Lysosomes, but not mitochondria, accumulate iron and porphyrins in porphyria induced by hexachlorobenzene

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In female rats with porphyria induced by hexachlorobenzene, the amounts of non-haem iron and porphyrins in liver mitochondrial fractions were increased almost 3-fold and > 500-fold respectively compared with that of untreated animals. A considerable fraction of both iron and porphyrins in this fraction was shown to be located in lysosomes. Thus mitochondrial preparations, which were further depleted of lysosomes by Percoll-density-gradient centrifugation, contained 2.78 ± 0.75 and 2.99 ± 0.49 nmol of non-haem iron/mg of protein when isolated from the liver of control rats and hexachlorobenzene-treated rats respectively. Mitochondria isolated from the liver of hexachlorobenzene-treated animals contained a pool of iron (about 1 nmol/mg of protein) that was available for haem synthesis *in vitro*. This pool is similar to that previously reported for mitochondria isolated from the liver of rats with normal haem synthesis. Hexachlorobenzene treatment, therefore, does not affect the iron status of the mitochondria.

INTRODUCTION

In eukaryotic cells four of the eight enzymes of the haem biosynthetic pathway are located in the mitochondria and the other four in the cytosol (see Granick & Beale, 1978). Ferrochelatase (protohaem ferro-lyase, EC 4.99.1.1) catalyses the final step in the pathway, i.e. insertion of Fe^{2+} into protoporphyrin IX, and is located in the mitochondrial inner membrane with its active site facing the matrix side (Jones & Jones, 1969; Harbin & Dailey, 1985). A regulated supply of iron and porphyrin to the mitochondria is therefore a prerequisite for normal haem synthesis.

We have previously shown that rat liver mitochondria contain a pool of iron that is available for haem synthesis *in vitro*, and we suggested that this iron pool may function as the proximate iron donor to ferrochelatase *in vivo* (Flatmark & Tangerås, 1977; Tangerås *et al.*, 1980; Tangerås, 1985). This iron pool represented about 1 nmol of iron/mg of protein, or about 25% of the total amount of iron in the mitochondria. In reticulocytes, Ponka and co-workers obtained evidence for the existence of a similar mitochondrial iron pool (Borova *et al.*, 1973; Ponka *et al.*, 1977), but the magnitude of this pool is not known. Relatively more iron was, however, present in this pool when haem synthesis in the reticulocytes was inhibited *in vitro*. Furthermore, an accumulation of iron in the mitochondria is also seen in reticulocytes from patients suffering from sideroblastic anemia, where haem synthesis is also inhibited (Bottomly, 1977). Whether or not the magnitude of the mitochondrial iron pool in liver cells is also increased when haem synthesis is inhibited is not known.

In porphyria induced by hexachlorobenzene, the activity of the enzyme uroporphyrinogen decarboxylase is decreased in the liver of both experimental animals and man (see Elder, 1978), and in several respects this form of porphyria resembles the human disease porphyria cutanea tarda (Stonard, 1974). Numerous studies have indicated that iron is involved in the pathogenesis of both porphyria cutanea tarda and porphyria induced by hexachlorobenzene, but the mechanism of its effect is not

known (see De Matteis & Stonard, 1977; Smith *et al.*, 1979).

Mitochondria isolated from the liver of hexachlorobenzene-treated rats have been reported to contain increased amounts of iron compared with that from control rats (Hanstein *et al.*, 1981; Masini *et al.*, 1984a,b, 1985), but the form of this extra iron is not known. In addition to enzyme-bound iron, so far only iron used for haem synthesis is known to be present in the mitochondria, but whether or not the magnitude of this iron pool is changed on hexachlorobenzene treatment is not known. A better understanding of how mitochondria handle iron used for haem synthesis should contribute to our understanding of how cells regulate their haem synthesis, and in the present study the iron status of mitochondria isolated from the liver of hexachlorobenzene-treated rats has been further characterized.

