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INHIBITION OF CERTAIN MITOCHONDRIAL OXIDATIVE ENZYMES BY PORPHYRINS AND METALLOPORPHYRINS

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Abstract—Liver mitochondria of mice administered hematoporphyrin parenterally showed inhibition of succinate-cytochrome *c* reductase, primary succinate dehydrogenase, *iso*citrate dehydrogenase, L-glutamate dehydrogenase and DPNH-cytochrome *c* reductase. Cytochrome oxidase was not inhibited. Porphyrin compounds in amounts in excess of those in the control animals were found in these mouse liver mitochondria and similar amounts of hematoporphyrin, as well as protoporphyrin and metalloporphyrins, were incorporated into mouse liver mitochondria *in vitro*. *iso*Citrate dehydrogenase, primary succinate dehydrogenase and DPNH-cytochrome *c* reductase were inhibited to a degree similar to that observed with the *in vivo*-administration of the inhibitor. Similar inhibitions also were observed with *iso*citrate and primary succinate dehydrogenases solubilized from the acetone powders. Highly purified preparations of enolase, aldolase and lactate dehydrogenase were not inhibited. The enzyme inhibitions noted, as well as others which may be present, appear to explain, at least in part, the toxicity of these compounds, and offer a possible mechanism for a biochemical pathogenesis of the loss of cellular function noted in some of the porphyrias.

RELATIVELY little information is available on the effects of porphyrins and metalloporphyrins on enzymes, although they are ubiquitously distributed throughout nature and are components of the heme proteins which are essential to most forms of life. Such information is of particular interest in view of the presence of abnormally large amounts of porphyrins in certain tissues in most of the porphyrias, in which sudden neurological deficits may appear, sometimes without apparent microscopic change at the sites of functional impairment. Hematoporphyrin administered parenterally to mice in sufficient dosage is known to induce a sharp fall in oxygen consumption and results in death.[1] The marked fall in oxygen utilization, together with the key presence of porphyrins in the prosthetic groups of the cytochromes in the oxidative chain of enzymes by means of which oxygen is utilized, suggests the possibility that hematoporphyrin may inhibit the oxidative enzymes, possibly by exchanging with or competing with the normal prosthetic groups of the cytochromes.

Porphyrins and hemins are also known to alter yeast oxygen consumption,[2, 3] to inhibit the growth of certain bacteria and protozoa[4–7] and to produce marked changes in the sensitivity of tumors and organisms to radiation.[8] Protoporphyrin and protohemin have been shown to overcome the inhibition produced by non-protoporphyrins.[1, 4–6] Serum cholinesterase, liver succinate cytochrome *c* reductase,

succinoxidase and yeast carboxylase have been reported to be inhibited by various porphyrins and metalloporphyrins.[9–14]

The present study was undertaken to obtain more information on the effect of porphyrins on enzyme systems in an attempt to explain their toxicity, and with the hope of possibly offering a clue to the mechanism of their radiosensitivity effects, as well as to the biochemical pathogenesis of porphyria. Since data in the mouse suggested a profound effect on biological oxidation, the possibility of formation of altered cytochromes with decreased activity or inhibition of associated enzymes was entertained. The oxidative chain of enzymes and some primary oxidative enzymes of mouse liver mitochondria and several non-mitochondrial enzymes were examined with respect to inhibition by several porphyrins and metalloporphyrins in experiments carried out both *in vivo* and *in vitro*, and some observations on the mechanisms of inhibition were made.

EXPERIMENTAL

Materials

Hematoporphyrin was obtained from Merck and Company, Inc. Hemin was obtained from Sigma Chemical Company and recrystallized twice from acetic acid. The following compounds were kindly supplied by Dr. Samuel Schwartz: protoporphyrin (chromatographically pure), Fe-hematoporphyrin BL 158-5 (one atom Fe/mole), Fe-hematoporphyrin BL 159-5 (2 atoms Fe/mole), Cu-hematoporphyrin SSE 26-2 (nitrated; tentatively, 1 atom Cu/mole), Cu-hematoporphyrin SSE 83-2 (2 atoms Cu/mole), Cu-protoporphyrin SSE 149-2 (2 atoms Cu/mole) and Cu-protoporphyrin (1 atom Cu/mole).* Male dark-coated ZBC strain mice weighing 20–30 g were kindly made available by Dr. John J. Bittner.

