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DECREASED CYTOCHROME P450 AND INCREASED PORPHYRIN CONCENTRATIONS IN THE LIVERS OF RATS ON A LOW IRON DIET GIVEN A SINGLE DOSE OF DESFERRIOXAMINE

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Abstract—The efficacy of the iron chelator desferrioxamine at a site of action in the liver was investigated. A single dose given to 48 hr starved male rats (1.56 m-moles/kg, i.p.) caused a time dependent decrease in liver ferritin iron concentrations to about 60 per cent of control values 4 to 8 hr after dosing. Concurrently with the decrease in ferritin iron levels, there was a small accumulation of porphyrins in the liver. By keeping male rats on a low iron diet for 10 days followed by 48 hr starvation, liver ferritin iron concentrations were reduced to about 5 per cent of control values. Administration of desferrioxamine to these animals caused a further decrease in liver ferritin iron levels and a marked increase in the accumulation of porphyrins in the liver. After 30 days on a low iron diet, liver ferritin iron could no longer be detected. Blood haematocrits and haemoglobin levels were severely depressed. Liver cytochrome b_5 and P450 values were similar to controls. In these animals desferrioxamine caused a substantial time dependent increase in the porphyrin content of the liver and a decrease in cytochrome P450 levels. Cytochrome b_5 concentrations were not significantly affected. It was suggested that these results were consistent with desferrioxamine blocking hepatic haem synthesis by making iron unavailable for inclusion into protoporphyrin at the ferrochelatase step. Relative to control animals, hepatic mitochondrial 5-aminolevulinic acid synthetase was significantly depressed in rats kept for 10 or 30 days on the low iron diet. Administration of desferrioxamine caused no marked increase in 5-aminolevulinic acid synthetase activities in either group of rats in the 24 hr after dosing. It was concluded that in addition to haem, iron may play a regulatory role in controlling the activity of 5-aminolevulinic acid synthetase.

Deferrrioxamine is a chelator with a high affinity for ferric ions (binding constant, 10^{31}; [1]). It is widely used in the treatment of iron overload such as occur in certain forms of thalassemia [2]. Administration of desferrioxamine to rats leads to the rapid excretion of chelated iron into the bile and urine [3, 4]. Little is known of the pools from which iron is removed, although the majority of iron in the body, i.e., in haemoglobin and myoglobin, is not available for chelation [1]. In the liver, iron of the parenchymal rather than the reticuloendothelial cells is the most readily depleted by desferrioxamine [5]. There is evidence to suggest that this iron does not originate from the catabolism of haem but from iron storage proteins such as ferritin [5]. Repeated administration of desferrioxamine to rats results in a marked reduction of liver ferritin iron [6]. Desferrioxamine given to *Chang* cells in tissue culture, also inhibits the uptake of iron by ferritin protein [7], while in cultured chick embryo liver cells it causes the accumulation of porphyrins by making iron unavailable for haem synthesis [8].

In this paper, rather than measuring overall urinary and biliary excretion of chelated iron, we report on criteria for determining the efficacy of desferrioxamine at a site of action in the liver. The effect of pre-lowering the iron content of the liver by keeping rats on a low iron diet for various lengths of time before the administration of desferrioxamine is also investigated.

MATERIALS AND METHODS

Animals and dosing. Male Sprague–Dawley rats housed in plastic cages with stainless steel grid floors were used. These were normally given Purina Lab Chow diet (Ralston Purina Co., St. Louis, iron content, 275–380 mg/kg) and deionised water to drink. In some instances, rats of 110–120 g or weanling animals (50–60 g) were fed 10 or 30 days respectively on a low iron diet (Teklad Test Diets, Madison, iron content, 1.4–2.8 mg/kg). These animals received glass distilled drinking water and were starved 48 hr before dosing unless indicated otherwise. To prevent bedding or faeces from being ingested during this period, animals were kept in stainless steel metabolic cages for 48 hr prior to being dosed. At the time of dosing all the rats weighed 130–160 g. Desferrioxamine mesylate (Ciba Pharmaceutical Co., Summit NJ) was dissolved in water and given intraperitoneally, normally at a dose of 1.56 m-moles/kg (1000 mg/kg) in a volume of 0.5–0.6 ml unless stated otherwise. Animals were killed by decapitation at various times after dosing as indicated.

