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Role of Iron in the Hydrogen Peroxide-Dependent Oxidation of Hexahydroporphyrins (Porphyrinogens): A Possible Mechanism for the Exacerbation by Iron of Hepatic Uroporphyria

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SUMMARY

The hypothesis that the accumulation of uroporphyrin, characteristic of uroporphyria, arises at least in part from oxidation of uroporphyrinogen and the molecular basis for the potentiation of the disorder by iron have been investigated. The iron chelates of ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid were very active at promoting the hydrogen peroxide-dependent oxidation of porphyrinogens, and a similar role of iron was found for the NADPH-dependent oxidation of porphyrinogens by liver microsomes *in vitro*. In contrast, neither the iron chelate of desferrioxamine (DES) nor ferritin iron possessed prooxidant activity, but the latter could be mobilized in an active form by incubation with EDTA. Iron was also found to promote further modification of the porphyrin pigment, leading to marked loss of its Soret absorbance. This latter effect, which could also be inhibited by DES, suggested further oxidative conversion of the accumulating uroporphyrin, but further work is necessary to establish the relevance of this (or similar) reaction to the inhibitor of uroporphyrinogen decarboxylase which has recently been reported. These results suggest a possible mechanism for the exacerbation of uroporphyria by excess iron and also for its marked improvement when the iron stores are diminished, for example, by DES treatment.

Uroporphyria, a metabolic disorder characterized by accumulation of uroporphyrin and other highly carboxylated porphyrins, can be induced in intact rodents and chicken embryo hepatocyte cultures by several polyhalogenated chemicals, among these hexachlorobenzene (reviewed in Ref. 1). This drug-induced alteration of porphyrin metabolism closely resembles PCT, the most common variety of human hepatic porphyria, as in both conditions the essential biochemical lesion is a defect in uroporphyrinogen metabolism which can be worsened by excess iron and relieved by reducing the iron body stores (reviewed in Ref. 2). Because of the potentiating role of iron and also on account of the variety of drugs which are effective uroporphyria-inducing agents in the chicken hepatocyte culture system, the hypothesis (3, 4) has been put forward that the precipitating drugs may interact with the NADPH-dependent reductase/cytochrome P-450 system of the hepatocytes, leading to mobilization of stored iron and to production of reduced oxygen species, such as hydrogen peroxide. Uroporphyria may then result from inhibition of uroporphyrinogen decarboxylase (porphyrinogen carboxylyase, EC 4.1.1.37) by some mechanism requiring interaction of reduced oxygen with non-heme iron. A contributing factor may be direct oxidation by hydrogen peroxide of the substrate of the decarboxylase, uroporphyrinogen (hexahydrouroporphyrin), since the oxidative derivative, uroporphyrin, cannot be metabolized and would therefore be expected to accumulate. Some support for this mechanism has recently come from three laboratories with the demonstration that liver microsomes from animals treated with polyhalogenated chemicals produce increased amounts of reduced oxygen metabolites when incubated with NADPH *in vitro* (5) and will catalyze the oxidation of porphyrinogens in the presence of NADPH, especially when challenged with small amounts of a uroporphyria-inducing chemical (6, 7). However, the molecular basis for the potentiation of the disorder by iron has not yet been clarified.

The main purpose of this communication is to describe a chemical model system for the interaction of hydrogen peroxide and iron in the oxidation of porphyrinogens and to show a similar role of iron in the NADPH-dependent oxidation of porphyrinogens by liver microsomes *in vitro*. Evidence will also be presented that, unlike the iron chelates of EDTA and NTA, which are very active at promoting the hydrogen peroxide-dependent oxidation of porphyrinogens, the iron chelate of DES and ferritin iron are both inactive. These results suggest a molecular basis for the exacerbation of drug-induced uroporphyria and human PCT by excess iron and provide an expla-

ABBREVIATIONS: PCT, porphyria cutanea tarda; EDTA, ethylenediaminetetraacetic acid; NTA, nitrilotriacetic acid; DES, desferrioxamine mesylate; 3,4-TCB, 3,4,3′,4′-tetrachlorobiphenyl; DMSO, dimethyl sulfoxide.

nation for their marked improvement when the iron stores are reduced, for example, by DES treatment.

