

THE EFFECT OF DESFERRIOXAMINE ON IRON METABOLISM AND LIPID PEROXIDATION IN HEPATOCYTES OF C57BL/10 MICE IN EXPERIMENTAL UROPORPHYRIA

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Abstract—The effects of the iron chelator desferrioxamine (DFx) on liver iron accumulation, malondialdehyde (MDA) production, porphyrin accumulation and uroporphyrinogen decarboxylase (URO-D; EC 4.1.1.37) activity were investigated over a period of 14 weeks in C57BL/10 mice, made porphyric by the administration of hexachlorobenzene (HCB) and iron–dextran (Imferon, IMF) or IMF alone. In addition, we measured the amount of low molecular weight (LMW) iron in liver tissue to determine a possible correlation with MDA production. These experiments showed that combined treatment with HCB + IMF, as well as IMF alone, resulted in porphyrin accumulation, increased MDA production and reduced URO-D activity, whereas HCB alone had no effect. DFx caused a reduction in hepatic porphyrins, this reduction being more distinct in the IMF group than in the HCB + IMF group. The effect of DFx on MDA production and URO-D activity was in agreement with the results on porphyrin accumulation. LMW iron pool measurements at 11 weeks correlated well with data on MDA production in all treated groups in that period ($r^2 = 0.84$), suggesting both variables are interdependent. In conclusion, these results suggest an important role for iron in porphyrin accumulation, probably through its catalytic role in the generation of oxygen-related free radicals, resulting in direct damage to URO-D. The effectiveness of DFx in reducing porphyrin accumulation is probably the result of a reduction in LMW iron, thus diminishing the amount of iron available for a catalytic role in the generation of oxygen-related free radicals.

Both experimental uroporphyrin and human porphyria cutanea tarda are characterized by a partial block in the haem biosynthetic pathway at the level of uroporphyrinogen decarboxylase (URO-D; EC 4.1.1.37) and by the accumulation of uroporphyrins and heptacarboxylporphyrins in the liver [1]. Uroporphyrin can be induced in rodents and humans by the administration of hexachlorobenzene (HCB), a polyhalogenated aromatic hydrocarbon [2].

As in human porphyria cutanea tarda, iron plays an important role in polyhalogenated aromatic hydrocarbon-induced porphyria; porphyria develops faster if animals are made siderotic [3–12]. In addition the administration of Imferon (IMF), an iron–dextran complex, to C57BL/10 mice, by itself leads to porphyrin accumulation [10–12]. Iron deficiency due to bleeding [5] or treatment with desferrioxamine (DFx) [13, 14], an iron chelator,

diminishes or completely prevents the development of porphyria in animals [6, 8, 15, 16]. The effect of iron has been explained by its ability to participate in iron-catalysed free radical-mediated processes [8, 10, 11, 17–19]. If in hepatocytes, either as a result of cytochrome P450 induction or as a result of (genetically determined) active oxidative metabolism in certain species, reactive oxygen species are produced and “free” iron is also present, highly reactive oxygen-related free radicals could be formed by the Haber–Weiss reaction [20, 21]. In addition, the intracellular presence of free ferrous iron itself can induce the formation of O_2^- (superoxide) [22, 23] and thus initiate the chain of reactions leading to the formation of oxygen-related free radicals. In the presence of oxygen-related free radicals a variety of reactions could be initiated, such as the peroxidation of membrane lipids [24]. Recently, we suggested that the process of lipid peroxidation is involved in the pathogenesis of experimental uroporphyrin [8, 18].

Since the effect of the iron chelator, DFx, on liver iron accumulation, malondialdehyde (MDA) production (as a marker of lipid peroxidation), porphyrin accumulation and URO-D activity in the course of time has not been studied extensively, we decided to investigate this in livers of C57BL/10 mice, made porphyric by the administration of

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§ Abbreviations: DFx, desferrioxamine; MDA, malondialdehyde; URO-D, uroporphyrinogen decarboxylase; HCB, hexachlorobenzene; IMF, iron–dextran; LMW, low molecular weight.