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Estimation of liver ferritin. Anti-rat ferritin was prepared as follows: liver ferritin from iron loaded rats was purified according to the procedure of Linder and Munro [9]. This ferritin gave a single immunoprecipitation line in agar double diffusion and cross-immunoelectrophoresis when tested against rabbit anti-ferritin (supplied by Dr. Ralph Green of this Foundation). A goat was injected weekly for 4 weeks into the lymph nodes of the leg with 110 μg of ferritin in 1.5 ml of Freund's complete adjuvant and then for a further 4 weeks subcutaneously with incomplete adjuvant. At this time antiferritin antibodies were detected in the blood serum. The dose of ferritin was reduced to 11 μg/week until exsanguination 7 weeks later. The IgG fraction of the goat serum was obtained by precipitation with 50% ammonium sulphate [10]. Only a single immunoprecipitation line was seen when this fraction was tested against purified rat liver ferritin or rat liver cytosol. Each batch of purified antibody was assayed against pure rat liver ferritin and diluted with 0.14 M NaCl so that 0.1 ml precipitated 30 μg of ferritin iron.

Estimation of the ferritin content of rat liver was carried out essentially according to the procedure described by Linder and Munro [9]. Portions of rat liver (2 g) were homogenised in 18 ml of 0.03 M NaCl, heated at 70° for 10 min, then cooled on ice for 20 min. The heat treated homogenate was centrifuged at 15,000 *g* for 20 min to give a clear pink supernatant. To 1 ml of the supernatant was added an equal volume of 0.14 M NaCl and 0.1 ml anti-rat ferritin (sufficient to quantitatively precipitate the ferritin) in a disposable polystyrene tube. After mixing, the tubes were incubated at 37° for 1 hr in a shaking waterbath (100 strokes/min), then left for 18 hr at 4°. The ferritin-antibody precipitates were recovered by centrifugation (3000 *g* for 15 min), washed with 2 ml 0.14 M NaCl and dissolved in 1 ml 0.01 M NaOH. The solubilised ferritin-antibody precipitates were analysed for protein content by the method of Lowry *et al.* [11] using bovine serum albumin as standard and for iron content by atomic absorption spectrophotometry. In some instances, the ferritin pellets were wet ashed by digestion with H_2SO_4, HNO_3 and $HClO_4$ using the method of Brumby and Massey [12]. The final digest was made up to 5 ml with water before determining the iron content. The ferritin iron content in the acid digested samples compared well with the non-digested ones. Glassware used in these experiments was soaked overnight in 2 M HCl and thoroughly washed in glass distilled water to remove traces of iron. All solutions used in the estimation of liver ferritin were prepared in high purity water (Harkco, Gibbstown, NJ, specific resistance > 10 MΩ cm^{-1}).

Preparation of rat liver microsomes. Estimation of cytochromes b_5 *and P450.* The livers of rats killed by decapitation were quickly removed, rinsed in ice-cold 1.15 per cent (w/v) KCl, blotted dry, weighed and 10 per cent (w/v) homogenates prepared in 0.25 M sucrose. Following centrifugation at 10,000 *g* for 20 min, 4 vol. of 8 mM $CaCl_2$ in 0.0125 M sucrose were added to the supernatant [13]. The microsomal fraction was precipitated by centrifugation at 10,000 *g* for 10 min, washed in Ca^{2+}–sucrose and was resuspended in 0.25 M sucrose so that 1 ml of microsomal suspension was equivalent to 0.5 g liver wet weight. Cytochrome b_5 was estimated from the NADH reduced versus oxidised difference spectrum (ΔE 424–409 nm) using a millimolar absorption coefficient of 185 $mM^{-1}cm^{-1}$. Cytochrome P450 was determined from the CO-dithionite versus dithionite reduced difference spectrum (ΔE 450–490 nm) using a millimolar absorption coefficient of 91 $mM^{-1}cm^{-1}$ [14].

