADH and succinate-cytochrome C reductases and also NADH and succinate-ferricyanide were assayed as described by Hatefi, Haavik and Jurtshuk (1961) and cytochrome oxidase (EC 1.9.3.1., ferrocytochrome C: oxygen oxidoreductase) by the method of Cooperstein and Lazarow (1951), except that solid dithionite was added until the optical density at 550 nm was maximal. The solution was then left to stand at room temperature for at least 30 min before assay. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49 D-glucose 6-phosphate: $NADP^+$ oxidoreductase) and 6-phosphogluconate dehydrogenase (Ec 1.1.1.44 6-phospho-D-gluconate: $NADP^+$ oxidoreductase) activities were measured by the mixed substrate procedure of Glock and McClean (1953) and cytochrome P450 assayed by the method of Omura and Sato (1964).

Determinations are expressed in enzyme units, *i.e.* the amount of enzyme catalysing the metabolism of 1 µmol of substrate per min and the activities of the various fractions expressed per g wet weight of liver. In the case of cytochrome oxidase, the unit used was the first order rate constant K sec^{-1} per mg protein.

Haematological data.—Haemoglobin concentration was determined by the cyanomethaemoglobin method (Dacie and Lewis, 1968) and serum iron and total iron binding capacity determined by the method of Young and Hicks (1965). Liver non-haem iron was measured by the method of Young and Hicks (1965) after a preliminary extraction (Bruckman and Zondek, 1940).

Statistical methods.—The difference between the results obtained from normal and iron deficient animals was examined for statistical significance by the Student's " *t* " test (Croxton, 1959); *P* values greater than 0·05 were considered not significantly different.

RESULTS

The haematological details of all animals are given in Table I. The serum iron concentration, transferrin saturation and liver non-haem iron were significantly decreased and the total iron binding capacity elevated in both groups of iron deficient animals compared with their controls.

The weights of the iron deficient animals were decreased at both 7 and 14 weeks. Similarly, the liver weight in iron deficient animals was also significantly lower although the liver weight:

TABLE I.—*Iron Status, Body Weight and Liver Weight in All Groups of Normal and Iron Deficient Animals*

Group	7 weeks			14 weeks		
	Normal (12)*	Iron deficient (12)	*P*	Normal (6)	Iron deficient (8)	*P*
Haemoglobin (g/100 ml)	15·5±0·80†	8·1±0·90	<0·001	14·1±0·29	6·6±0·90	<0·001
Serum iron (μg/100 ml)	173±11·0	52±10·7	<0·001	150±10·0	41±7·7	<0·001
Total iron binding capacity (μg/100 ml)	453±31	711±32	<0·001	460±49	790±33	<0·001
Transferrin saturation (%)	38·0±1·4	7·2±0·3	<0·001	32·0±1·5	5·2±0·5	<0·001
Liver non-haem iron (μg/100 g)	8·75±0·73	1·3±0·42	<0·001	10·4±0·35	1·12±0·55	<0·001
Weight (g)	184±8	109±6	<0·001	276±12	129±8	<0·001
Weight of liver (g)	5·22±0·27	3·33±0·27	<0·001	6·90±0·25	3·35±0·30	<0·001
Liver weight: body weight ratio	0·028±0·002	0·031±0·002	NS	0·025±0·0007	0·027±0·0013	NS

* No. of animals.
† S.e. mean.
NS = Not significant.

body weight ratios were not significantly changed.

Enzyme activities

There was no decrease in mitochondrial NADH-cytochrome C or NADH-ferricyanide reductase activities in the iron deficient group after 7 or 14 weeks when compared with those in the iron supplemented group. However, after 14 weeks the microsomal activities were both significantly reduced by about 35% compared with those in the normal animals.

Neither of the mitochondrial succinate reductase activities was reduced in the iron deficient group after 7 weeks but at 14 weeks succinate-cytochrome C reductase values in the iron deficient animals were about half those in the normal group. There was no decrease in succinate-ferricyanide reductase activity.

Cytochrome oxidase activity was not significantly decreased in the iron deficient rats either at 7 or 14 weeks. Cytochrome P450 concentrations were similar in the iron deficient and control animals.

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were present only in the soluble fraction of the cell. The effect of iron deficiency on their activities is shown in Table II. At 7 weeks both 6-phosphogluconate and glucose 6-phosphate dehydrogenase activities were decreased by 51 and 53% respectively. However, at 14 weeks there was no difference in the levels of 6-phosphogluconate dehydrogenase as the activity in normal animals had decreased to the level previously found in the iron deficient group. The activity of glucose 6-phosphate dehydrogenase in the iron deficient group was about 45% lower than that of the normal animals at 14 weeks.

DISCUSSION

A good deal of evidence has accumulated suggesting that components of the mitochrondrial electron transport pathway are affected by iron deficiency although the magnitude of these effects seems to vary considerably from one tissue to another. According to some workers, levels of cytochrome C were markedly decreased in the kidney and in skeletal muscle whereas no changes were observed in the heart and liver (Salmon, 1962).

