Induction of Liver Cell Haem Oxygenase in Iron-Overloaded Rats

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Rats were chronically iron-overloaded by intraperitoneal injections of iron-dextran. Electron microscopy revealed that the excess iron was deposited in ferritin-like particles packed in lysosomes and scattered in hepatic cytoplasm. No mitochondrial iron deposition or damage was seen. Furthermore, mitochondrial preparations from chronically iron-overloaded animals were found to be contaminated with lysosomes, which could explain previously reported increases in mitochondrial iron by chemical analysis. Mitochondrial function, as measured by cytochromes *a-a₃*, *b* and *c* concentrations as well as activity of the rate-limiting enzyme of haem synthesis, δ -aminolaevulinate synthetase, was not diminished by chronic iron-overloading. Microsomal haem was decreased by 30% at the time that haem oxygenase, the rate-limiting enzyme of haem degradation, was increased approx. 3-fold. Animals were given a single intraperitoneal injection of iron-dextran and the activities of δ -aminolaevulinate synthetase and haem oxygenase were measured over 24 h. δ -Aminolaevulinate synthetase activity increased approx. 2-fold in these acutely iron-overloaded rat livers, but at a time after the increase in haem oxygenase. These results suggest that an early consequence of excess iron in liver is acceleration of the rate of haem degradation, possibly by haem oxygenase.

Abnormal liver function is a well recognized complication of clinical states of iron overload seen in many haematological diseases and often resulting from multiple erythrocyte transfusions. Little is known, however, about the cellular metabolic consequences of excess iron that lead to hepatic cell abnormal function. Peters & Seymour (1976) showed that in iron-overloaded human liver, the iron accumulated in lysosomes. These investigators suggested that iron accumulation damages the lysosomal membrane, releasing acid hydrolases into the cytoplasm and thus initiating cell damage. In contrast, Hanstein *et al.* (1975) have reported that parenteral iron-overload in rats increased the liver iron content mainly by deposition of iron in mitochondria. These investigators found that the increased concentrations of non-haem iron decreased mitochondrial respiratory control with pyruvate/malate, but not with 3-hydroxybutyrate or succinate, as substrates, and, furthermore, had no effects on substrate oxidation and phosphorylation. They also reported a 3-fold increase of malonaldehyde, which suggests that there is continuous lipid peroxidation of mitochondrial membranes.

If indeed there is mitochondrial damage as suggested by Hanstein *et al.* (1975), one might

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expect to see abnormalities in haem metabolism, as these organelles contain three major enzymes of haem synthesis, δ -aminolaevulinate synthetase, coproporphyrinogen oxidase and ferrochelatase (Maines & Kappas, 1977). The present study was undertaken, therefore, to investigate if iron overload decreases liver cell haem content by decreasing mitochondrial function and haem synthesis. In agreement with Peters & Seymour (1976), we found that in parenterally iron-overloaded rats the iron deposition was in cytoplasmic lysosomes, and that there was no mitochondrial iron deposition or damage detectable by electron microscopy.

Although microsomal haem content was decreased in hepatic cells, this appears to result from increased haem degradation rather than from decreased haem synthesis or mitochondrial function. There was no evidence that the activity of δ -aminolaevulinate synthetase or the amount of haem synthesis were decreased in chronically iron-overloaded animals.

Materials and Methods

Preparation of iron-overloaded rat liver

Male Sprague-Dawley rats initially weighing 100–125 g were used. The rats were given six intraperitoneal injections of iron-dextran (12.5 mg/100 g body wt.) evenly distributed over a 6-week period (chronic iron overload). Control rats were injected

with an equal volume of dextran at the same time. The dextran-treated rats were identical to non-treated animals in all parameters studied. Rats were killed 3 days after the last injection. The livers were removed and homogenized with a Teflon homogenizer in 3 vol. of 0.25 M-sucrose in 0.01 M-Tris/HCl, pH 7.4. Samples of this suspension were taken for assay of δ -aminolaevulinate synthetase activity. In some experiments (acute iron overload) animals were given a single intraperitoneal injection (12.5 mg/100 g body wt.) of iron-dextran.