HCb + IMF or IMF alone. The nature of the iron pool involved in experimental porphyria is not clear. On the basis of the finding of a morphological co-occurrence of uroporphyrin crystals and ferritin iron in hepatocytes of porphyric C57BL/10 mice, we suggested a role for ferritin-bound iron in the pathogenesis of experimental porphyria [12]. However, ferric iron in ferritin is sequestered in a non-toxic oxyhydroxide, complexed with phosphate. The release of iron from ferritin requires reduction [25]. Although *in vitro* release of ferrous iron from ferritin by liver microsomes has been described [9, 26–29], it is not clear whether this also occurs *in vivo*. An alternative possibility is an intracellular pool of low molecular weight (LMW) iron, as suggested by Jacobs [30] and others [31–33]. A role for this LMW iron has been demonstrated in free radical formation in iron-loaded cells [23, 34, 35]. A recently developed technique [35] to measure the amount of LMW iron in tissue homogenates was used to elucidate this problem further.

MATERIALS AND METHODS

Chemicals. HCB was purchased from Merck AG (Darmstadt, Germany), ferrihydroxide–dextrane (IMF) was purchased from Fisons Pharmaceuticals (Leusden, The Netherlands), Ethrane (Enfluraan) was purchased from Abbott B.V. (Amstelveen, The Netherlands) and deferoxaminmesilate (Desferal) was purchased from Ciba-Geigy B.V. (Arnhem, The Netherlands). All other chemicals were of the highest purity commercially available.

Animals and treatment. This study was performed in accordance with the “Regulations for the use of laboratory animals at the Erasmus University Rotterdam”, laid down by the Laboratory Animal Committee of the Erasmus University. Male C57BL/10 mice, 9–11 weeks of age and weighing 20–25 g, were purchased from Centraal Proefdier Bedrijf (Zeist, The Netherlands).

HCb treatment consisted of two intraperitoneal (i.p.) injections of 8 mg HCB (400 mg/kg body weight) dissolved in 0.25 mL warm corn oil, with 1 week interval. Mice treated with IMF received a single i.p. injection of 12 mg (600 mg/kg body weight).

Treatment with DFX consisted of daily i.m. injections of 5 mg DFX (250 mg/kg body) in 0.05 mL sterile water, administered alternately to the hind legs. When both HCB and IMF were given, IMF was given 3 days after the second injection of HCB. HCB and IMF were injected under Ethrane anaesthesia.

Twenty mice were given HCB alone (HCB), 25 mice the combination of HCB and IMF (HCB + IMF) and 25 mice IMF alone (IMF). DFX administration started either immediately (HCB + IMF + DFX; 25 mice and IMF + DFX; 25 mice) or at 7 weeks (HCB + IMF + DFX7; 15 mice and IMF + DFX7; 15 mice) to provide information on the effectiveness of DFX in reversing the changes induced by IMF and/or HCB. Twenty mice were not treated at all and served as controls (control group).

In order to obtain blood-free livers, mice were

anaesthetized with Ethrane, the vena cava inferior was cannulated and the liver was perfused with a 0.15 M NaCl solution at room temperature until the fluid leaving the liver was clear (the average perfusion time was 3 min). Livers were removed, weighed and processed for further measurements. Measurements were performed before treatment (0 weeks) and at 4, 7, 11 and 14 weeks after treatment with HCB and IMF and with IMF alone.

Hepatic porphyrins. These were measured using a modification of the method described by Lim *et al.* [36]. Briefly, 300 mg of liver tissue were homogenized (glass on glass, manual) to yield a 10% homogenate in water, which was then centrifuged at 10,000 g for 4 min. The supernatant was stored at –20° until further processing. To 0.1 mL of the homogenate, 0.1 mL of Tris–HCl (50 mmol/L, pH 8.0) and 0.8 mL of a “mix” were added. The “mix” contained: 25 mL dimethyl sulphoxide (100% w/v), 10 mL trichloric acid (50% w/v), 10 mL HCl (1 mol/L), *p*-Benzoquinon (2.3 mmol/L) (Merck-Schuchardt, Hohenbrunn, Germany) [37] and 35 mL of distilled water. This was stirred and centrifuged for 15 min at 1800 g. The porphyrins in the supernatant were measured by reversed-phase HPLC [37]. Protein was measured according to Lowry *et al.* [38], and the amount of porphyrins expressed in picomoles per milligram protein.