MATERIALS AND METHODS

Materials

Hexachlorobenzene (organic analytical standard grade) was obtained from BDH (Poole, Dorset, U.K.). A porphyrin standard marker kit containing 8-, 7-, 6-, 5-, 4- and 2-carboxy-porphyrins was obtained from Porphyrin Products (Logan, UT, U.S.A.; lot no. 70) and uroporphyrin III octamethyl ester was from Sigma Chemical Co. (Poole, Dorset, U.K.). Protein dye reagent and Chelex-100 were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and Percoll (lot no. 2665) was from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

Animals

Female Wistar rats obtained from Møllegaard breeding laboratory (Ejby, Denmark) at a body weight of about 160 g were fed a pellet diet (R3 from Ewos A/B, Södertälje, Sweden) with 0.1% (w/w) hexachlorobenzene added and tap water *ad libitum*. The pellets, which contained 270 p.p.m. of iron (typical analysis given by the manufacturer), were soaked in a solution of hexachlorobenzene in acetone, and the acetone was removed by evaporation under reduced pressure. Control animals

received the same pellet food but without hexachlorobenzene. After 8–10 weeks the animals were killed.

Preparation of mitochondria

The mitochondrial fraction was isolated by differential centrifugation in 0.25 M-sucrose/2 mM-Hepes, pH 7.4, and the final pellet resuspended in the same buffer at a protein concentration of about 30–40 mg/ml (Romslo & Flatmark, 1973; Tangerås *et al.*, 1980). When indicated, the mitochondria were further purified by density-gradient centrifugation in an iso-osmotic medium consisting of Percoll and sucrose (Tangerås, 1983). A solution of 55% (v/v) Percoll containing 0.21 M-sucrose to make the solution iso-osmotic (Vincent & Nadeau, 1984) was adjusted to pH 7.4 with HCl, and 38 ml portions were centrifuged at 19000 rev./min for 20 min at 4 °C in a Sorvall RC 5B centrifuge equipped with a SS-34 rotor (Sorvall Instruments, Newtown, CT, U.S.A.) to form the gradient. On top of the gradient was layered 1 ml of the mitochondrial fraction isolated by differential centrifugation, and the tubes were centrifuged at 19000 rev./min for another 20 min. For analytical purposes the gradients were fractionated from the top into 1.4 ml portions. For isolation of mitochondria from the gradients the mitochondrial band was collected and diluted with 0.25 M-sucrose/2 mM-Hepes, pH 7.4. The mitochondria were pelleted by centrifugation at 6000 rev./min for 17 min in a HB-4 rotor (Sorvall) at 4 °C and then resuspended in the same buffer at a protein concentration of about 30 mg/ml.

The mitochondrial preparations were shielded from light as much as possible during isolation and further manipulations in order to avoid photodamage due to the presence of porphyrins (Sandberg & Romslo, 1982).

Analytical methods

Chelation of Fe(II) with bathophenanthroline sulphonate was measured in a dual-wavelength spectrophotometer (Tangerås *et al.*, 1980). Total non-haem iron was quantified after solubilization of the sample in a buffer consisting of 1% (w/v) SDS, 10 mM-sodium acetate, pH 4.5, and reduction of any Fe(III) with dithionite (Tangerås *et al.*, 1980). Total iron was measured as described by Beinert (1978), except that interfering iron in the reagents was removed by using a Chelex-100 column. Sodium dithionite (1.4 M) was prepared under N₂ in a Thunberg tube in a 50 mM-Hepes buffer, pH 7, and freed from interfering iron on a column of Chelex-100.

The stock solution of Percoll used for preparing the density gradients contained iron at a concentration of 0.269 ± 0.002 mM ($n = 6$). Even though this iron was chelated only slowly in the assay of non-haem iron, the presence of Percoll in the samples gave a background in this assay that was corrected for by running a blank containing the same amount of Percoll as the sample.

Uro- and heptacarboxyl-porphyrins were measured in an h.p.l.c. system similar to that developed for dicarboxyl-porphyrins and dicarboxyl-metalloporphyrins (Tangerås, 1984). The tetrapyrroles were separated on a Supelcosil LC-18 column by using a mobile phase containing 70% (v/v) methanol, 10 mM-acetic acid, 5 mM-tetrabutylammonium hydrogen sulphate and adjusted to pH 5.5 with aq. NH₃. The flow rate was 1 ml/min. The porphyrins were detected from their fluorescence by using an excitation wavelength of 400 nm and an emission wavelength of 622 nm. Total porphyrins represent the

sum of uro- and heptacarboxyl-porphyrins. Uroporphyrin III was prepared by hydrolysis of its octamethyl ester in 6 M-HCl at room temperature for 24 h and, in the standard solutions, porphyrins were quantified spectrophotometrically in aq. HCl (Falk, 1964).