Assay of δ -aminolaevulinate synthetase

This enzyme was assayed as previously described (Spieler *et al.*, 1978) by incubating the homogenate for 1 h at 37°C in a medium optimum for its activity. The amount of δ -aminolaevulinate formed was estimated in 0.4 ml of the supernatant obtained from precipitation by trichloroacetic acid, by converting δ -aminolaevulinate to the pyrrole compound by condensation with acetylacetone in sodium acetate, pH 4.9. After the aminoacetone was removed by methylene chloride, the colour was developed by addition of an equal volume of Ehrlich/mercury reagent. The peak for δ -aminolaevulinate was scanned on an Aminco-Chance DW 2 spectrophotometer from a wavelength of 500 to 650 nm. The peak at 553 nm represents δ -aminolaevulinate and the millimolar absorption coefficient used was $58 \text{ mm}^{-1} \cdot \text{cm}^{-1}$.

Preparation of mitochondria and microsomal fraction

These were prepared by differential centrifugation as previously described (Maines *et al.*, 1977). The homogenate was centrifuged twice at 800g for 10 min. The resultant supernatant was then centrifuged at 10000g for 10 min to yield the mitochondrial pellet. The remaining supernatant was then centrifuged at 105000g for 90 min to yield the microsomal pellet. The mitochondria were washed twice in 0.25 M-sucrose in 0.01 M-Tris/HCl, pH 7.4. After each wash the pellet was obtained by centrifugation at 8500g for 10 min. Mitochondria were then suspended in the same sucrose/Tris buffer to a final concentration of 6–10 mg of protein/ml. The microsomal fraction was suspended in 0.05 M-potassium phosphate, pH 7.4, to a final concentration of 15–20 mg of protein/ml. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Electron microscopy

Small pieces of freshly excised rat livers were fixed by immersion in 4% (v/v) glutaraldehyde, 0.1 M-sodium cacodylate buffer, pH 7.4. The tissue was washed in buffer, post-fixed in 2% OsO_4 in water, dehydrated in ethanol and embedded in low-viscosity

epoxy resin. Mitochondrial pellets were fixed by pouring the above fixative on the intact pellet. After hardening, the pellets were removed, sliced and their orientation maintained throughout processing so that they could be sectioned and examined across the thickness. Ultra-thin sections were made with a diamond knife and viewed in a Zeiss EM9S.

Haem oxygenase assay

Haem oxygenase was measured by the method of Maines & Kappas (1975). The reaction mixture (6.0 ml) contained 17 μM -haemin, 2 mM- MgCl_2 , 10 mg of microsomal protein, 10 mg of protein of the microsomal supernatant fraction from 105000g centrifugation supplemented with an NADPH-generating system (glucose 6-phosphate, 0.85 mM; glucose 6-phosphate dehydrogenase, 3 units; and NADP^+ , 0.8 mM). One unit of glucose 6-phosphate dehydrogenase activity is the amount of enzyme required to convert 1 μmol of substrate/min. The incubation was performed for 10 min at 37°C. At the end of the incubation period, the mixture was scanned between 400 and 540 nm. Bilirubin formation was calculated by using an absorption coefficient of $32 \text{ mm}^{-1} \cdot \text{cm}^{-1}$, which was obtained by using authentic bilirubin. All spectral studies were carried out with an Aminco-Chance DW 2 spectrophotometer.

Microsomal haem determinations

Microsomal haem was determined as the pyridine haemochromogen by using the reduced-minus-oxidized difference spectrum between 557 and 575 nm, with an absorption coefficient of $32.4 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Rieske, 1967).

In acute iron-overload experiments radioactive microsomal haem was extracted and crystallized by the method of Labbe & Nishida (1957). In these experiments the rats were given an intraperitoneal injection of 10 μCi of δ -amino[4- ^{14}C]laevulinate (sp. radioactivity 55.1 mCi/mmol) to prelabel hepatic haem. After 9 h the animals were given injections of iron-dextran (12.5 mg/100 g body wt.) or dextran. The extraction of haem was performed as described (Labbe & Nishida, 1957) except that 1 ml of rabbit erythrocyte haemolysate was added as a source of haem carrier to each 1 ml of microsomal fraction. In these experiments the mixture of SrCl_2 with acetic acid and acetone was always prepared freshly.