Materials and Methods

Source of special chemicals. NADPH (type III), *o*-dianisidine (dihydrochloride), horse spleen ferritin (type I), lactoperoxidase (70 units/mg), and NTA (trisodium salt) were obtained from Sigma Chemical Co., Poole, Dorset, UK, and glucose oxidase (grade I, 280 units/mg) was from Boehringer, Lewes, East Sussex, UK; DES was obtained from Ciba Laboratories, Horsham, West Sussex, UK. Uroporphyrin III and heptacarboxylate porphyrin I were from Porphyrin Products, Logan, VT; 3,4-TCB was from Ultra Scientific, Hope, RI.

Preparation of iron chelates. These were prepared by mixing a 100 mM solution of either EDTA, NTA or DES with an equal volume of freshly prepared 40 mM $FeCl_3$, and serial dilutions of these stock iron chelates were made with 50 mM solutions of the appropriate chelators without iron. In this way the concentration of each chelator was kept constant at 50 mM, while varying the concentration of the chelated iron.

Treatment of animals. Chicken embryos, 16 days of age, of the Rhode Island Red × White Rock strain, were obtained from Orchard Farm, Pinner, Middlesex, UK, and liver microsomes were prepared 24 hr after a single dose of 3,4-TCB (145 μg/embryo) dissolved in 10 μl of 1,4-dioxan given by injection through the air sac into the fluids surrounding the embryo. The microsomes were prepared by differential centrifugation as described (8) and were stored at −70° as a pellet overlayed with 0.5 ml of 1.15% KCl, until used. The enzyme system under study was found to be stable under these conditions of storage.

***In vitro* oxidation of porphyrinogens.** Uroporphyrin III or heptacarboxylate porphyrin I (both free carboxylate) were fully reduced to the corresponding porphyrinogen by treatment with sodium amalgam under N_2. Addition of the porphyrinogen did not alter the pH of the incubation mixture, as monitored by a pH electrode, either during or after addition. The oxidation of the porphyrinogens was followed in disposable plastic cuvettes (incubated in the presence of air in the thermostatically controlled, 28 or 37° compartment of a Cary 2200 spectrophotometer) by monitoring the increase in absorbance at the Soret maximum (396 and 396.5 nm, respectively, for the two porphyrins) against a blank containing all components except the prophyrinogen. The samples were scanned at the end of the incubation in several cases to verify that the recorded increase in absorbance was in fact due to the Soret maximum and the increase in porphyrin fluorescence was visually checked in a few typical experiments as a further confirmation. In the experiments with liver microsomes, the incubation mixture contained in a total volume of 3.5 ml the following components, with final concentrations in parentheses: Na/potassium phosphate buffer, pH 7.4 (0.1 M); EDTA, added either independently or as a component of the buffer (see Results), (1 mM); NADPH (73 μM); liver microsomes corresponding to a concentration of cytochrome P-450 of 65 pmol/ml; and DMSO (3 μl/ml), containing 3,4-TCB (2.8 μg/ml). When the effect of glucose oxidase (or hydrogen peroxide) was investigated, glucose (9.5 mg/ml) and glucose oxidase (35.3 μg/ml) or various concentrations of authentic hydrogen peroxide were added to the buffer instead of microsomes, NADPH, and DMSO. The effect of chelators other than EDTA and of various concentrations of iron on the rate of reoxidation of the porphyrinogens was also studied by substituting for EDTA either NTA or DES (again at a final concentration of 1 mM) and by adding increasing amounts of chelated iron. Any departure from these incubation conditions will be specifically referred to under Results.

After a preliminary incubation of 5 min, the porphyrinogens (5 or 10 μl) was added to the sample cuvette with rapid mixing and the rate of increase in absorbance was monitored. A lag phase was observed (especially in the absence of iron when low oxidation rates were seen), and this was followed by a period in which the rate increased gradually until the maximum and approximately linear rate was seen: the rates given in the tables refer to the maximum rates observed. A period of progressively decreasing rate was then observed and, in some cases, a subsequent slow decline in Soret absorbance indicative of progressive loss of porphyrin pigment. These two latter phases were also studied in some experiments.

To investigate whether EDTA would donate iron to DES, thereby rendering it inactive, Fe-EDTA (5 mM Fe in 50 mM EDTA) was incubated with a 10-fold excess (by volume) of 50 mM DES in plastic disposable test tubes for 3 hr at 37° followed by 16 hr standing at room temperature. The chelation of Fe by DES was monitored by the increase in absorbance at 440 nm, characteristic of the Fe-DES chelate and found to be essentially complete after 3 hr incubation at 37°. The prooxidant activity of this sample was then compared to unincubated Fe-EDTA or to a control sample of Fe-EDTA of identical final composition and similarly incubated, but lacking DES. The prooxidant activity of ferritin was also measured using either freshly diluted ferritin or ferritin allowed to stand in the dark for 8 days at 4° under sterile conditions with either 7 vol of 50 mM EDTA or 19 vol of 0.9% NaCl. The ferric iron content of the unincubated ferritin micelle (21.4 μg of Fe/100 μg of ferritin) was calculated using the 420-nm extinction coefficient given in Ref. 9.