Liver porphyrins. These were determined by following the procedure of Abbritti and De Matteis [15]. To 4.5 ml ice-cold 1 M $HClO_4$/methanol (1:1 v/v) was added 0.5 ml of 10 per cent (w/v) liver homogenate in 0.25 M sucrose. After mixing and centrifuging (3000 *g* for 20 min) the porphyrin content of the supernatant was estimated in an Aminco–Bowman spectrofluorimeter (excitation wavelength 405 nm, emission 600 nm) using a protoporphyrin IX standard.

Liver mitochondrial 5-aminolevulinic acid synthetase. Rat liver mitochondria were prepared from 10 per cent (w/v) homogenates in ice-cold 0.25 M sucrose containing 0.1 mM EDTA [16]. The washed mitochondrial pellets were resuspended in sucrose-EDTA so that 1 ml of suspension corresponded to 1 g liver wet weight. 5-Aminolevulinic acid synthetase was measured by the procedure of Narisawa and Kikuchi [17]. Incubation mixtures of 2 ml contained: ATP, 5 μmoles; CoA 0.05 μmoles; pyridoxal phosphate, 0.2 μmole; $MgCl_2$, 5 μmoles; mercaptoethanol, 5 μmoles; sodium succinate, 60 μmoles; glycine, 100 μmoles and potassium phosphate buffer, pH 7.0, 100 μmoles. After equilibration at 37°, 0.5 ml of the mitochondrial suspension was added. The reaction was stopped after incubation for 30 min in a shaking waterbath (100 strokes/min) in air with 0.5 ml ice-cold 12.5 per cent (w/v) trichloroacetic acid. The mixture was centrifuged at 10,000 *g* for 15 min and the 5-aminolevulinic acid estimated in the supernatant by means of a modified Ehrlich reagent [18] after conversion to an Ehrlich reactive pyrrole and column fractionation on Dowex AG-1 X8 acetate. Blank values ($t = 0$) were obtained by adding trichloroacetic acid to the assay mixture kept on ice before addition of the mitochondria.

Estimation of blood haematocrits and haemoglobin. Rats were lightly anaesthetised with diethylether. The abdominal aorta was cannulated in the region of the left renal artery and approximately 3 ml of blood was collected in a heparinised tube. Haematocrits and haemoglobin concentrations were determined according to conventional clinical laboratory methods.

RESULTS

Effects of desferrioxamine on liver ferritin iron and the concentration of porphyrins in the liver. In preliminary experiments using male rats fed a normal Purina Lab Chow diet we were unable to obtain any consistent effect of desferrioxamine on the ferritin iron content of the liver. However, in male rats starved for 48 hr before the administration of desferrioxamine, there was a dose dependent decrease in the levels of liver ferritin iron measured 8 hr after dosing (Fig. 1). Relatively high doses of desferrioxamine had to be used to achieve this effect. Rats appeared subdued for 2–4 hr after being given desferrioxamine at dose