A portion (approx. 0.5 mg) of haem was dissolved in 0.2 ml of 0.1 M-NaOH and bleached with 0.2 ml of 30% (v/v) H_2O_2 . 'Aqueous counting scintillant' (complete xylene-surfactant-based liquid-scintillation 'cocktail'; Amersham Corp., Des Plaines, U.S.A.) was added and the radioactivity determined in a Beckman scintillation counter (efficiency 82%). In the calculations of c.p.m./10 mg of carrier haem it was assumed that the contribution of microsomal haem

EXPLANATION OF PLATE I

Liver parenchymal cells from control (a), 2 weeks (25 mg of iron-dextran/100 g body wt.) (b), and 6 weeks (75 mg of iron-dextran/100 g body wt.) (c) iron-overloaded animals and mitochondrial pellets from control (d), 2 weeks (25 mg of iron-dextran/100 g body wt.) (e), and 6 weeks (75 mg of iron-dextran/100 g body wt.) (f) iron-loaded animals

In the liver parenchymal cells, only in the 6 weeks specimen has sufficient iron accumulated to be visible in lysosomes (arrows) and cytoplasm (asterisk). Magnification, 32000 $\times$. In mitochondrial pellets, a small amount of iron is visible in (e) (arrows), but much more is visible in (f) (arrows). Mitochondria are not significantly different morphologically in any of the preparations. Magnification, 32000 $\times$.

was negligible in relation to the erythrocyte carrier haem added. Haem concentrations were determined as the pyridine haemochromogen as described above.

Measurement of mitochondrial cytochromes

The mitochondria were suspended at a concentration of 10 mg of protein/ml and solubilized with 10% (w/v) sodium deoxycholate. This suspension was then divided into two equal parts; to one part a few grains of $\text{Na}_2\text{S}_2\text{O}_4$ were added to reduce it, whereas to the other part $\text{Na}_3\text{Fe}(\text{CN})_6$ was added to oxidize it. To measure the cytochromes, the differences in spectra between the oxidized and reduced samples were analysed by using the Aminco-Chance DW 2 spectrophotometer (Rieske, 1967). For cytochromes *a*–*a*₃ scanning was performed between 605 and 630 nm, with a molar absorption coefficient of $16 \text{ mm}^{-1} \cdot \text{cm}^{-1}$. Cytochrome *b* was determined by scanning between 560 and 577 nm, with a molar absorption coefficient of $22 \text{ mm}^{-1} \cdot \text{cm}^{-1}$. For cytochrome *c* scanning was between 540 and 550 nm and the molar absorption coefficient used was $19.1 \text{ mm}^{-1} \cdot \text{cm}^{-1}$.

Materials

NADP^+ , glucose 6-phosphate, glucose 6-phosphate dehydrogenase, cycloheximide and haemin were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Iron-dextran ('Imferon') was obtained from Lakeside Laboratories Inc., Milwaukee, WI, U.S.A., and Dextran 40 was from Coulter Laboratories, Berkeley, CA, U.S.A. δ -amino-[4- ^{14}C]laevulinate was obtained from New England Nuclear Corp., Boston, MA, U.S.A., and the 'aqueous counting scintillant' was from Amersham Corp.

Results

Ultrastructural studies

Specimens of liver were examined by electron microscopy 2 and 6 weeks after beginning iron-overloading. At 2 weeks small particles resembling ferritin were seen scattered in the cytoplasm and clustered in the lysosomes of Kupfer cells. None were seen in mitochondria. Parenchymal cells at this time were entirely free of iron particles and were indistinguishable from control cells (Plates 1a and 1b). By 6 weeks ferritin-like particles were scattered throughout the cytoplasm of parenchymal cells and were packed within lysosomes, which were more numerous than at 2 weeks (Plate 1c). Mitochondria were still normal in appearance.