In one experiment the rate of hydrogen peroxide production by the glucose oxidase system was measured in the presence or absence of EDTA by monitoring the oxidation of *o*-dianisidine (56 μg/ml of incubation, added in DMSO, 3 μl/ml) in spectrophotometric cuvettes by the increase in 436-nm absorbance in the presence of lactoperoxidase (6 μg/ml). The concentrations of the phosphate buffer and glucose oxidase were identical to those used for the oxidation of the porphyrinogen, but much lower concentrations of glucose were used (ranging from 10 to 100 μg/ml), so as to maintain the production of hydrogen peroxide limiting in the overall reaction and the rate of *o*-dianisidine oxidation directly proportional to glucose concentrations. When the peroxidatic activity of Fe-EDTA was measured, similar incubation conditions were adopted except that authentic hydrogen peroxide was added instead of glucose and glucose oxidase, and Fe-EDTA was added in place of lactoperoxidase.

Results and Discussion

Role of the iron chelates of EDTA and NTA in the hydrogen peroxide-dependent oxidation of porphyrinogens. Previous work has shown that liver microsomes isolated from chick embryos induced with 3,4-TCB can rapidly oxidize uroporphyrinogen and other porphyrinogens to the corresponding porphyrins in the presence of NADPH and 3,4-TCB *in vitro* (7), but the identity of the oxidizing species involved has not been established. In the experiments described below we show that authentic hydrogen peroxide can likewise promote oxidation of porphyrinogens *in vitro* and, in addition, provide evidence that in both the enzymic and chemical oxidation processes certain forms of chelated iron are involved as catalysts.

When EDTA was omitted from the phosphate buffer which had been used to resuspend the microsomes and which was added as a component of the incubation mixture, very much lower rates of reoxidation were observed (Table 1). Omission of EDTA from the buffer also markedly decreased the rate of oxidation of porphyrinogens by the glucose oxidase system, but the rate of production of hydrogen peroxide, as measured by the oxidation of *o*-dianisidine in presence of lactoperoxidase, was unaffected. This latter finding, and the observation that authentic hydrogen peroxide could also oxidize porphyrinogens much more effectively when the buffer contained EDTA, indicated clearly that the latter was in some way required for the oxidative interaction of hydrogen peroxide with the porphyrinogens.

TABLE 1

Rate of oxidation of porphyrinogens by chicken embryo 3,4-TCB-induced liver microsomes, glucose oxidase, and hydrogen peroxide and rate of oxidation of *o*-dianisidine by glucose oxidase: Effect of adding EDTA as a component of the phosphate buffer

Uroporphyrinogen III (7.4 nmol) or heptacarboxylate porphyrinogen I (3.7 nmol) was added to a spectrophotometric cuvette containing, in a total volume of 3.5 ml, the various components indicated under Materials and Methods, including phosphate buffer, with EDTA present as a component of the buffer, where shown. The rate of oxidation of the porphyrinogens was recorded from the increased absorbance of the Soret maximum and the values given refer to the linear portion of the rate of oxidation seen in individual experiments (with averages). The incubations were carried out at 28°.

Oxidizing system	Type of buffer	Rate of oxidation of	
		Porphyrinogen[a] (pmol/min)	*o*-Dianisidine[b]
		pmol/min	*rate/min/100 μg glucose added*
Induced microsomes + NADPH and 3,4-TCB	Phosphate, no EDTA	8.57 (8.22, 8.92)	
	Phosphate, + EDTA	23.3 (22.2, 24.3)	
Glucose oxidase	Phosphate, no EDTA	7.52 (5.77, 9.27)	0.300
	Phosphate, + EDTA	85.2 (80.9, 82.2, 90.8)	0.312
Hydrogen peroxide[c]	Phosphate, no EDTA	6.2 (5.6, 6.96)	
	Phosphate, + EDTA	46.1 (43.0, 49.2)	

[a] Uroporphyrinogen was used with induced microsomes and glucose oxidase; heptacarboxylate porphyrinogen was used with hydrogen peroxide.
[b] Under these conditions the rate of increase of A_{436nm} was proportional to the amount of glucose added; values given are averages of four different rates obtained with different glucose concentrations and are expressed as rate (increase in A_{436nm}/min) per 100 μg glucose added.
[c] Final concentration, 2.5 mM.