Malate dehydrogenase (EC 1.1.1.37) activity was measured spectrophotometrically in the presence of 0.1% (v/v) Triton X-100 (Bergmeyer & Bernt, 1974). Sulphite dehydrogenase (EC 1.8.2.1) activity was assayed in the presence of 0.04% (w/v) deoxycholate with cytochrome *c* as the electron acceptor, as described by Cohen *et al.* (1972). β -N-Acetyl-D-glucosaminidase (EC 3.2.1.30) activity was measured fluorimetrically (Robinson & Willcox, 1969) at 37 °C in 0.1 M-sodium citrate, pH 4.3, and 0.05% (w/v) Triton X-100. Protein was determined by the method of Bradford (1976), with bovine serum albumin as the standard.

RESULTS

At the end of the feeding period the animals treated with hexachlorobenzene weighed 224.8 ± 12.8 g ($n = 6$), which was slightly less than the control animals (243.7 ± 3.2 g, $n = 6$). The liver was enlarged in the treated rats compared with the controls, with a wet weight of 14.70 ± 1.19 g ($n = 6$) and 9.16 ± 0.49 g ($n = 6$) in the hexachlorobenzene-treated and control animals respectively.

The concentration of porphyrins in the liver was markedly increased (> 500-fold) in the hexachlorobenzene-treated rats (Table 1). Only uroporphyrin and heptacarboxyl-porphyrins were detected, and the former represented $87.5 \pm 2.3\%$ of the total ($n = 6$).

The concentration of iron in the liver of hexachlorobenzene-treated animals was slightly, but significantly, lower than that found in the control animals (Table 1).

The mitochondrial fractions isolated by differential centrifugation from the liver of the hexachlorobenzene-treated rats contained large amounts of porphyrins (Table 2), and the porphyrin pattern was identical with that found in the total liver; i.e. $88.5 \pm 2.9\%$ ($n = 4$) was uroporphyrin and the rest was heptacarboxyl-porphyrin. In the control animals, no porphyrins were detected at the sensitivity of the assay used.

The content of total non-haem iron in the mitochondrial fraction from hexachlorobenzene-treated rats was significantly increased compared with that from control animals (Table 2). Such mitochondrial fractions are, however, inevitably contaminated with lysosomes, which may largely contribute to the measured non-haem iron in

Table 1. Effect of hexachlorobenzene treatment of rats on the total amounts of iron and porphyrins in the liver

The values given are means $\pm$ S.D. for the number of livers indicated in parentheses. *indicates significantly different from the control as determined by the Student's *t* test ($P < 0.05$).

	Iron (μ mol/g of liver)	Porphyrins (nmol/g of liver)
Hexachlorobenzene-treated	3.25 ± 0.68 (6)*	306 ± 83 (6)
Control	4.11 ± 0.72 (6)	< 0.2

Table 2. Amounts of non-haem iron and porphyrins in mitochondrial fractions isolated by differential centrifugation and after purification by Percoll-density-gradient centrifugation

The values given are means $\pm$ S.D. for the number of individual preparations indicated in parentheses. Significance of differences between groups were determined by the Student's *t* test. * indicates significantly different from the control ($P < 0.0005$). ** indicates significantly different from the preparations obtained after density-gradient centrifugation ($P < 0.005$). Abbreviation used: N.D., not determined.

	Control (nmol/mg of protein)	Hexachlorobenzene-treated (nmol/mg of protein)
Non-haem iron		
Before density-gradient centrifugation	5.5 ± 1.1 (6)**	14.7 ± 4.1 (4)*, **
After density-gradient centrifugation	2.78 ± 0.75 (5)	2.99 ± 0.49 (3)
Porphyrins		
Before density-gradient centrifugation	< 0.005	2.59 ± 0.98 (6)**
After density-gradient centrifugation	N.D.	0.39 ± 0.28 (3)

Fig. 1. Chelation of Fe(II) with bathophenanthroline sulphonate in mitochondrial fractions isolated by differential centrifugation

(a) Mitochondrial fraction isolated from the liver of a hexachlorobenzene-treated rat was suspended in 0.25 M-sucrose/10 mM-Hepes, pH 7.4, in the presence of 28 mM-sodium dithionite at a protein concentration of 0.18 mg/ml. The temperature was 25 °C. Bathophenanthroline sulphonate (BPS) was then added at a concentration of 50 μ M, followed by digitonin (20 μ M). The Fe(II)-bathophenanthroline sulphonate complex-formation was measured spectrophotometrically. (b) Same as (a), except that the mitochondrial fraction was suspended in 1% (w/v) SDS/10 mM-sodium acetate, pH 4.5.