Examination of mitochondrial pellets of normal 2 week iron-overloaded and 6 week iron-overloaded livers showed mitochondria that were morphologically indistinguishable (Plates 1d–1f). What did distinguish the three kinds of mitochondrial preparations was the extent and kind of lysosomal contamination. Iron-containing lysosomes were numerous in mitochondrial preparations from animals subjected to 6 weeks of overload. Indeed the pellet was rust-coloured before processing for electron microscopy. Lysosomes were less common in preparations from control and 2-week animals, and iron-containing lysosomes were absent from the first, and scarce in the second. The latter probably were from Kupfer cells.

Effect of chronic iron-overload on haem metabolism

To study the chronic effect of iron-overload on rat liver haem metabolism, we measured microsomal haem concentrations, the activity of the rate-limiting enzyme of haem synthesis, δ -aminolaevulinate

Table 1. *Effect of chronic iron-overload on rat liver δ -aminolaevulinate synthetase, haem oxygenase, microsomal haem and cytochromes a–a₃, b and c*

Iron-dextran was given intraperitoneally to 15 rats in divided doses to a total of 75 mg of iron-dextran/100 g body wt. The same number of control rats were given similar injections of dextran alone. The animals were killed 3 days after the last injection and the livers prepared as described in the Materials and Methods section. Assays for δ -aminolaevulinate synthetase and haem oxygenase were performed as described in the Materials and Methods section. These assays were carried out on three separate days with livers from five animals in each group on each day. All experiments were performed in triplicate with less than 10% variation between samples. The results shown are expressed ± 1 s.d. for the results obtained on the three days. Microsomal haem and mitochondrial cytochrome assays were performed in triplicate with five livers pooled from animals in each group. Variation between triplicate samples was less than 10%. The assays are described in the Materials and Methods section.

	δ -Aminolaevulinate synthetase (nmol of δ -aminolaevulinate formed/g of liver per h)	Haem oxygenase (pmol of bilirubin formed/mg of microsomal protein per min)	Microsomal haem (nmol/mg of microsomal protein)	Cytochromes (nmol of cytochrome/mg of mitochondrial protein)		
				<i>a</i> – <i>a</i> ₃	<i>b</i>	<i>c</i>
Control	23.3 ± 3.2	281 ± 13.1	1.34	0.096	0.072	0.19
Iron-overloaded	48.3 ± 5.5	807 ± 139.0	0.94	0.101	0.069	0.18

synthetase, and the activity of the rate-limiting enzyme of haem degradation, haem oxygenase (Table 1). Microsomal haem concentrations were decreased by 30%. δ -Aminolaevulinate synthetase and haem oxygenase activities were increased in chronically iron-overloaded animals by 2.1- and 2.9-fold respectively. Mitochondrial cytochromes a - a_3 , b and c contents, on the other hand, were not significantly different from control values.

Effect of a large single dose of iron-dextran on haem metabolism (acute iron-overload)

In these experiments with acute iron-overload (Fig. 1) livers were pooled from two animals for each time point shown. After the injection of a single dose of iron-dextran (12.5 mg/100 g body wt.) microsomal haem oxygenase activity increased at 6 h and reached a maximum at 15 h. δ -Aminolaevulinate synthetase activity initially decreased by 25% and did not increase significantly until 9 h and reached a maximum at 18 h. Cycloheximide (1.8 mg/100 g body wt.), an inhibitor of cytoplasmic protein synthesis, was

Fig. 1. Time course of elevation of δ -aminolaevulinate synthetase and haem oxygenase activities in rat liver from animals injected with 12.5 mg of iron-dextran/100 g body wt. (acute iron-overload)

Each point represents livers pooled from two rats. Each assay was performed in triplicate with less than 10% variation between samples. Control animals (dextran-injected) showed no significant alterations in either δ -aminolaevulinate synthetase or haem oxygenase activities during this time. Cycloheximide was dissolved in 25% ethanol and injected intraperitoneally at 1.8 mg/100 g body weight to one group of animals at 6 h after iron-dextran administration. Two rats from this group were killed at each time indicated, and δ -aminolaevulinate synthetase and haem oxygenase were assayed in triplicate on the pooled liver. The heights of the vertical arrows indicate δ -aminolaevulinate synthetase activities. $\blacktriangle$ — $\blacktriangle$, Change in δ -aminolaevulinate synthetase activity after injection (at 6 h) of cycloheximide; $\bullet$ — $\bullet$, haem oxygenase activity; $\bullet$ — $\bullet$, change in haem oxygenase activity after injection (at 6 h) of cycloheximide.