Hepatic URO-D activity. To 0.1 mL of a 10% homogenate of liver tissue (as described above), 0.05 mL of DL-dithiothreitol (30 mmol/L) in Tris–HCl (50 mmol/L, pH 8.0) (Sigma Chemical Co., St Louis, MO, U.S.A.) was added to restore the activity of the enzyme uroporphyrinogen-III-synthase (EC 4.2.1.75) [37], followed by incubation for 30 min at 4°. Then, 0.05 mL of porphobilinogen (0.8 mg) in 5 mL of EDTA (4 mmol/L) in Tris–HCl (50 mmol/L, pH 8.0) was added, or followed by incubation for 60 min at 37° [39]. The reaction was stopped by adding 0.8 mL of the “mix” (as described above). This was stirred and centrifuged for 15 min at 1800 g. The porphyrins in the supernatant were measured by reversed-phase HPLC [36]. Protein was measured according to Lowry *et al.* [38] and the amount of porphyrins formed was expressed in picomoles per milligram protein.

Total liver iron content. This was measured by a modification [12] of the method described by Harris [40]. Liver tissue was dried at 110° overnight. The dried liver tissue was weighed, 0.5 mL perchloric acid (70% w/v) was added and the solution was heated until colourless. After cooling, distilled water was added to a final volume of 2.0 mL. From this solution 0.2 mL was taken and 0.1 mL HCl (1 mol/L), 0.2 mL L-ascorbic acid (0.14 mol/L), 0.1 mL sodium acetate (saturated) and 0.2 mL Ferrozine (10 mmol/L) (Sigma) were added. This solution was mixed thoroughly and after 10 min the absorbance was measured at 562 nm against three standard iron solutions. The amount of iron was expressed in micromoles per gram dry weight.

Total protein measurements [41]. A small sample of liver tissue was homogenized (Ultrathurax, Janke and Kunkel, Germany), followed by stepwise dilution. To 100 µL of the 0.1% homogenate, 5 mL of Coomassie brilliant blue was added and after

10 min the absorbance was measured at 595 nm. The results were compared with four different standard solutions of bovine serum albumin (Pierce, Rockford, IL, U.S.A.) in the range of 100–400 $\mu\text{g}/\text{mL}$. The amount of protein was expressed in micrograms per gram wet weight.

MDA. This was measured using the method described by Kornbrust and Mavis [42] and Wilson *et al.* [43]. Four aliquots (0.25, 0.5, 0.75 and 1.0 mL) of a 10% (wet w/v) liver tissue homogenate in a KCl/Tris-HCl buffer (0.15 mol/L, 5 mmol/L, pH = 7.8) were filled up to a final volume of 1.0 mL with the same buffer solution. To each of these samples, 0.3 mL trichloric acid (20% v/v), 0.6 mL 2-thiobarbituric acid (0.05 mol/L) and 0.1 mL 2,6-di-*tert*-butyl-4-methylphenol (BHT) (0.2% in ethanol, w/v) were added. The solution was mixed and centrifuged (10 min, 2800 g). The supernatant was heated for 8 min at 100° and immediately cooled. Subsequently, the absorbance was measured (535 nm, $\epsilon = 156,000$) and the results were compared to a standard solution of MDA, treated in the same manner.

LMW iron. Amounts of LMW iron were measured after 11 weeks of treatment, by a method developed by Voogd *et al.* [35]. Some modifications were necessary, due to the limited quantity of liver tissue available for analysis.