Enzyme preparations and assays

Soluble *iso*citrate dehydrogenase was prepared from mouse liver mitochondrial acetone powder[16] essentially by the shorter method of Siebert *et al.* and assayed by their method.[17] Fractions which were precipitated between 40% and 50% saturation with ammonium sulfate were used. Soluble primary succinate dehydrogenase was prepared as a tris buffer extract of mouse liver mitochondrial acetone powder, essentially by the method of Singer *et al.*[18] Dr. Clarence Suelter kindly supplied aldolase, which was prepared and assayed as described by Drechsler *et al.*[19] Yeast enolase was a gift of Dr. Edward Westhead, and was prepared and assayed according to the method of Bücher.[20] Beef heart lactate dehydrogenase (turnover number, 10,600/min) was prepared by the method of Meister[21] and assayed by the method of Neilands.[22]

Other enzyme activity measurements were made essentially as follows: cytochrome oxidase was assayed by the method of Cooperstein and Lazarow,[23] and activity calculated as described by Smith;[24] succinate-cytochrome *c* reductase was assayed by the method of Green *et al.*,[25] succinate dehydrogenase (using methylene blue) by the spectrophotometric method of Bonner,[26] primary succinate dehydrogenase by the manometric phenazine methosulfate method of Singer and Kearney,[27] DPNH-cytochrome *c* reductase by the method of Mahler;[28] L-glutamate dehydrogenase by the method of Strecker,[29] and *iso*citrate dehydrogenase of intact mitochondria by the

* The finding of porphyrins having 1, 2, and possibly more than 1 atoms of metal per molecule has recently been described in preliminary form.[8,15]

method of Ochoa.[30] For the last two assays, a 5-min incubation at room temperature, prior to addition of substrate, was used in order to eliminate interfering changes in optical density due to mitochondrial swelling on dilution.

Porphyrin administration and preparation of mitochondria

In the *in vivo* experiments, mice were injected intraperitoneally or subcutaneously with 10 or 15 mg of hematoporphyrin (approximately the MLD_{100}) in 0·5 ml of 0·1 M phosphate buffer, pH 7·4, while an equal number of control animals were injected with buffer alone. All prophyrin-injected animals showed an immediate, marked decrease in normal movement and usually had occasional convulsions. Animals were killed by decapitation from 1 to 3 hr after injection. The livers were removed, blotted dry, weighed and homogenized in 0·25 M sucrose, usually containing 1×10^{-4} M ethylenediamine-tetra-acetate at 0 °C in a Potter–Elvehjem homogenizer. A mitochondrial fraction was prepared,[31] washed two to three times with, and then suspended in, 1 ml of 0·25 M sucrose usually containing 1×10^{-4} M ethylenediamine-tetra-acetate. If some assays were to be performed at a subsequent time, aliquots of 0·2 ml were stored at −20 °C. In most cases, mitochondria were prepared from individual livers and assayed separately.

Estimation of porphyrin and hemin in fractions

The porphyrin and hemin content of the mitochondrial and nuclear fractions of pooled livers of five untreated mice and five mice injected subcutaneously, 1 to 1·5 hr previously, with 10 mg of hematoporphyrin were estimated, using modifications of the method of Basford *et al.*[32] and Connelly *et al.*[33] As was always the case, the hepatic mitochondria of mice given hematoporphyrin were more deeply brown than were control mitochondria. Both fractions were washed consecutively with 0·25 M and 0·58 M sucrose and distilled water. Two 10-ml acetone extractions were made and pooled. The extracts originating from animals given hematoporphyrin were pink and fluorescent, while extracts from control animals were colorless. The absorption spectra of the acetone extracts were obtained in a recording Cary Spectrophotometer. The amount of porphyrin present was determined by comparison with the absorbance produced by a measured amount of hematoporphyrin added to the acetone extract.

The residue from acetone extraction was extracted with 10 ml of chloroform–methanol (v/v, 2/1, extracts discarded), and then four times with 5 ml of acetone–HCl (1·8 ml 37% HCl/100 ml of solution) to give an extract containing hemins. Acetone–HCl extracts were evaporated in a rotated flask under vacuum for 6–12 hr, and the brown oily residue was dissolved in 2 ml of pyridine to which 2 ml of 0·2 N KOH were then added to give the pyridine hemochromogens. The liquid was then filtered through Whatman no. 50 filter paper and a difference spectrum obtained between experimental and control mitochondrial and nuclear extracts. The difference in hemin content between experimental and control fractions was derived by using the molar absorbancy index $a_{M,\ 400m\mu} = 6519$, which was obtained using recrystallized hemin in pyridine—0·2 N KOH (v/v, 1/1).