Table 2. Effect of acute iron-overload on prelabelled microsomal haem

Six rats were given intraperitoneal injections of $10 \mu\text{Ci}$ of δ -amino[4- ^{14}C]laevulinate to prelabel hepatic haem. After 9 h three animals were given a single injection of iron-dextran (12.5 mg/100 g of body wt.) and three animals were given dextran (control). At 16 h after these injections the animals were killed and the livers assayed individually for ^{14}C -labelled haem extracted and haem oxygenase as described in the Materials and Methods section. All assays were performed in triplicate with less than 10% variation between samples from the same animal. The results shown are means $\pm$ 1 s.d. between the individual animals.

	^{14}C -labelled haem extracted (c.p.m./10 mg of carrier haem)	Haem oxygenase (pmol of bilirubin formed/mg of protein per min)
Control	12261 $\pm$ 1438	269 $\pm$ 47.9
Iron-overloaded	8516 $\pm$ 447	877 $\pm$ 40.8

injected into some animals 6 h after the iron-dextran. This has previously been reported to decrease the activity of δ -aminolaevulinate synthetase, which is synthesized in cytoplasmic microsomal fraction and transferred to the mitochondria in a subsequent step (Marver *et al.*, 1966; Beattie & Stuchell, 1970; Sassa & Kappas, 1977). In our hands, indeed within 2 h, the activity of δ -aminolaevulinate synthetase decreased. This effect of cycloheximide on δ -aminolaevulinate synthetase confirms previous results showing that this liver enzyme has a short half-life (60–80 min) (Beattie & Stuchell, 1970; Sassa & Kappas, 1977). Haem oxygenase activity, in contrast, was not decreased until 8 h after the injection of cycloheximide. Thus haem oxygenase appears to have a much longer half-life than δ -aminolaevulinate synthetase. Pimstone *et al.* (1971) reported a 6 h half-life for rat kidney haem oxygenase, which is consistent with our data.

Evidence of increased haem degradation in acute iron-overload

δ -Amino[4- ^{14}C]laevulinate is a specific precursor of haem, and its radioactivity when lost from haem degradation cannot be reutilized for new haem synthesis. This precursor has also been shown to label hepatic haem much more than haemoglobin (Schwartz *et al.*, 1964). Rats were given this precursor to label haem. After 9 h, the animals were given a single dose (12.5 mg/100 g body wt.) of iron-dextran. At 16 h after administration of the iron-dextran the rats were killed. As shown in Table 2, there was a significant 30% loss of microsomal haem radioactivity/10 mg of carrier haem compared with control animals. At this time haem oxygenase activity

was increased 3.3-fold. These results suggest that the rate of liver haem degradation is responsible for the decreased microsomal haem concentrations in iron-overloaded animals.

Discussion

In the present study rats were chronically iron-overloaded by intraperitoneal injection of iron-dextran. Electron microscopy revealed that cellular iron accumulation was in the form of ferritin in cytoplasmic lysosomes, and not in mitochondria. This is in agreement with the report of Peters & Seymour (1976) in iron-overloaded human liver. In contrast, Hanstein *et al.* (1975) reported that the major cellular increase of iron was due to accumulation of iron in mitochondria, from 429 to 679 ng-atoms/g wet wt. These investigators, however, determined the iron content by chemical analysis of subcellular fractions and did not exclude the possibility that their mitochondrial preparations were contaminated by lysosomes. It has been reported that rat liver lysosomes cannot be separated from mitochondria by differential centrifugation (Davies, 1975). In agreement with this, electron microscopy of our mitochondrial preparations showed considerable contamination by iron-containing lysosomes. Any study measuring iron content in mitochondria, therefore, must exclude lysosomal contamination before any conclusions can be drawn.