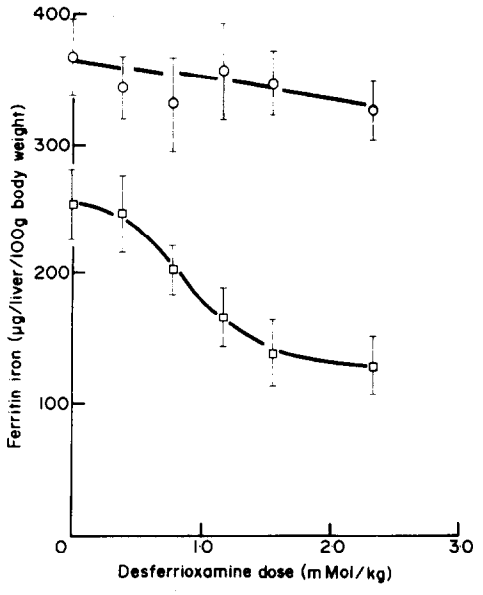

Fig. 1. Effect of desferrioxamine at various dose levels on liver ferritin iron concentrations of starved male or female rats. Rats were kept on a normal Purina Lab Chow diet and starved 48 hr before being given desferrioxamine mesylate intraperitoneally at the doses indicated. Animals were killed 8 hr after dosing and the liver ferritin iron content estimated as described in the Methods section. Each point represents the mean ± S.E. of eight animals. ○, Female rats; □, male rats.

levels of 1.56 m-moles/kg (1000 mg/kg) or greater but subsequently behaved normally. In 48 hr starved female rats, the ferritin iron concentrations of the liver were higher than in males and showed no significant decrease with increasing doses of desferrioxamine.

Figure 2 shows the loss of liver ferritin iron with time following a single intraperitoneal dose of desferrioxamine (1.56 m-moles/kg) to starved male rats. After an initial lag period of about 2 hr there was a rapid fall to about 50 per cent of the saline-dose controls. This was maintained for about 14 hr before returning to control values. At the same time as the fall in hepatic ferritin iron concentrations, there was a transient increase in the levels of porphyrins in the liver lasting 4–6 hr (Fig. 3).

Action of desferrioxamine in rats fed for 10 days on a low iron diet. Rats were fed on a low iron diet to reduce the amount of iron in the body available for chelation and thus potentiate the effects of desferrioxamine. After 10 days on such a diet, the liver ferritin iron content had fallen to about 5 per cent of control values. Desferrioxamine administered to these rats (starved 48 hr before dosing) caused a further fall in the ferritin iron levels in the liver, reaching about 50 per cent of the $t = 0$ values 2 hr after dosing (Fig. 2). In absolute terms, the amount of liver ferritin iron chelated was very much less than in control animals. Relative to control rats, desferrioxamine caused a large increase in the accumulation of porphyrins in the liver (Fig. 3).

Rats on this low iron diet which were not starved showed no consistent decrease in liver ferritin iron as a result of the desferrioxamine treatment. To investigate if iron absorbed from the diet replaces by that chelated by desferrioxamine, rats were fed powdered glucose (Aristar glucose, BDH Ltd., Poole, Dorset, U.K. Iron content < 0.2 mg/kg) for 48 hr, in place of the usual 48 hr starvation. In the glucose fed rats there was no significant further decrease in the ferritin iron content of the liver measured 4 hr after dosing with desferrioxamine (1.56 m-moles/kg).

The response of individual rats kept for 10 days on the low iron diet followed by 48 hr starvation to a dose of desferrioxamine showed considerable variation in terms of the accumulation of porphyrins in the liver. The possibility of obtaining animals with a more uniform low iron status was attempted by keeping rats on a low iron diet for 30 days from the time of weanling.

Effects of desferrioxamine on rats kept for 30 days on a low iron diet. Unlike rats kept for 10 days on a low iron diet, in rats kept for 30 days on the low iron diet, there was a marked reduction in the blood haematocrit values and in red blood cell haemoglobin levels (Table 1). The concentration of cytochromes b_5 or P450 in the liver were not depressed. No liver ferritin iron could be detected in these animals and the ferritin liver protein precipitated was less than 25 per cent that of the controls. This suggested that the majority of the apoferritin protein may be degraded in the absence of iron. A lower stability of the apoferritin protein has been reported by other workers [19]. Relative values for ferritin protein have to be treated with caution since in these experiments, no attempt was made to purify the liver ferritin. In supernatants