When EDTA itself was added directly to the reaction mixture (at the same final concentration as that added with the EDTA-containing buffer) and the porphyrinogen was added after a preliminary incubation of 5 min as usual, only a small stimulation of the rate of porphyrinogen oxidation was seen: the rate then observed was 12 pmol/min (Table 2), instead of 5.6 ± 0.3 pmol/min in the absence of EDTA (see Table 4). This degree of stimulation was much less marked than that seen when EDTA was added as a component of the phosphate buffer (Table 1), in which case, of course, EDTA had been in contact with the buffer for a much longer time. This suggested that, when mixed to phosphate buffer, EDTA might slowly chelate iron present in the buffer as a contaminant and the resulting iron-EDTA chelate—rather than EDTA itself—might be the active catalyst. In agreement with this, it was shown (Table 2) that, unlike EDTA itself, its iron chelate (Fe-EDTA) was markedly effective at stimulating the H_2O_2-dependent oxidation of the porphyrinogen: when H_2O_2 or Fe-EDTA was added separately, very little oxidation took place, but a marked synergism was seen when the two were added together. A clear synergism between Fe (at two different concentrations) and hydrogen peroxide (added at a final concentration of either 2.5 mM or 25 μM) is shown in Table 2. Lower concentrations of H_2O_2 (2.5 μM) also stimulated the rate of porphyrinogen oxidation in the presence of either concentration of Fe, but the effect was small and short-lasting.

Further experiments were carried out with 2.5 mM H_2O_2 to investigate the stimulatory effect of Fe-EDTA in more detail and to compare Fe-EDTA to other iron chelates. Fig. 1 shows

TABLE 2

Oxidation of the heptacarboxylate porphyrinogen I by hydrogen peroxide: Role of iron, added as the EDTA chelate, as a catalyst

The incubation mixture contained in a total volume of 3.5 ml the following components (final concentration): phosphate buffer (0.1 M); EDTA added independently of the buffer immediately before incubation (1 mM) containing, where appropriate, chelated iron at the concentration indicated; and, finally, hydrogen peroxide. After 5 min preincubation, 3.7 nmol of heptacarboxylate porphyrinogen I were added and the rate of porphyrinogen oxidation was monitored at 37°. Values given are means with individual observations in parentheses.

Addition and final concentration		Rate of oxidation of porphyrinogen	
Fe	H_2O_2	pmol/min	increase in rate due to H_2O_2 (vs. corresponding control with same Fe concentration)
μM			
		7.5 (5.9, 7.3, 9.3)	
	25	9.0 (8.3, 9.7)	1.5
	2500	12.0 (10.3, 12.3, 13.3)	4.5
20		6.4 (6.3, 6.5)	
20	25	33.0 (31.3, 34.8)	26.6
20	2500	132.0 (125, 140)	125.6
100		10.9 (9.3, 11.5, 11.9)	
100	25	49.0 (46, 52)	38.1
100	2500	226.0 (215, 238)	215.1

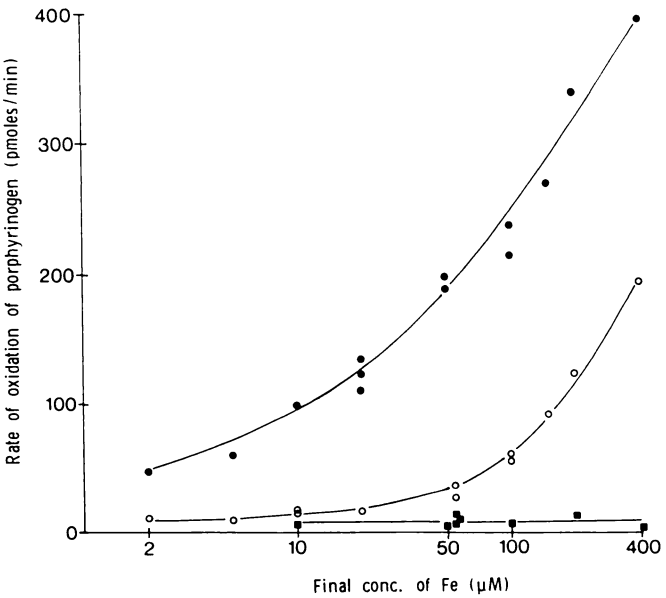

Fig. 1. Log dose response curve for porphyrinogen oxidation obtained with various concentrations of three different iron chelates. The rate of oxidation of heptacarboxylate porphyrinogen I was determined under the conditions described in the legend to Table 3 after addition of a 1 mM concentration of either EDTA (●), NTA (○), or DES (■), each containing various concentrations of iron as indicated. Note that when Fe-DES was tested, EDTA (350 μM) was also present to minimize the iron-independent prooxidant activity of DES, as discussed in the text.