It is certainly possible that iron could have a metabolic effect on mitochondria before accumulation is visible by electron microscopy. Additional evidence against mitochondrial damage, however, were our findings that the mitochondrial cytochrome contents were not affected, which is in agreement with the work of Hanstein *et al.* (1975), who reported that mitochondrial capacity for phosphorylation and substrate oxidation is normal in iron-overloaded rat liver.

In the chronic iron-overloaded rat, microsomal haem was decreased by 30% at a time when haem oxygenase was increased 3-fold and δ -aminolaevulinate synthetase was increased 2-fold. To investigate the mechanism of decreased microsomal haem in the chronic iron-overloaded rat, we studied the effect of a single dose of iron (acute iron-overload) on prelabelled haem, as well as a time course of induction of haem oxygenase and δ -aminolaevulinate synthetase. In these experiments we assume that the changes occurring after acute iron-overload are related to those observed in chronic iron-overload. This assumption appears justifiable on the basis of similar findings in both the acute and chronic situations. In addition, it seems reasonable to assume that the changes initiated by iron are related to those sustained by iron.

Our findings strongly suggest that the decrease in

microsomal haem results from an increase in haem degradation rather than from a decrease in the mitochondrial portion of haem synthesis. First, in the experiments where haem was prelabelled with radioactive δ -aminolaevulinate followed by acute iron-overload there was a loss of radioactivity from the haem. If haem synthesis was decreased by iron-dextran, one would expect to see no change (or even a slight rise) in radioactivity. In fact, haem synthesis in iron-overloaded animals may be even greater than in control animals. The decrease in microsomal haem radioactivity is best explained by both increased haem breakdown together with dilution of the radioactivity by increased synthesis of unlabelled haem in iron-overloaded animals. Secondly, when haem oxygenase was studied in acute iron-overload it was found that this enzyme increased in activity, and that the rise in activity occurred before that of δ -aminolaevulinate synthetase. Haem oxygenase is the rate-limiting enzyme of haem degradation and can be induced in the liver by a number of substances including haem (Tenhunen *et al.*, 1970), cobalt (Maines & Kappas, 1975) and others (Tenhunen *et al.*, 1970; Maines & Kappas, 1975; Kappas & Maines, 1976; Maines, 1977). The mechanism by which haem oxygenase is induced in the present study is not known. Evidence has been presented that iron itself is a direct inducer of haem oxygenase (Maines & Kappas, 1976; Kappas & Maines, 1976; Maines, 1977), which could explain the increase in haem oxygenase activity that we observed in the iron-loaded rat.

Another possibility is that the effect of the iron is indirect. Iron salts have previously been implicated in microsomal (Wills, 1969; Hochstein *et al.*, 1964) and mitochondrial (Hunter *et al.*, 1963) phospholipid peroxidation. DeMatteis & Sparks (1973) and Levin *et al.* (1973) have observed significant lipid peroxidation *in vitro* and loss of cytochrome *P*-450 haem in rat liver microsomal fraction in the presence of NADPH and iron. The loss of cytochrome *P*-450 in association with microsomal lipid peroxidation has recently been challenged by Horn *et al.* (1976). These investigators used liver microsomal fraction from vitamin E-deficient rats and could not find a decrease in cytochrome *P*-450 haem despite lipid peroxidation *in vivo*. It is not clear, however, if these two systems are directly comparable.

Even though this controversy is not settled, it is conceivable that in our system the excess iron accumulated in the liver increased lipid peroxidation, destabilized the haemoprotein cytochrome *P*-450, and released the haem moiety. Thus, the increase in the free haem might have caused induction of haem oxygenase (Tenhunen *et al.*, 1970). Indeed, Bissell & Hammaker (1976a) have suggested that stimulation of haem oxygenase in the liver results from disso-

ciation of cytochrome *P*-450 haem. They postulated that as haem dissociates from cytochrome *P*-450 it induces haem oxygenase, but also enters into the pool that can regulate δ -aminolaevulinate synthetase by feedback control (Bissell & Hammaker, 1976b).