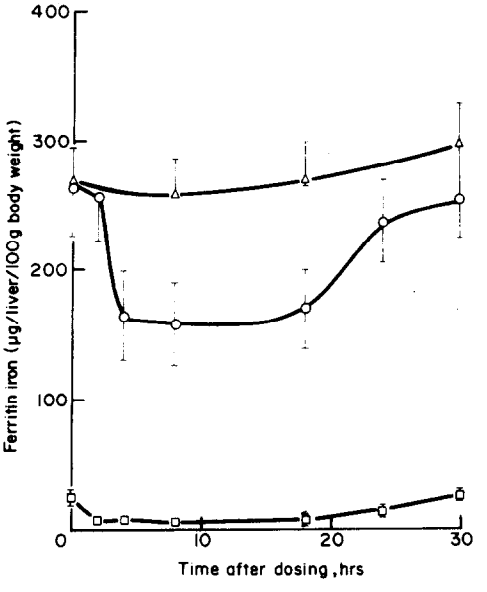

Fig. 2. Dependence with time of liver ferritin iron content after an intraperitoneal dose of desferrioxamine (1.56 m-moles/kg) to starved male rats. Animals were killed at various times as indicated after being dosed with desferrioxamine mesylate. Controls received 0.5 ml of isotonic saline (i.p.) only. Rats were fed either a normal Purina Lab Chow diet or were kept for 10 days on a low iron diet. All rats were starved 48 hr before dosing and for the duration of the experiment. Rat liver ferritin iron concentrations were determined as described in Methods. Each point represents the mean ± S.E. of eight animals. △, Normal diet, controls; ○, normal diet, desferrioxamine dosed; □, 10 day low iron diet, desferrioxamine dosed.

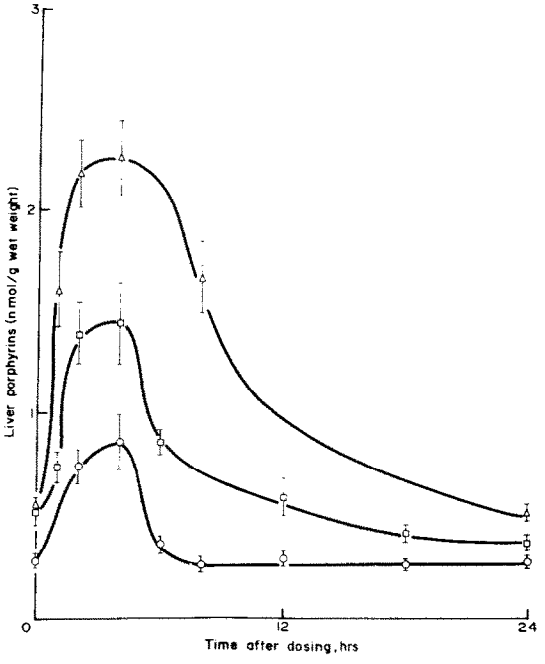

Fig. 3. Dependence of porphyrin concentrations in the livers of starved male rats with time after an intraperitoneal dose of desferrioxamine (1.56 m/moles/kg). Rats were fed either a normal Purina Lab Chow diet or kept for 10 or 30 days on a low iron diet. All animals were then starved for 48 hr before the administration of desferrioxamine. Rats were killed at various times after dosing as indicated and the liver porphyrin levels determined as described in Methods. Each point represents the mean ± S.E. of eight animals. ○, Normal diet; □, 10 days low iron diet; △, 30 days low iron diet.

from heat-treated homogenates, the estimation of ferritin protein could be unreliable due to co-precipitation of non-ferritin proteins with the antibody–antigen complex [9].

Treatment of chronically iron deficient rats with desferrioxamine resulted in a 4-fold increase in the levels of porphyrins in the liver, 4 hr after dosing (Fig. 3). The degree of inhibition of ferrochelatase activity caused by removal of iron from the liver by the desferrioxamine treatment was not directly measured. That haem synthesis was substantially reduced in these animals could be inferred from the time dependent decrease in the cytochrome P450 levels in the liver (Fig. 4) during the time porphyrins accumulated. Cytochrome b_5 was not significantly affected.