TABLE II.—*Enzyme Activities in Normal and Iron Deficient Animals (Enzyme Units are as Described in the Text)*

		7 weeks			14 weeks		
Enzyme	Fraction	Control (12)	Iron deficient (12)	*P*	Control (6)	Iron deficient (8)	*P*
NADH-cytochrome C reductase	Mitochondrial	28·0±1·0	30·3±1·1	NS	36·0±3·2	33·0±1·66	NS
	Microsomal	45·0±2·9	43·0±2·8	NS	53·0±5·1	38·0±1·10	<0·02
NADH-ferricyanide reductase	Mitochondrial	208±9·3	194±8·6	NS	206±11·9	188±8·2	NS
	Microsomal	221±2·4	191±14·5	NS	251±25·0	196±10·0	<0·05
Succinate-cytochrome C reductase	Mitochondrial	7·4±0·5	6·7±0·77	NS	10·8±0·84	5·5±0·45	<0·001
Succinate-ferricyanide reductase	Mitochondrial	18·5±0·9	16·1±1·0	NS	15·1±2·5	14·4±1·5	NS
Cytochrome oxidase	Mitochondrial	57±1·87	47±5·0	NS	54±0·9	59±1·3	NS
Cytochrome P 450	Microsomal	0·81±0·03	0·80±0·75	NS	0·98±0·05	0·76±0·01	NS
6-phosphogluconate dehydrogenase	Supernatant	9·4±0·3	6·5±0·3	<0·001	7·16±0·65	6·49±0·34	NS
Glucose 6-phosphate dehydrogenase	Supernatant	4·65±0·32	2·17±0·12	<0·001	6·58±1·3	3·6±0·68	<0·05

However, other workers have claimed that levels of the cytochrome were decreased in the liver (Beutler, 1957; Dallman and Goodman, 1971). Similarly, cytochrome oxidase activity seems to be variously affected. For example, while the activity of the enzyme in the alimentary canal is considerably reduced in iron deficiency (Richmond *et al.*, 1972; Jacobs, 1961; Dallman, Sunshine and Leonard, 1967), possibly related to the rapid turnover of these tissues, cytochrome oxidase levels in other tissues is less affected (Beutler, 1959). This divergent pattern has also been observed with succinate dehydrogenase (Richmond *et al.*, 1972; Beutler and Blaisdell, 1960).

The more detailed work described in this present paper also suggests that iron deficiency has a variable effect on the liver mitochondrial reductases. Neither NADH-cytochrome C reductase nor NADH-ferricyanide reductase was decreased by iron deficiency, suggesting that none of the constituents of the electron transport complexes I and III were reduced. Similarly, the succinate-ferricyanide reductase was not altered but the enzyme activity using cytochrome C as the electron acceptor was about 50% lower in the iron deficient mitochondria. Cytochrome oxidase activity was essentially normal in the presence of severe iron deficiency.

Clearly, iron deficiency does not bring about a general decrease in the levels of the mitochondrial constituents despite the fact that the condition is known to result in marked changes in the structure of the liver mitochondria, including changes in the cristae and marked swelling (Goodman, Warshaw and Dallman, 1970). However, there is no clear indication that such alterations take place *in vivo* during iron deficiency and it may be that iron deficient mitochondria are more prone to disruption by the fixation procedure employed. These workers found no change in ATP production or respiration in iron deficient rats although any changes in the membrane structure might be expected to become evident in an impairment of iron or substrate transport.

Unlike the mitochondrial enzymes, a marked decrease was observed in the activities of the microsomal reductases. The NADH-cytochrome C and NADH-ferricyanide systems, which can utilize NADPH and NADH as reducing substrates, form part of the NADPH-oxidase system (Gillette, 1971) and involve the iron containing cytochromes b_5 and P450 as well as a non-haem iron containing component. Levels of both these reductases were reduced by about 35% in the iron deficient animals but the concentration of cytochrome P450 was unaffected. Dallman and Goodman (1971) also show that iron deficiency has no effect on the levels of cytochrome P450, indeed induction of the reductase system by treatment with phenobarbitone produced a three-fold increase in levels of both cytochrome b_5 and P450 in the iron deficient and normal animals. These workers interpreted their results to indicate a high priority of the microsomal system for the available iron. The abnormalities observed in our microsomal fractions might not result directly from a shortage of iron but from a secondary consequence of the iron deficient state.

Further weight is added to this possibility by the fact that levels of both the pentose-phosphate pathway dehydrogenase enzymes, which are not thought to be iron dependent, were decreased in 7-week old rats on the iron deficient regimen. After 14 weeks the activity of only glucose 6-phosphate dehydrogenase was lower in the iron deficient animals. Decreased levels of this enzyme were also observed by Strivastava, Sanwal and Tewar (1965). Increases in the activity of the enzyme glycogen phosphorlyase observed by these workers were not found in our experiments. In the liver, about half the metabolized glucose enters the pentose-phosphate pathway and is an important factor in the maintenance of NADPH levels. The decreased dehydrogenase activities may reflect a

reduction in other synthetic functions of the liver as a response to iron deficiency.

It is of interest that myocardial mitochondrial preparations from the same animals used in this study showed a greater range of abnormality than the liver mitochondria (Blayney *et al.*, 1974). There was a marked diminution in both NADH and succinate-cytochrome C reductases in iron deficient animals together with a reduction of cytochrome C oxidase at 14 weeks. In addition, the concentrations of cytochromes a_3, b and c were all significantly reduced at 14 weeks. It appears that in the face of severe iron depletion within the body the various iron containing compounds are not affected uniformly, nor does the depletion of particular compounds occur at the same rate in different organs. The effectiveness of haem compounds at different sites in competing for the limited amounts of available iron may depend on the metabolic activities of the organ and its reserve capacity. In the case of cytochrome P450, the normal concentrations found in the present study and the observation of increased concentrations by Dallman and Goodman (1971) following phenobarbitone administration suggest that induction of the cytochrome may be an important factor. The demonstration of a reduced hexobarbitone sleeping time in iron deficient rats (Becking, 1972) suggests not only that this may occur but that there may be functional consequences.

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