Alternatively, Schacter *et al.* (1972) have suggested that the iron-induced microsomal lipid peroxidation process results in the destruction of haem, probably through mechanisms not related to haem oxygenase activity. Thus, it is also possible that the loss of microsomal haem that we noted may be due to lipid peroxidation induced by iron-dextran, and the noted increase in haem oxygenase could be unrelated to increased haem concentrations (related instead to iron induction) (Maines & Kappas, 1976; Kappas & Maines, 1976; Maines, 1977). It is conceivable that cytochrome *P*-450 might not have been decreased. Recently, Paine & Legg (1978) have shown that high concentrations of cytochrome *P*-450 can be maintained in the face of high haem oxygenase activity. This might have resulted from an increased rate of haem synthesis in these liver cells in tissue culture.

Maines & Kappas (1977, 1978) have presented evidence that iron is a repressor of δ -aminolaevulinate synthetase formation. Maines *et al.* (1976) have also shown in whole animals that metal ions produce a late 'rebound' increase in synthesis of δ -aminolaevulinate synthetase (at 8 h) after early enzyme repression (at 2 h), as has also been shown with haem (Waxman *et al.*, 1966). Our results are compatible with and confirm these previous reports of cyclic oscillations on this enzyme as well as haem oxygenase (Schacter *et al.*, 1976). Thus, we found a decrease of approx. 25% in δ -aminolaevulinate synthetase activity at 3 h in the acutely iron-overloaded animals, which is compatible with iron or haem repression, and a later increase in enzyme activity beginning at approx. 9 h, which is compatible with the 'rebound'. It is thus noteworthy that iron salts potentiate the induction of δ -aminolaevulinate synthetase where the enzyme activity was assayed at 4–24 h after iron injection (Stein *et al.*, 1969).

We would, therefore, postulate the following pattern of events to explain our results. The effect of iron is to initially repress the activity of δ -aminolaevulinate synthetase and increase the activity of haem oxygenase. As haem oxygenase activity rises (and/or as a result of lipid peroxidation destroying haem) intracellular haem is depleted. δ -Aminolaevulinate synthetase activity is then induced by release of haem feedback repression (Granick & Sassa, 1971) and/or inhibition (Ibrahim *et al.*, 1978). At the time that this enhanced δ -aminolaevulinate synthetase activity is seen, it appears that the metal is no longer available at the cellular sites for enzyme repression.

The increase in δ -aminolaevulinate synthetase

might explain the previously reported slightly increased amounts of urinary uro- and coproporphyrin in dietary iron-overloaded rats (Shanley *et al.*, 1970). These investigators, however, did not observe an increase in δ -aminolaevulinate synthetase unless alcohol was also added to the diet. This could be due to their method or time of assaying the enzyme, or perhaps there are differences between dietary and parenteral iron overload.

Further studies on iron-overloaded tissues are necessary to examine the other haem synthetic enzymes and their relationship to other enzymes in cells. Thus these types of study might prove useful in the understanding of the pathophysiology of organ damage due to excess iron, as well as in the study of pathological conditions with aberrant haem synthesis and/or degradation.