the log dose-response curves obtained with Fe-EDTA, Fe-NTA, and Fe-DES; extension of the curves over higher doses of Fe was not possible because of the high color of the chelates and/or the near saturation of the chelator by higher concentrations of Fe. Like Fe-EDTA, Fe-NTA could stimulate significantly the H_2O_2-dependent rate of porphyrinogen oxidation (as compared with either NTA alone or control samples with no additions) (Table 3) but appeared to be a less effective prooxidant than Fe-EDTA (Fig. 1). The behavior of DES and of its iron chelate was quite different, as discussed below.

The iron chelate of DES and ferritin iron are both inactive in the H_2O_2-dependent oxidation of porphyrinogens; ferritin iron can be mobilized in an active form. Unlike EDTA and NTA, DES was significantly active at stimulating the H_2O_2-dependent porphyrinogen oxidation when added as obtained commercially (in a putative Fe-free form), and this prooxidant activity was decreased—rather than increased—by chelating Fe (table 3). This prooxidant activity of DES itself was entirely dependent on addition of H_2O_2 (results not shown) and could be significantly decreased by EDTA (Table 3), suggesting, at least in part, the involvement of a metal (other than Fe) which might be present as an impurity in the commercial preparation of DES and which, unlike Fe, might be inactivated by EDTA chelation. This aspect will require further work. Use has been made of the ability of EDTA to decrease the basal (iron-independent) prooxidant activity of DES in the dose response experiment of Fig. 1, where the various concentrations of Fe-DES were all tested in the presence of EDTA. The results show clearly that when chelated to DES, Fe is completely inactive. Omission of EDTA led to basically similar results, with the exception, of course, that DES showed slightly higher rates of porphyrinogen oxidation, especially at low Fe concentrations (data not shown).

When Fe-EDTA was incubated with a 10-fold molar excess of DES and the prooxidant activity of the incubated and unincubated samples was compared (Table 4), it was found that EDTA could donate its chelated iron to DES (as shown by a gradual increase in absorbance at 440 nm, characteristic of the Fe-DES chelate) and, after such preincubation, the prooxidant activity of Fe was markedly reduced. It was also shown that ferritin iron had no prooxidant activity of its own, but activity appeared in the presence of EDTA, especially when preincubation with EDTA was allowed to proceed for several days (Table 4). The results are consistent with the conclusion that Fe-DES cannot participate in porphyrinogen oxidation and also suggest that ferritin iron is similarly inactive but can support the oxidation once it has been mobilized. Mobilization of iron from ferric lactoferrin by incubation with chelators and without the use of reducing agents has also been reported (10).

TABLE 3

Rate of oxidation of heptacarboxylate porphyrinogen I in the presence of NTA and DES added on their own or with chelated iron: Effect of adding EDTA together with DES

The porphyrinogen (3.7 nmol) was added to an incubation mixture containing phosphate buffer (0.1 M), hydrogen peroxide (2.5 mM), and, in addition, the various components indicated below. The rate of oxidation of the porphyrinogen was monitored at 37° by the increase in Soret absorbance, and values given are means ± standard error of the number of observations in parentheses or averages of the individual results in parentheses.

Additions and final concentration (μM)		Rate of porphyrinogen oxidation (pmol/min)
Chelator(s)	Fe	
Experiment 1		
None	none	7.6 ± 0.5 (4)
NTA (1000)	none	9.5 ± 0.8 (4)
NTA (1000)	55.0	32.3 (27.9, 36.8)
DES (1000)	none	38.2 ± 2.5 (5)[a]
DES (1000)	55.0	25.2 ± 0.4 (3)[b]
Experiment 2		
DES (1000)	none	29.7 ± 1.6 (4)
DES (1000) + EDTA (350)	none	16.4 ± 0.3 (4)[c]

[a] $p < 0.001$ when compared to value of samples without any addition.
[b] $p < 0.01$ when compared to corresponding DES value without Fe.
[c] $p < 0.001$ when compared to corresponding DES value without EDTA.