Additions of porphyrins to mouse liver mitochondria in vitro

The following compounds were used in *in vitro*-experiments with mitochondria: hematoporphyrin, protoporphyrin, Fe-hematoporphyrin BL 158-5, hemin, and

Cu-hematoporphyrin SSE 83-2. Of each compound 1 mg was dissolved in 0·1 ml of 0·2 N KOH, following which 4·9 ml of 0·313 M potassium phosphate buffer, pH 7·8, were added. Absorption spectra from 350–700 mμ of each solution were obtained in a Cary recording spectrophotometer. Each solution was then added to a one-sixth aliquot of liver mitochondria pooled from six mouse livers, and incubated for 1·5 hr at 0 °C. The mitochondrial suspensions were then centrifuged and the spectra of the supernatant solutions obtained. The amounts of porphyrins retained by the mitochondrial fractions which were deeply colored were calculated from the difference in absorbance of the solutions before and after addition of mitochondria. Estimates at different wavelengths gave closely comparable values. The mitochondria were washed once with 0·25 M sucrose (wash contained only trace amounts of porphyrin) and suspended in 1 ml of 0·25 M sucrose for use in enzyme assays.

TABLE 1. EFFECT OF HEMATOPORPHYRIN ADMINISTERED *in vivo* ON ENZYME ACTIVITIES OF MOUSE LIVER MITOCHONDRIA

Mitochondria were isolated from control mice injected with phosphate buffer solvent and from mice given 10–15 mg of hematoporphyrin (Merck) parenterally 1–3 hr before sacrifice, and kept at 0°C. Mice were of the ZBC strain and weighed 20–30 g. Enzyme activities are expressed per mitochondria obtained from 1 g of liver. Similar amounts of mitochondria were obtained from control and experimental animals. Assays were done at 24°C, unless otherwise indicated. Figures within parentheses indicate the number of animals assayed.

		Enzymes							
Experimental series	Type of mitochondria	Cytochrome oxidase‡ k	Succinate cytochrome c reductase (ΔA_{550} mμ/min)	Primary succinate dehydrogenase (μl O_2/hr)	DPNH-cytochrome c reductase (ΔA_{550} mμ/min) mitochondrial dilution 1/10	1/20	1/40	L-Glutamate dehydrogenase (ΔA_{340} mμ/min)	*iso*Citrate dehydrogenase (ΔA_{340} mμ/min)
1	Control	503* (3)	3·4† (3)	9650† (2)		12·3 (2)	7·4 (2)	0·73 (2) (61 days)§	
	Porphyrin	608* (3)	0† (3)	900† (2)		10·3 (2)	3·0 (2)	1·09 (2)	
2	Control			7850* (3)	18·2 (4)			1·42 (4) (2 days)§	4·9 (4)
	Porphyrin			560* (4)	9·6 (4)			0·72 (4)	1·1 (4)
3	Control							1·01 (4) (10 days)§	
	Porphyrin							0·66 (4)	

* Reaction temperature, 30°C.

† Reaction temperature, 38°C.

‡ k = first-order rate constant = $\frac{\log (A - A_\infty)t_1 - \log (A - A_\infty)t_2}{t_2 - t_1} \times 2.3 \text{ sec}^{-1}$

§ Time of ageing at −20°C.

RESULTS

Enzyme activities following administration of hematoporphyrin in vivo

The activities of various mitochondrial enzymes of control animals and those receiving hematoporphyrin are summarized in Table 1. No inhibition of cytochrome oxidase was observed. Evidence of inhibition in the oxidative chain between dehydrogenation of succinate and the reduction of cytochrome *c* was indicated by the strong inhibition of succinate cytochrome *c* reductase. The inhibition of the reduction of methylene blue, which was observed in the presence of succinate, suggested that the

inhibition was between succinate dehydrogenation and cytochrome *b*. Using phenazine methosulfate as electron acceptor, primary succinate dehydrogenase was then found to be strongly inhibited.

Since primary succinate dehydrogenase is an iron-flavoprotein, another enzyme of this type, DPNH-cytochrome *c* reductase, was studied and found to be inhibited, although not as strongly. To test another mitochondrial oxidative enzyme which is neither an iron flavoprotein nor a cytochrome, L-glutamate dehydrogenase was studied; this enzyme was inhibited fairly strongly, except in a preparation which was stored for 2 months at −15 °C. The striking inhibition of primary succinate dehydrogenase prompted the investigation of another tricarboxylic acid cycle enzyme, *iso*citrate dehydrogenase; this enzyme also was markedly inhibited.