Effects of a low iron diet on 5-aminolevulinic acid synthetase. Rats kept on a low iron diet for 10 days had an apparently lower liver mitochondrial 5-aminolevulinic acid synthetase activity than that of control animals. After 30 days on a low iron diet, the apparent activity of this enzyme was even more depressed (Table 1). Administration of desferrioxamine to normal or iron deficient rats caused no significant change in the activities of liver mitochondrial 5-aminolevulinic acid synthetase in the 24 hr period after dosing.

DISCUSSION

Effect of desferrioxamine on liver ferritin iron. Our results showed there to be a marked sex difference in the response of starved rats to desferrioxamine. In males, a single dose was effective in causing a decrease in the liver ferritin iron content whereas a similar dose had no significant effect in starved female rats. Since female rats have a higher turnover of iron than do males [20] it is suggested that in females, iron chelated by desferrioxamine can be more readily replaced from sources other than ferritin iron. However, repeated doses of desferrioxamine to female rats over a 10 day period do result in a substantial reduction of liver ferritin iron concentration [6].

The ferritin iron content of the liver was determined in preference to total or to non-haem iron since it was considered this would give the best estimate of the iron reserves of the liver. Values for the total or non-haem iron levels would include some hepatic iron already chelated by desferrioxamine. This factor would be particularly important at early time points after dosing prior to the ferrioxamine being excreted into the bile or urine [1].

In male rats treated with desferrioxamine, ferritin levels in the liver returned to normal 24 hr after dosing, in spite of continued starvation. This indicated that the hepatic ferritin iron concentration is maintained at the expense of other iron reserves of the body. The source of the iron for the newly formed ferritin is not known. Iron released as a result of erythrocyte turnover may play a part in this process.

Table 1. Effects of feeding a low iron diet to male rats for 10 or 30 days on some liver and blood parameters*

	Controls	10 Day low iron diet	Significance†	30 Day low iron diet	Significance†
Liver weight (% of body weight)	3.08 ± 0.071 (16)	3.09 ± 0.078 (16)	N.S.	3.10 ± 0.051 (16)	N.S.
Liver mitochondrial 5-aminolevulinic acid synthetase (nmoles/30 min/mg protein)	0.815 ± 0.055 (8)	0.300 ± 0.049 (4)	$P < 0.001$	0.190 ± 0.030 (4)	$P < 0.001$
Liver cytochrome P450 (nmoles/g liver)‡	17.08 ± 0.77 (16)	17.86 ± 1.25 (10)	N.S.	19.10 ± 1.5 (4)	N.S.
Liver cytochrome b_5 (nmoles/g liver)‡	6.01 ± 0.18 (16)	5.54 ± 0.44 (10)	N.S.	5.60 ± 0.70 (4)	N.S.
Blood haematocrit (%)	45.6 ± 0.8 (16)	43.4 ± 0.7 (16)	N.S.	27.5 ± 1.0 (16)	$P < 0.001$
Blood haemoglobin (mg/100 ml)	15.91 ± 0.3 (16)	15.01 ± 0.4 (16)	N.S.	8.70 ± 0.3 (16)	$P < 0.001$

* All rats were starved 48 hr before death. Results represent the mean ± S.E. Number of animals are given in parentheses.

† Probability of significance of difference between control and low iron diet animals. N.S. = not significant.

‡ The content of cytochrome/g liver is an operational value which does not take into account the cytochrome content which was removed in the first 10,000 *g* sediment (see Methods).