TABLE 4

Effect of preincubating Fe-EDTA with DES and of ferritin with EDTA on the iron-catalyzed reoxidation of heptacarboxylate porphyrinogen I by hydrogen peroxide

Porphyrinogen (3.7 nmol) was added to an incubation mixture containing phosphate buffer (0.1 M), H_2O_2 (2.5 mM), and, in addition, the various components shown below (some preincubated as indicated under Materials and Methods) and the rate of oxidation was monitored at 37°. Values given are means ± standard errors of the number of observations in parentheses or averages with individual observations in parentheses.

Additions and final concentration	Rate of porphyrinogen oxidation	Increase in rate attributable to Fe added as Fe-EDTA or as ferritin	
	pmol/min		
Experiment 1			
EDTA (88 μM)	12.9 (12.3, 13.5)		79.3
Fe (8.8 μM) in EDTA (88 μM)	92.2 ± 8.8 (4)		
EDTA (88 μM) preincubated with DES (885 μM)	17.0 (17.0, 17.0)		11.7
Fe (8.8 μM) in EDTA (88 μM) preincubated with DES (885 μM)	28.7 ± 0.7 (4)[a]		
Experiment 2			
None	5.6 ± 0.3 (3)		0.4
Ferritin (3 μg of Fe/ml)	6.0 ± 0.07 (3)		
EDTA (50 μM)	15.7 ± 0.6 (3)	5.8	24.2
EDTA (50 μM) + ferritin (3 μg of Fe/ml) preincubated with NaCl	21.5 ± 0.4 (3)[b]		
Ferritin (3 μg Fe/ml) preincubated with EDTA (50 μM)	39.9 ± 0.3 (3)[c]		

[a] $p < 0.001$ when compared to corresponding value containing no DES.
[b] $p < 0.01$, when compared to corresponding value obtained with EDTA alone.
[c] $p < 0.01$, when compared to value obtained with ferritin preincubated with NaCl.

Role of iron in promoting loss of the characteristic porphyrin spectrum. The changes in Soret absorbance so far discussed have only been interpreted in terms of production of porphyrins by oxidation of the corresponding porphyrinogens, without considering the possible contribution of further chemical change of the porphyrin system leading to loss of the characteristic spectrum. Evidence is presented below that iron also catalyzes loss of the porphyrin pigment. While the contribution of this loss to the overall changes in Soret absorbance is probably small in the first phase of the oxidation reaction (particularly when measuring initial rates), it becomes significant in the later stages, especially at high Fe concentrations, accounting for: (a) deviation from linearity of the rate of increase in Soret absorbance; (b) lower yields of porphyrin than expected from quantitative conversion of porphyrinogen; and (c) subsequent net decline in Soret absorbance. These changes, indicative of porphyrin destruction (Fig. 2, Table 5), were not only related to the amount of iron added, but also were dependent on the type of chelator, greater porphyrin loss being observed with Fe-NTA than with Fe-EDTA (results not shown).

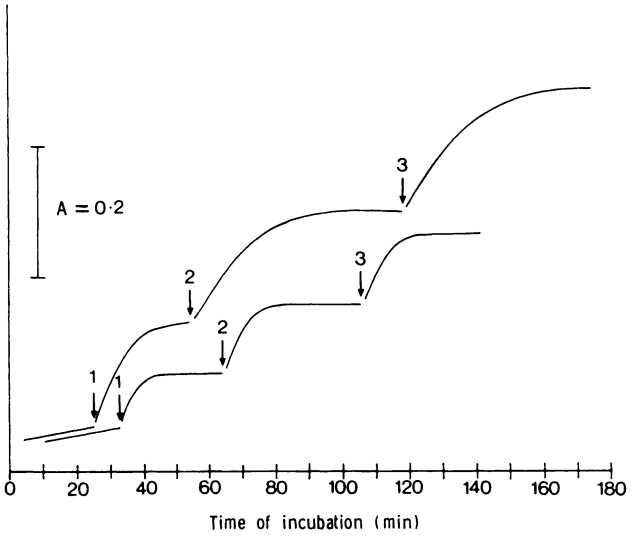

Fig. 2. Rate of heptacarboxylate porphyrinogen I oxidation and yields of the resulting porphyrin after addition of H_2O_2 and Fe-EDTA in the presence or absence of DES. The composition of the incubation mixture was as indicated in the legend to Table 5, except that in this experiment the concentration of EDTA was raised to 1.5 mM and one sample (*upper tracing*) also contained DES (500 μM). The reaction was started by adding Fe-EDTA to a final concentration of 20 μM Fe (at *arrow 1*); the rate of oxidation was then monitored at 37° by the absorbance at 396.5 nm and, when completed, started by addition of identical amounts of porphyrinogen (at *arrows 2* and *3*). Note that in the *upper tracing* the rate of oxidation lasts longer and also yields greater amounts of Soret-absorbing material.