A 10% (w/v) liver homogenate in Tris-HCl (100 mmol/L, pH 7.4) was centrifuged for 15 min at 10,000 g, 0°. To 1 mL of the supernatant, 100 μL DFX (22 mmol/L) were added and the samples were incubated for 5, 15, 30 and 60 min at 37°. To stop the reaction between the available iron and DFX, samples were passed through a SEPPAK C18 cartridge (Millipore Corp., Milford, U.S.A.) immediately after incubation. Prior to use, the cartridges were conditioned by means of pre-elution with 5 mL methanol, followed by 5 mL distilled water. After adding the samples, cartridges were flushed with

5 mL distilled water. In this way ferrioxamine (Fx) and DFX were retained, while most other compounds were eluted. Fx and DFX were subsequently eluted from the SEPPAK with 1 mL methanol and the effluent was applied to a HPLC RP 18 column (250 \times 4 mm i.d., Merck). The mobile phase consisted of $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, acetonitrile (88%, 20 mmol/L, 12%), sodium-EDTA (2 mmol/L) and ammonium acetate (1 mol/L). HPLC analysis was performed on a dual pump LKB system with automatic sample injector and two variable wavelength detectors, to measure Fx (430 nm) and DFX (229 nm) simultaneously in the effluent. The results (duplicate samples, 4-fold measurements per sample) were compared to three standard amounts of iron, treated in the same manner as the liver samples.

Statistical data analysis. All measurements are presented as means \pm SD. Differences within and between groups were evaluated by one-way analysis of variance. As multiple groups were compared, we used a one-way analysis procedure with a Bonferroni correction option in STATA release 2 (Computing Resource Center, Los Angeles, CA, U.S.A.). Results were considered significant if $P < 0.05$.

RESULTS

Total liver iron content (Fig. 1)

The liver iron content in the IMF group was significantly increased at 4 weeks, but even in the following 10 weeks a distinct rise could be measured. Liver iron content in the IMF + DFX group was significantly lower at all times, compared to the IMF group ($P < 0.001$). In the IMF + DFX7 group, liver iron content decreased after starting DFX administration at 7 weeks, resulting in about a 50% liver iron reduction at 14 weeks. The increase in liver iron content in the HCB + IMF group was equal to that in the IMF group. However, in the HCB + IMF + DFX group, liver iron contents were

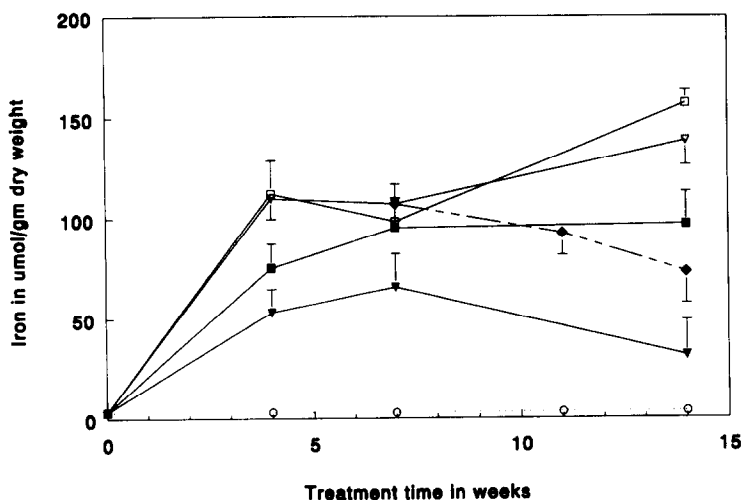


Fig. 1. Liver iron concentration during the experimental period. Iron concentrations were measured in duplicate samples of liver tissue of C57BL/10 mice. Open circles: control group. Open triangles: IMF group. Closed triangles: IMF + DFX group. Open squares: HCB + IMF group. Closed squares: HCB + IMF + DFX group. Closed diamonds: IMF + DFX7 group. Values shown represent the means \pm SD.