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References

- Beattie, D. S. & Stuchell, R. N. (1970) *Arch. Biochem. Biophys.* **139**, 291–297
- Bissell, D. M. & Hammaker, L. E. (1976a) *Arch. Biochem. Biophys.* **176**, 91–102
- Bissell, D. M. & Hammaker, L. E. (1976b) *Arch. Biochem. Biophys.* **176**, 103–112
- Davies, M. (1975) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Dean, R. T., eds.), vol. 4, pp. 305–348, American Elsevier, New York
- DeMatteis, F. & Sparks, R. G. (1973) *FEBS Lett.* **29**, 141–144
- Granick, S. & Sassa, S. (1971) in *Metabolic Pathways* (Vogel, N. J., ed.), vol. 5, pp. 77–141, Academic Press, New York
- Hanstein, W. B., Sacks, P. V. & Müller-Eberhard, U. (1975) *Biochem. Biophys. Res. Commun.* **67**, 1175–1184
- Hochstein, P., Hardenbrand, K. & Erster, L. (1964) *Biochem. Biophys. Res. Commun.* **14**, 323–328
- Horn, L. R., Machlin, L. J., Barker, M. O. & Brin, M. (1976) *Arch. Biochem. Biophys.* **172**, 270–277
- Hunter, F. E., Gebicki, J. M., Hoffstein, P. E., Weinstein, J. & Scott, A. (1963) *J. Biol. Chem.* **238**, 828–835
- Ibrahim, N. G., Gruenspecht, N. R. & Freedman, M. L. (1978) *Biochem. Biophys. Res. Commun.* **80**, 722–728
- Kappas, A. & Maines, M. D. (1976) *Science* **192**, 60–62
- Labbe, R. F. & Nishida, G. (1957) *Biochim. Biophys. Acta* **26**, 437
- Levin, W., Lu, A. Y. H., Jacobson, M., Kuntzman, R., Payer, J. L. & McCay, P. B. (1973) *Arch. Biochem. Biophys.* **158**, 842–852
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

- Maines, M. D. (1977) in *The Year in Haematology* (Gordon, A. S., Silber, R. & LoBue, J., eds.), pp. 1-45, Plenum Publishing Corp., New York
- Maines, M. D. & Kappas, A. (1975) *J. Biol. Chem.* **250**, 4171-4177
- Maines, M. D. & Kappas, A. (1976) *Biochem. J.* **154**, 125-131
- Maines, M. D. & Kappas, A. (1977) *Science* **198**, 1215-1221
- Maines, M. D. & Kappas, A. (1978) *J. Biol. Chem.* **253**, 2321-2326
- Maines, M. D., Janousek, V., Tomio, J. M. & Kappas, A. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1499-1503
- Maines, M. D., Ibrahim, N. G. & Kappas, A. (1977) *J. Biol. Chem.* **252**, 5900-5903
- Marver, H. S., Collins, A., Tschudy, D. P. & Rechcigl, M. J. (1966) *J. Biol. Chem.* **241**, 4323-4329
- Paine, A. J. & Legg, R. F. (1978) *Biochem. Biophys. Res. Commun.* **81**, 672-679
- Peters, T. J. & Seymour, C. A. (1976) *Clin. Sci. Mol. Med.* **50**, 75-78
- Pimstone, N. R., Engel, P., Tenhunen, R., Seitz, P. T., Marver, H. S. & Schmid, R. (1971) *J. Clin. Invest.* **50**, 2042-2050
- Rieske, J. S. (1967) *Methods Enzymol.* **10**, 488-493
- Sassa, S. & Kappas, A. (1977) *J. Biol. Chem.* **252**, 2428-2436
- Schacter, B. A., Marver, H. S. & Meyer, U. A. (1972) *Biochim. Biophys. Acta* **279**, 221-227
- Schacter, B. A., Yoda, B. & Israels, L. G. (1976) *Arch. Biochem. Biophys.* **173**, 11-17
- Schwartz, S., Ibrahim, G. & Watson, C. J. (1964) *J. Lab. Clin. Med.* **64**, 1003
- Shanley, B. C., Zail, S. S. & Joubert, S. M. (1970) *Br. J. Haematol.* **18**, 79-87
- Spieler, P. J., Ibrahim, N. G. & Freedman, M. L. (1978) *Biochim. Biophys. Acta* **518**, 366-379
- Stein, J., Berk, P. & Tschudy, D. (1969) *Life Sci.* **8**, 1023-1031
- Tenhunen, R., Marver, H. S. & Schmid, R. (1970) *J. Lab. Clin. Med.* **75**, 410-421
- Waxman, A. D., Collins, A. & Tschudy, D. P. (1966) *Biochem. Biophys. Res. Commun.* **24**, 675-683
- Wills, E. D. (1969) *Biochem. J.* **113**, 325-332