such preparations (Tangerås, 1983). The lysosomal iron can, however, be selectively measured by chelation of Fe(II) with bathophenanthroline sulphonate after disruption of lysosomes with digitonin and reduction of iron with dithionite (Tangerås, 1985). From Fig. 1 it is seen that, in the absence of digitonin, the hydrophilic chelator, which does not cross biological membranes, chelated only a small fraction of the iron present in mitochondrial preparations from hexachlorobenzene-treated rats. When digitonin was added, the amount of iron chelated increased drastically. Digitonin (20 μ M) disrupted the lysosomal membrane, whereas the mitochondrial outer membrane, which is more sensitive to digitonin than the inner membrane (Schnaitman & Greenawalt, 1968), was

left intact. Thus $89.4 \pm 3.3\%$ ($n = 4$) of the total activity of the lysosomal marker enzyme β -N-acetyl-D-glucosaminidase in the preparations was released during the incubation, whereas only $8.1 \pm 5.6\%$ ($n = 4$) of the total activity of sulphite dehydrogenase, known to be localized in the mitochondrial intermembrane space (Cohen *et al.*, 1972), was released. A major part of the non-haem iron present in mitochondrial fractions isolated by differential centrifugation from the liver of hexachlorobenzene-treated rats was therefore present in contaminating lysosomes.

When such mitochondrial preparations were subjected to density-gradient centrifugation in an iso-osmotic medium of Percoll and sucrose, mitochondria were separated from the main lysosomal peak, as revealed by the distributions of the activities of the mitochondrial marker enzyme malate dehydrogenase and the lysosomal marker enzyme β -N-acetyl-D-glucosaminidase in the gradient (Fig. 2). The distribution of non-haem iron in the Percoll gradient showed two main peaks, one coinciding with the mitochondrial peak and one located with the lysosomal peak. Also, the distribution of porphyrins in the gradient showed that only a minor fraction of uro- and heptacarboxyl-porphyrins was located with the mitochondrial peak, whereas the major fraction was present with the lysosomal peak (Fig. 2).

Mitochondrial preparations obtained from the Percoll gradient contained less non-haem iron than the preparations applied to the gradient, which were isolated by differential centrifugation (Table 2). A significant decrease in the amount of non-haem iron was also found for mitochondrial fractions from control animals after density-gradient centrifugation (Table 2). In the purified mitochondrial fractions no significant difference in content of non-haem iron was found between fractions isolated from hexachlorobenzene-treated animals and control animals (Table 2).

The mitochondrial preparations obtained after the Percoll-gradient-centrifugation step also contained significantly less porphyrins than the preparations isolated by differential centrifugation alone (Table 2). However, the amounts of porphyrins in the preparations were still higher than those found in preparations from control animals.

Fig. 2. Density-gradient centrifugation of a mitochondrial fraction isolated by differential centrifugation

A mitochondrial fraction isolated from the liver of a hexachlorobenzene-treated rat was subjected to density-gradient centrifugation in an iso-osmotic medium consisting of sucrose and Percoll. Distribution of malate dehydrogenase activity (a), β -N-acetyl-D-glucosaminidase activity (b), non-haem iron (c), and total porphyrins (d) in the gradient is shown. In (a) the density profile of the gradient is also given (○).

DISCUSSION

In the present study, uroporphyrin and heptacarboxylporphyrins accumulated in the liver of female rats fed on a diet containing hexachlorobenzene for 8–10 weeks. The total amount of porphyrins accumulated, as well as the pattern of porphyrin species, were similar to that already reported by others (see Elder, 1978).

The concentration of iron in the liver was slightly less in the hexachlorobenzene-treated animals compared with the controls (Table 1). However, owing to the hepatomegaly induced by hexachlorobenzene, the total amount of iron in the liver was about 25% higher in these animals than in the untreated animals. An increase in the total amount of iron in the liver after hexachlorobenzene treatment has also been reported by others (Saunders *et al.*, 1963; Smith *et al.*, 1979, 1985; Hanstein *et al.*, 1981; Masini *et al.*, 1984b). Smith *et al.* (1985) also found a significant decrease in the concentration of iron in the liver after treating the animals with 0.02% hexachlorobenzene for 90 weeks, whereas others have found an increase in the iron concentration after giving 0.2% hexachlorobenzene for about 8 weeks (Hanstein *et al.*, 1981; Masini *et al.*, 1984b), and no explanation for this discrepancy can be offered.