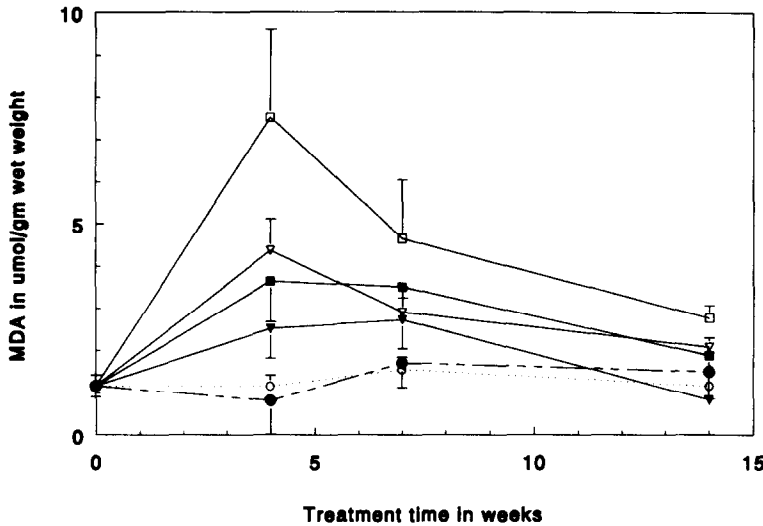


Fig. 2. Liver MDA concentration during the experimental period. MDA concentrations were measured 4-fold in samples of liver tissue of C57BL/10 mice. Open circles: control group. Closed circles: HCB group. Open triangles: IMF group. Closed triangles: IMF + DFX group. Open squares: HCB + IMF group. Closed squares: HCB + IMF + DFX group. Values shown represent the means \pm SD.

significantly elevated at all times, compared to the iron results in the IMF + DFX group ($P = 0.008$). In contrast, liver iron content in the non-treated control mice was very low, with an average of $3 \mu\text{mol/g}$ dry wt liver tissue.

Liver MDA concentration (Fig. 2)

In all treated groups the highest MDA contents were found after 4 weeks of treatment, followed by a reduction in liver MDA, depending on the nature of the treatment. All DFX-treated groups showed a distinct reduction in MDA production compared to those groups receiving the same treatment without

DFX. In the IMF + DFX group, MDA production at 14 weeks was even lower than that in the control groups. Treatment with HCB + IMF resulted in higher MDA concentrations than treatment with IMF alone; DFX reduced these differences.

Total liver porphyrins (Fig. 3)

Significant porphyrin accumulation was not detected during the experimental period in livers of the control group, nor in livers of the HCB group. The IMF group showed a continuous increase in hepatic porphyrin accumulation during the 14 weeks. Apart from the results at 14 weeks, the hepatic

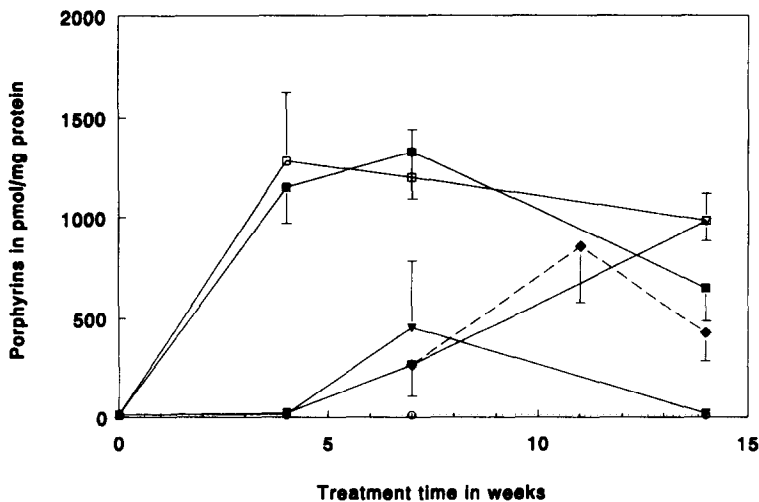


Fig. 3. Liver porphyrin accumulation during the experimental period. Porphyrin concentrations were measured as described in Materials and Methods. Open circles: control group. Open triangles: IMF group. Closed triangles: IMF + DFX group. Open squares: HCB + IMF group. Closed squares: HCB + IMF + DFX group. Closed diamonds: IMF + DFX7 group. Values shown represent the means \pm SD.