TABLE 5

Effect of DES, added alongside (but not preincubated with) Fe-EDTA, on the rate of H_2O_2-dependent oxidation of heptacarboxylate porphyrinogen I, on the yield of the corresponding porphyrin and on the subsequent loss of porphyrin pigment

The porphyrinogen (3.7 nmol) was added to an incubation mixture containing phosphate buffer (0.1 M), hydrogen peroxide (2.5 mM), and, where indicated, DES (1 mM). The reaction was started by adding Fe in EDTA to a final concentration of 20 μM and 1 mM, respectively, and the rate of oxidation was monitored at 37°. At 2 hr and again at 24 hr the spectrum was scanned and the amount of porphyrin present was calculated from the difference in absorbance between 396.5 and 450 nm and compared to the yield expected from quantitative conversion of the porphyrinogen. Results given are those of individual experiments.

Further addition	Rate of porphyrinogen oxidation	Per cent recovery of porphyrinogen as porphyrin at	
		2 Hr	24 Hr
	pmol/min		
None	179	40	32
None	170	35	27
None	202		36
None	188		37
DES	180	78	79
DES	194	75	74
DES	233		81
DES	237		90

Addition of DES to the incubation mixture alongside Fe-EDTA did not decrease the initial rate of oxidation (at these DES/EDTA ratios chelation of Fe by DES was not sufficiently rapid to affect the prooxidant activity of iron significantly); however, DES markedly decreased the subsequent loss of pigment as shown by all three criteria (a, b, and c) listed above (compare the two tracings in Fig. 2 and see also Table 5).

These findings suggest that even though both accelerated oxidation of porphyrinogen and loss of the resulting porphyrin require iron (and hydrogen peroxide), these two effects can, under certain conditions, be dissociated from each other. It is possible that different mechanisms are involved in the two effects of iron, one involving Fe^{3+} acting similarly to a peroxidase (11) and resulting in porphyrinogen oxidation. This would in theory require 3 mol of H_2O_2 (per mol of porphyrinogen) to reach completion. (Support for a peroxidatic activity of Fe-EDTA was obtained in one experiment by showing that this iron chelate could catalyze the H_2O_2-dependent oxidation of *o*-dianisidine in a dose-dependent fashion.) However, if iron was to some extent reduced by the porphyrinogen, H_2O_2 might also produce on interaction with Fe^{2+} the hydroxyl radical [·OH, a very reactive species (12, 13)], and this might conceivably be responsible for porphyrin loss. It is also possible that Fe^{2+} might be chelated by the newly formed porphyrin, producing the corresponding heme: this might also account for the porphyrin loss as heme is known to suffer rapid breakdown on interaction with H_2O_2 (14). These mechanisms are at present still speculative and more work is required to clarify them and the inhibition by DES of porphyrin loss which has now been described.

Role of the iron chelate of EDTA in the microsomal-dependent oxidation of porphyrinogens. In the last set of experiments the involvement of iron in the oxidation of porphyrinogens by microsomal membranes was investigated. Microsomal incubations pose special problems with respect to iron-catalyzed reactions, as the iron stored as ferritin might be expected to be mobilized, especially if reductive conditions are employed and a suitable chelator (such as EDTA) is present in the incubation mixture. The approach taken, therefore, was to keep the concentration of EDTA to a minimum and to compare the rate of oxidation obtained with EDTA alone with that obtained when exogenous iron was added as an EDTA chelate. The results (Table 6) indicate a stimulation by iron of the rate of porphyrinogen oxidation also with the microsomal system.