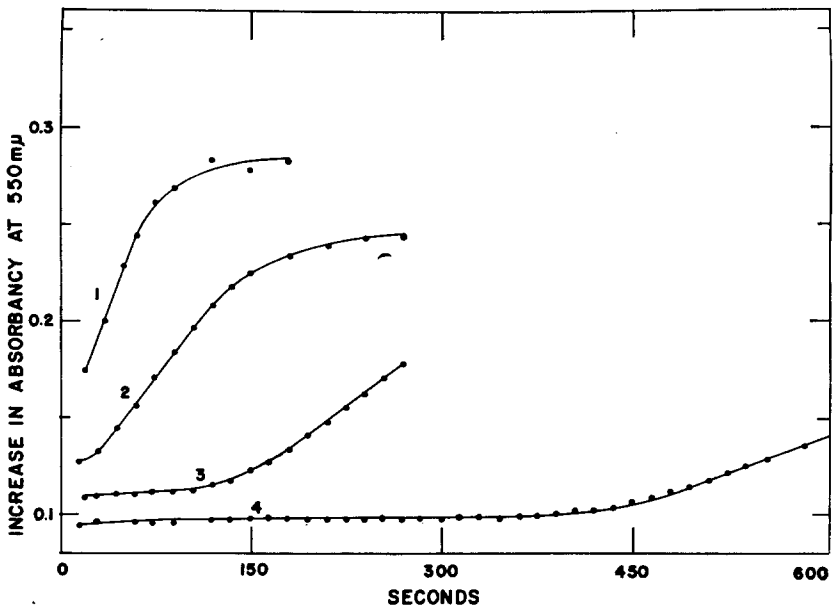

FIG. 1. Inverse relationship between rat liver mitochondrial concentration and the lag in increase in absorbancy at 550 mμ in the succinate–cytochrome *c* reductase assay. Absorbance at 500 mμ of the mitochondria in 3-ml reaction volume: (1) 0·067, (2) 0·0313, (3) 0·0143, (4) 0·0077 (based on absorbancies of mitochondrial concentrations three times higher). Reaction mixtures contained 30 mg of serum albumin, 15 μmoles of succinate, 0·5 mg of cytochrome *c*, 3 μmoles of KCN, 30 μmoles of K phosphate buffer (pH 7·4), 2·4 μmoles of sucrose, and 9×10^{-7} μmoles of ethelenediamine-tetra-acetate.

Peculiarities related to mitochondrial dilution in two of the assays deserved mention. In the DPNH–cytochrome *c* reductase assay, a decrease in specific activity with dilution of mitochondria was observed. At all dilutions, however, less activity was observed in the mitochondria from animals given hematoporphyrin (Table 1). In the succinate–cytochrome *c* reductase assay, a lag was observed in the onset of spectral change (Fig. 1). The lag periods were approximately proportional to the inverse squares of the mitochondrial concentrations. This suggests that the lag period may be related to mitochondrial interaction. For the assays reported in Table 1, very weak activity appeared in the porphyrin mitochondria after from 1 to 4 hr.

Also of interest is an observation that upon storage of mitochondria at −20° in 0·25 M sucrose for 61 days, a release of glutamate dehydrogenase into the medium occurred. The aged preparation from animals receiving hematopophyrin showed slightly less release to the medium and had slightly greater activity than control. Total activity tended to decrease with ageing (Table 1).

Since porphyrins are photodynamic compounds, and cause inhibition of certain enzymes only in the presence of intense light,[34] it was of interest to see whether diffuse room light had any effect on the inhibitions of the mitochondrial enzyme of mouse liver. No essential difference in inhibition of primary succinate dehydrogenase and of DPNH–cytochrome *c* reductase was observed between mitochondria of mice kept in the dark and those remaining in ordinary diffuse room light after porphyrin injection.