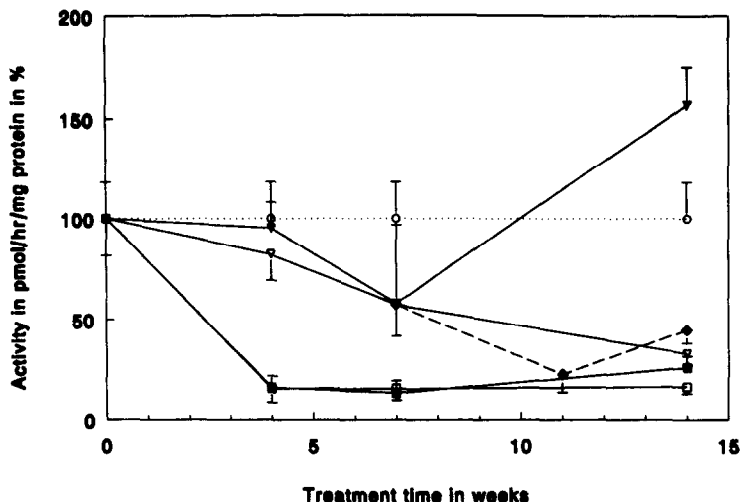


Fig. 4. Liver URO-D activities during the experimental period. URO-D activities were measured as described in Materials and Methods and expressed as percentages of activity of the control group (100%: 161 pmol/hr/mg protein). Open circles: control group. Open triangles: IMF group. Closed triangles: IMF + DFx group. Open squares: HCB + IMF group. Closed squares: HCB + IMF + DFx group. Closed diamonds: IMF + DFx7 group. Values shown represent the means \pm SD.

porphyrin concentration in the HCB + IMF group was significantly higher than in the IMF group ($P < 0.001$). The reduction in porphyrin accumulation was more pronounced in livers of the IMF + DFx group than in the HCB + IMF + DFx group.

Liver URO-D activities

Apart from livers in the control group, a strong negative correlation (-0.80 to -0.92) was found between porphyrin accumulation and URO-D activity data in all treated groups. Liver URO-D activity showed an increase at 14 weeks in the IMF + DFx and IMF + DFx7 group. In the first 4

weeks, URO-D activity in all HCB + IMF treated groups diminished to $\pm 15\%$ of control group values and did not recover during the latter part of the experimental period (Fig. 4).

LMW iron pool content (Fig. 5)

The results on LMW iron pool content at 11 weeks were compared with data on MDA production around that time. Calculations yielded a correlation coefficient of 0.84, thus indicating that the two variables are interdependent.