Mitochondrial fractions isolated by differential centrifugation from the liver of hexachlorobenzene-treated rats contained 2.7 times the amount of non-haem iron present in that from control animals (Table 2), and similar observations have been reported by Hanstein *et al.* (1981) and Masini *et al.* (1984a). However, in the present work it is shown that a considerable fraction of this iron was located in lysosomes contaminating the mitochondria when the latter were prepared by differential centrifugation. Thus no difference in non-haem iron content in mitochondrial preparations from hexachlorobenzene-treated animals and controls was found after a major fraction of the lysosomes was removed from the mitochondria by Percoll-density-gradient centrifugation (Table 2).

The amount of non-haem iron in mitochondrial preparations from control animals was also decreased when subjected to density-gradient centrifugation (Table 2). This is due to the fact that, also, in such preparations from untreated rats, some of the non-haem iron was present in contaminating lysosomes (Tangerås, 1985).

We have previously shown that mitochondria from rats with an undisturbed haem synthesis contain a pool of iron that was not bound in haem or in iron-sulphur centres (Flatmark & Tangerås, 1977; Tangerås *et al.*, 1980). This iron pool could function as an iron source for haem synthesis in isolated mitochondria, and the possibility that it functions as the proximate iron donor to ferrochelatase *in vivo* was therefore considered (Tangerås, 1985). This iron pool was also present in mitochondria from hexachlorobenzene-treated rats and represented about 1 nmol of iron/mg of protein, which is the same amount as that previously reported for mitochondria from untreated rats (Tangerås, 1985). Thus, in contrast with previous results (Hanstein *et al.*, 1981; Masini *et al.*, 1984a), the present work shows that the iron status of the mitochondria was not influenced by hexachlorobenzene treatment of the animals.

Mitochondrial preparations isolated by differential centrifugation of liver homogenates from the hexachlorobenzene-treated animals contained considerable amounts of porphyrins, and values similar to those reported by Hanstein *et al.*, (1981) and Masini *et al.* (1984a, 1985) were found (Table 2). However, from subcellular fractionation studies Sandberg & Romslo (1982) concluded that lysosomes, and possibly also peroxisomes, contained a considerable fraction of the porphyrins accumulated in the liver. These organelles are always present as contaminants in mitochondrial fractions prepared by differential centrifugation, and when lysosomes were separated from mitochondria by Percoll-density-gradient centrifugation, it was evident that most of the porphyrins present in mitochondrial preparations was located in lysosomes (Fig. 2). A peroxisomal localization of these porphyrins can be excluded, since, in Percoll gradients, peroxisomes have a buoyant density of about 1.06 g/ml (Neat *et al.*, 1980) and should therefore be located at the top, rather than at the bottom, of the gradient.

Mitochondrial preparations obtained from the Percoll gradient contained larger amounts of porphyrins than that obtained from control animals (Table 2). This is most likely due to a small, residual contamination of the mitochondria by lysosomes, which contain porphyrins. Thus a complete separation of lysosomes and mitochondria cannot be achieved by Percoll centrifugation, since

lysosomes in liver cells represent a very heterogeneous population, and some of them show a buoyant density similar to mitochondria in iso-osmotic Percoll gradients (Fig. 2; see also Tangerås, 1983; Hultcrantz *et al.*, 1984).

Even though inhibition of hepatic uroporphyrinogen decarboxylase and accumulation of porphyrin derived from its porphyrinogen substrate are the most striking effect seen in porphyria induced by hexachlorobenzene, the data presented here show that it also promotes an increase in a lysosomal population containing large amounts of iron. Also, ultrastructural studies have shown iron-containing particles in hepatocytes and Kupffer cells, possibly within lysosomes, after hexachlorobenzene treatment (Kuiper-Goodman *et al.*, 1974). Whether or not the observed increase in lysosomal iron could account for all the extra iron found in the liver after hexachlorobenzene treatment is not known.

No change in the amount of mitochondrial iron available for haem synthesis was found after hexachlorobenzene treatment. Other porphyria-inducing chemicals provoke inhibition of the enzyme ferrochelatase and accumulation of large amounts of its porphyrin substrate, protoporphyrin, in the mitochondria (Sandberg & Romslo, 1981). How the inhibition of ferrochelatase affects the mitochondrial handling of iron, i.e. the metal-ion substrate of the enzyme, is, however, not known.

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