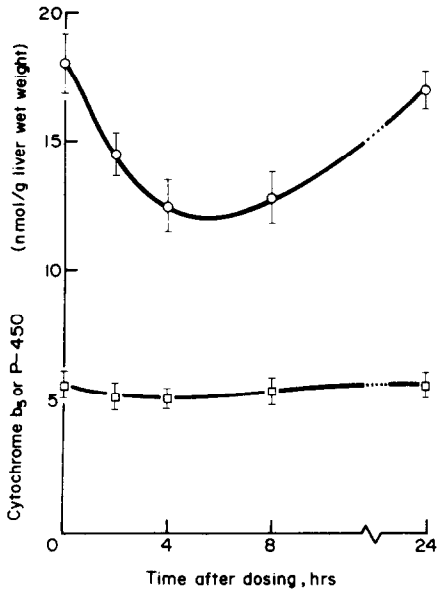

Fig. 4. Dependence of cytochrome b_5 and P450 levels in the livers of starved male rats with time after an intraperitoneal dose of desferrioxamine (1.56 m-moles/kg). Rats were kept for 30 days on a low iron diet and starved for 48 hr before dosing with desferrioxamine mesylate. Animals were killed by decapitation at various times after dosing and the liver microsomal cytochrome b_5 and P450 concentrations determined as described in Methods. Each point represents the mean ± S.E. of four animals. ○, Cytochrome P450; □, cytochrome b_5.

Effects of desferrioxamine on liver porphyrins. Desferrioxamine given to rats on a normal diet caused only a transitory increase in the concentration of porphyrins in the liver (Fig. 3), while the reduction in liver ferritin iron content was more prolonged (Fig. 2). This suggested that desferrioxamine was initially causing a loss of iron from sources other than liver ferritin and the loss was subsequently made good by replacement from the hepatic ferritin iron reserves. The increase in porphyrin levels which occurred in the liver after the administration of desferrioxamine indicated that this compound also caused a decrease in the iron available for haem synthesis. A similar effect is seen when desferrioxamine is added to cultured chick embryo cells *in vitro* [8].

In rats kept for 30 days on a low iron diet, the iron reserves of the body were sufficiently low so as to significantly reduce the blood haemoglobin content (Table 1). The amount of iron in the liver however was sufficient to allow the synthesis of haem for the maintenance of cytochrome P450 and b_5 concentrations. This is in agreement with the findings of Becking [21] that the liver microsomal cytochrome concentrations are maintained in chronic iron deficiency. The loss of hepatic cytochrome P450 following the administration of desferrioxamine to these rats was not inconsistent with a normal breakdown of this cytochrome in the absence of new synthesis caused by the lack of haem production. Cytochrome b_5 has a considerably longer half life than the faster turning over cytochrome P450 components [22, 23] and therefore might be expected to be less easily affected.

Effects of a low iron diet and desferrioxamine on hepatic mitochondrial 5-aminolevulinic acid synthetase. The levels of rat liver mitochondrial 5-aminolevulinic acid synthetase decreased progressively as rats were kept for 10 or 30 days on a low iron diet (Table 1). This suggests that iron may have a direct regulatory role in the synthesis of 5-aminolevulinic acid. In the experimental procedure used for determining 5-aminolevulinic acid synthetase activities, one of the substrates of this enzyme, succinyl-CoA was generated in the mitochondria from succinate, CoA and ATP added to the incubation mixture (see Methods). We cannot therefore distinguish if the effects of iron is a direct one on 5-aminolevulinic acid synthetase or the preceding enzyme, succinyl-CoA synthetase. There is little evidence for a regulatory role of iron at this level of the haem synthetic pathway although iron citrate administration does cause an induction of 5-aminolevulinic acid synthetase and potentiates the induction of this enzyme by 2-allyl-2-isopropylacetamide [25] or hexachlorobenzene [26].

In rats on a 30 days low iron diet there is good evidence, from the accumulation of porphyrins in the liver and the decrease in cytochrome P450 levels, that haem synthesis in the liver had been severely depressed following the administration of desferrioxamine. We had expected that this depression of haem synthesis would be sufficient to cause an increase in 5-aminolevulinic acid synthetase activity since haem is thought to control the activity of this enzyme by a negative feedback mechanism [27]. Such an induction occurs for example, in rats after the administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine, which inhibits the ferrochelatase enzyme of the liver [28]. The apparent absence of induction of 5-aminolevulinic acid synthetase following treatment of rats with desferrioxamine is indicative that iron may be required for the expression of 5-aminolevulinic acid synthetase activity.

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