DISCUSSION

In accordance with previous studies, we confirmed

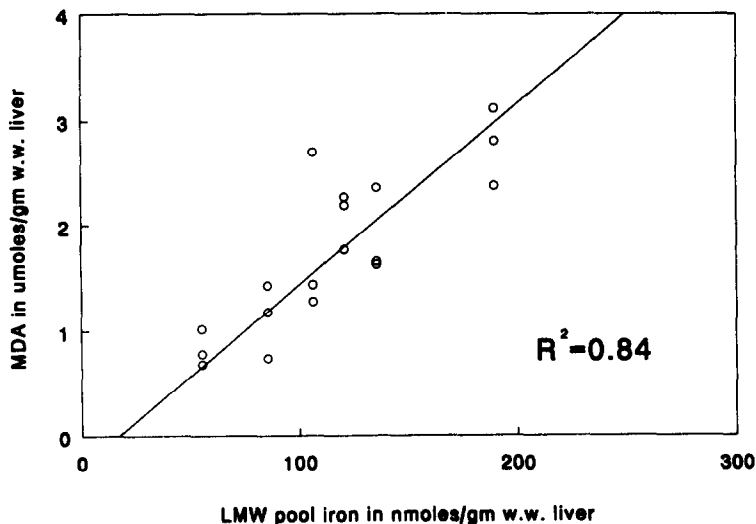


Fig. 5. Liver LMW pool iron concentrations compared to liver MDA concentrations after 11 weeks of treatment. Results of LMW iron pool measurements after 11 weeks were compared to MDA concentration results from that period. Open circles represent individual results. Calculations yielded a correlation coefficient of 0.84.

that treatment of C57BL/10 mice with HCB and IMF, but also with IMF alone, resulted in the accumulation of (uro)porphyrins in the liver. In this strain of mice, HCB alone did not produce uroporphyrin [10–12]. These results (amongst others) point to an important role for iron in the pathogenesis of this form of uroporphyrin.

Three major mechanisms for the role of iron in experimental uroporphyrin have been proposed. (i) Iron (Fe^{2+}) binds directly to one or more sulfhydryl groups of URO-D [44–46]. This would inactivate the enzyme, which needs free sulfhydryl group(s) for its catalytic activity [44, 47]. When (under aerobic conditions) URO-D is incubated with iron in the presence of an electron donor, a reduction in enzyme activity can be measured [44]. (ii) This could be the result of free radical-mediated damage to URO-D itself [44], or (iii) the result of oxidation of uroporphyrinogens to uroporphyrins that inhibit enzyme activity [11, 15, 48].

In general, it is not clear whether the direct interaction of ferrous iron and URO-D also occurs *in vivo*. Mukerji *et al.* [44, 45] found that Fe^{2+} concentrations in the range of 0.1–1.0 mM were required *in vitro* to reduce the activity of partially purified URO-D. In our view, these Fe^{2+} concentration requirements cannot be met *in vivo* [49]. However, our experimental results do not preclude the possibility of direct interaction between ferrous iron and URO-D. In fact, we found that IMF alone reduced URO-D activity and caused concomitant porphyrin accumulation. The relatively slow onset of porphyrin accumulation (as compared to the rapid accumulation of iron, cf. Fig. 1 to Fig. 3) could be explained by the results of a morphological examination of the liver tissue of IMF-treated mice [50]. Iron-positive granules were present in Kupffer cells shortly after administration of IMF, but it took another 2 weeks before iron accumulation could be detected in the hepatocytes. As a rule, the accumulation of ferritin iron preceded porphyrin accumulation in hepatocytes [50]. Liver iron measurements do not detect this intrahepatic “iron shift” and give the sum of the iron content of both cell types. Therefore, these results are not an actual representation of the amount of iron accumulated in hepatocytes. Experimental results in the IMF + DFX group, however, partially contradict the possible interaction of ferrous iron and URO-D. At 14 weeks, URO-D activity was completely restored and accumulated porphyrins had disappeared, despite the fact that the liver iron concentration was still 10 times higher than in the control group.

In recent years, there has been a growing interest in the role of iron and other trace metals as mediators in the production of oxygen-related free radicals. In our experiments, treatment with IMF resulted in increased MDA production, which was maximal at 4 weeks, but was still significantly increased at 14 weeks ($P = 0.004$, Fig. 2), despite the fact that most iron will be stored in ferritin by this time. MDA production at 14 weeks in the IMF + DFX group is significantly lower than in the IMF group ($P < 0.001$). This could explain why URO-D activity was restored at that time, despite the presence of an iron excess in the liver. Moreover, the LMW iron results in the

IMF + DFX group [55 ± 12.6 nmol/g wet wt liver tissue (mean \pm SD)] were also lower than those in the IMF group (120.8 ± 15.1 nmol/g wet wt) and confirmed our data on MDA production (Fig. 5). These results support the theory of URO-D inactivation as a result of free radical-mediated damage.