Previous work (6, 7) has shown that liver microsomes from chick embryos induced *in vivo* with either 3-methylcholanthrene or 3,4-TCB produce, on incubation with NADPH *in vitro*, accelerated oxidation of a porphyrinogen, especially when small amounts of 3,4-TCB are also added. The present results demonstrate many similarities between the microsomal oxidiz-

TABLE 6

Rate of oxidation of heptacarboxylate porphyrinogen I by liver microsomes from chicken embryos induced with 3,4-TCB: Effect of adding exogenous iron as the EDTA chelate

The porphyrinogen (7.4 nmol) was added to a spectrophotometric cuvette containing the various components indicated under Materials and Methods, including phosphate buffer (without EDTA), microsomes resuspended in the same buffer (also in the absence of EDTA), 3,4-TCB (added in DMSO), and NADPH. The rate of oxidation was followed at 28° by the increase in absorbance at the Soret maximum. Once a linear rate was obtained, EDTA was added to a final concentration of 38 μM, carrying chelated iron to a final concentration of 3.8 μM, where indicated. Values are rates of oxidation observed before and after addition of EDTA ± Fe and differences between the two rates seen in each individual experiment, and are given as means ± standard errors of the number of observations in parentheses.

Addition	Rate of porphyrinogen oxidation		
	A) Before addition	B) After addition	C) B − A
	pmol/min		
EDTA	10.9 ± 1.8 (4)	14.4 ± 0.5 (4)	3.4 ± 1.3 (4)
Fe-EDTA	11.6 ± 0.4 (6)	28.4 ± 1.6 (6)*	16.8 ± 1.3 (6)*

* $p < 0.001$, when compared to corresponding values obtained with EDTA alone.

ing system and the effect of the hydrogen peroxide itself, suggesting that the latter may be the main oxidizing species produced by the microsomes. This is in keeping with previous findings that hydrogen peroxide is produced by microsomes incubated aerobically with NADPH (15, 16) and that drugs which act as uncouplers of the microsomal electron transport system (17, 18) will increase production of hydrogen peroxide. These findings therefore provide further support for the view (3–7) that uroporphyria-inducing agents, such as 3,4-TCB, may act as uncouplers of the cytochrome P-450 system, leading to oxidative escape of uroporphyrinogen III from the pathway of heme biosynthesis.

The exact role of cytochrome P-450 in this reaction (whether only acting as a source of hydrogen peroxide or also exerting some other, more specific function) requires further study. Other points which need elucidation are: (a) the nature of the postulated endogenous iron pool involved in the reaction; (b) the way in which the oxidizing species escape inactivation by the cellular defenses, such as glutathione peroxidase and catalase; and (c) the site within the cell where oxidation of uroporphyrinogen takes place *in vivo*. With isolated microsomes, catalase inhibited the oxidation reaction only slightly (7), and in another similar study (6), both catalase and superoxide dismutase were completely ineffective. For this reason Sinclair *et al.* (6) have suggested that the oxidation reaction might take place within the environment of cytochrome P-450. The results of our present work are also compatible with a reaction proceeding outside the environment of cytochrome P-450, but they do not exclude the possibility that the porphyrinogen may undergo oxidation while bound to the membranes of the endoplasmic reticulum or in close proximity to the membranes. In either case interception and inactivation of hydrogen peroxide by catalase and other protective enzymes may well be minimized *in vivo*.

General Conclusion

The ability of iron to worsen the course of human PCT and of the related uroporphyrias of the experimental animals is well documented (2, 19–21), as is the protective effect of depleting the iron stores by low iron intake, phlebotomy, or treatment with DES (2, 22, 23). Iron stored as ferritin would be expected to be inactive, and mobilization of iron in an active form, complexed to an unknown endogenous ligand, has therefore been suggested to be required (3, 24) before iron can potentiate the characteristic metabolic lesions of uroporphyria, the block in uroporphyrinogen metabolism. The present results suggest a molecular basis for the role of iron, as Fe-EDTA is now found to be required for accelerated oxidation of porphyrinogens by authentic hydrogen peroxide and also by microsomes "uncoupled" by a uroporphyria-inducing drug. In contrast, ferritin iron and iron chelated with DES are both shown to be inactive, as anticipated from the points which have been discussed above.

These studies do not provide information on the nature of the inhibitor of uroporphyrinogen decarboxylase which has been isolated from porphyric livers (25–27). However, the present finding that iron will not only stimulate the peroxide-dependent porphyrinogen oxidation but also lead to further modification of the resulting porphyrin (with loss of the characteristic spectrum) suggests that an oxidative derivative of the porphyrin might be formed which could act as a targeted inhibitor of the enzyme. This could be a modified porphyrin. However, the possibility that an open-chain degradation product, such as a polycarboxylate oligopyrrole, might exhibit high affinity for the active site of the decarboxylase is attractive and should be explored.

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