HCB causes the induction of microsomal cytochrome P450IA2 [51, 52] and this enzyme could promote oxidation of uroporphyrinogens. However, it is more likely that cytochrome P450IA2 induction leads to the generation of reactive oxygen species [21, 53]. HCB alone did not induce porphyrin accumulation in C57BL/10 mice [12], neither did it affect MDA production. Combined treatment with HCB + IMF, however, resulted in increased MDA production, porphyrin accumulation and decreased URO-D activity as compared to the IMF group (Fig. 2). These differences between the IMF and HCB + IMF groups were statistically significant for all results except those at 14 weeks ($P < 0.004$). In contrast, liver iron measurements (Fig. 1) as well as morphological studies [50] showed more or less equal results in both groups. The effects of DFX in the HCB + IMF + DFX group were less pronounced than in the IMF + DFX group as compared to their non-DFX treated counterparts. Still, the results at 14 weeks in the HCB + IMF + DFX group are significantly different from those in the HCB + IMF group ($P = 0.008$), with the exception of the results on URO-D activity. In Fig. 4 it appears that URO-D activity in the HCB + IMF + DFX group increases, but measurements over a longer period of time will be necessary to confirm this trend. Our results support the idea that HCB stimulates the production of oxygen free radicals in the presence of iron. This is further illustrated by the fact that HCB alone will not induce porphyrin in C57BL/10 mice, probably due to the very low levels of total liver iron in the non-treated animals [5, 7, 54]. In our view, DFX reduces the amount of iron available for a catalytic role in the generation of oxygen free radicals. Our data on LMW iron are in agreement with this theory: treatment with HCB + IMF resulted in a 50% increase in LMW iron (189.6 ± 14.5 nmol/g wet wt liver tissue) as compared to IMF-treated mice. DFX reduced LMW iron in the HCB + IMF + DFX group (135.5 ± 8.5 nmol/g wet wt), which is confirmed by our data on MDA and porphyrin accumulation. The discrepancy in LMW iron between the IMF and HCB + IMF groups could be the result of a reductive release of iron from ferritin, due to the generation of O_2 by HCB [26, 27]. Whether radical-mediated processes damage URO-D itself, or cause the oxidation of uroporphyrinogens, is still an open issue, as our porphyrin measurements do not discriminate between porphyrins and porphyrinogens. However, the results on porphyrin accumulation and URO-D activity in the HCB + IMF + DFX group at 14 weeks are indicative of direct damage to URO-D, for the amount of porphyrins is already significantly diminished ($P < 0.001$) whereas URO-D activity is only slightly increased as compared to the results at 7 weeks (Figs 3 and 4).

In general, there seems to be a discrepancy

between the results on MDA production and iron accumulation. After 4 weeks MDA production decreased, while iron was still accumulating in the hepatocytes. Iron storage in ferritin reduces the availability of catalytic iron for the generation of oxygen free radicals [25]. Ferritin synthesis is an iron-regulated process at the mRNA level [55] and, as mentioned before, morphological studies show that transport of iron from the injection site to the hepatocytes takes a considerable amount of time. Once iron reaches the hepatocytes, ferritin synthesis will be stimulated, but it is reasonable to assume that ferritin synthesis will not be able to accommodate this iron excess immediately. Eventually, most iron will be stored in ferritin, but in the meantime the amount of "free" iron (LMW iron) will probably be elevated and enhance the generation of oxygen free radicals.

We therefore conclude that iron is an important factor in porphyrin accumulation in C57BL/10, probably through its catalytic role in the generation of oxygen-related free radicals, resulting in direct damage to URO-D. The possible direct interaction between iron and URO-D, rendering the enzyme inactive, cannot be excluded, but seems hardly likely with regard to the very high iron concentrations necessary for this inactivation to occur. DFX's effectiveness in reducing porphyrin accumulation is most likely the result of a reduction in LMW iron, thus diminishing the amount of iron available for a catalytic role in the generation of oxygen-related free radicals.

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