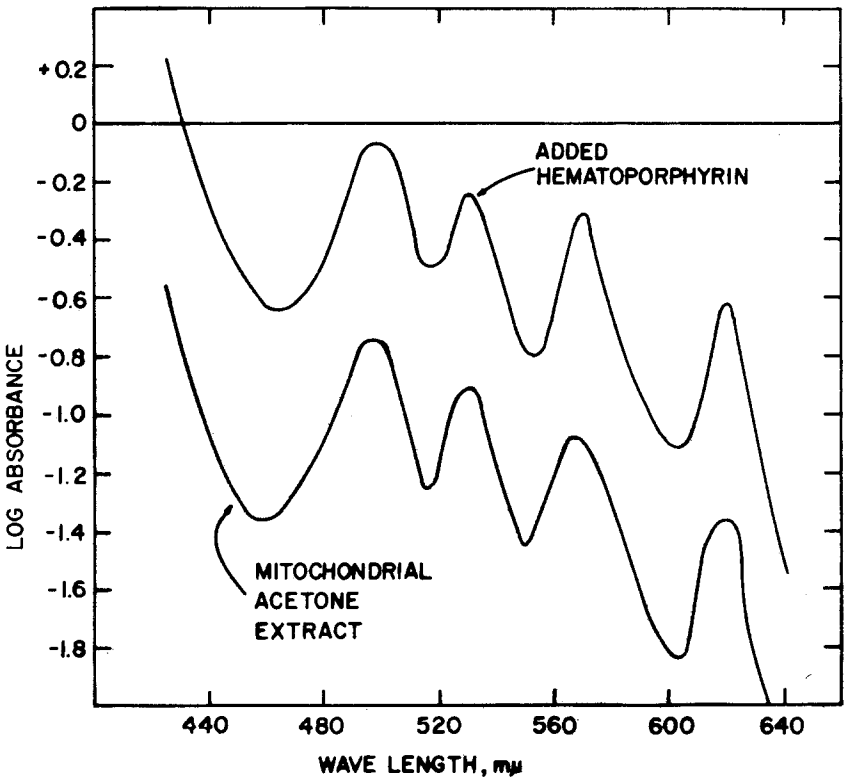

FIG. 2. Log absorbance plotted against wavelength in mμ for the mitochondrial acetone extract absorption spectrum and for the difference spectrum of hematoporphyrin added to extract (0·291 mg/ml).

Porphyrin and hemin in liver mitochondria and nuclei after hematoporphyrin administration in vivo

It was of interest to identify the subcutaneously administered porphyrin in mitochondria isolated from the liver and to estimate it, in order that a similar amount could be incorporated into isolated liver mitochondria by *in vitro*-addition of porphyrin and the data on the enzymic activities of these mitochondria compared to those obtained following *in vivo*-administration. Fig. 2 gives a plot of the log of the absorbancies vs.

wavelength for (1) a mitochondrial acetone extract containing porphyrin, and (2) the hematoporphyrin preparation used. The latter was obtained as the difference spectrum of the mitochondrial acetone extract and a portion of the same extract to which 0·291 mg. of hematoporphyrin was added per ml. Such a plot gives superimposable curves for identical materials. The very close resemblance of the absorbance of the extract to that of the added porphyrin suggests that little or no chemical change had occurred in the administered hematoporphyrin preparation which was extractable as porphyrin. Peak absorbancies occurred at 660–1, 622, 568–9, 532–3, 499 and 394 mμ. The small peak at 660–1 mμ may indicate the presence of a small amount of oxyporphyrin.[35]

Results of estimations of the amount of porphyrin in the acetone extracts and of hemin in the acid–acetone extracts (see experimental section) are given in Table 2. Approximately 3·9 per cent of the total administered hematoporphyrin is accounted for as porphyrin and hemin in the mitochondrial and nuclear fractions. The hemin in excess of control value may represent Fe-porphyrin newly formed from administered porphyrin with the aid of iron-inserting enzyme.[36] The porphyrin in excess of the control value in the nuclear fractions may be attributable to contamination with porphyrin-containing mitochondria, since in other experiments contaminating mitochondria, rather than the nuclei of the nuclear fraction, showed fluorescence.[37]

TABLE 2. PORPHYRIN AND HEMIN IN EXCESS OF CONTROL VALUES IN LIVER MITOCHONDRIAL AND NUCLEAR FRACTIONS OF MICE GIVEN HEMATOPORPHYRIN

Nuclear and mitochondrial fractions were isolated from five ZBC male mice injected subcutaneously with 10 mg of hematoporphyrin (Merck) 1–1·5 hr prior to sacrifice, and from five control mice injected with phosphate buffer solvent only. The amount of prophyrin and hemin in excess of control was determined spectrophotometrically. Hemin was estimated on the basis of $a_{M,400m\mu} = 6519$, obtained with recrystallized hemin in pyridine—0·2 N KOH (v/v, 1/1), which also was used as solvent for mitochondrial hemin estimation. See Experimental section for details.

Fraction	Amount of porphyrin and hemin in excess of control (mg/g of fresh liver)		
	Porphyrin	Hemin	Total porphyrin compounds
Mitochondrial	0·258	0·053	0·311
Nuclear	0·082	0·045	0·127

Enzyme activities and porphyrin and metalloporphyrin concentration following the addition of porphyrins and metalloporphyrins to mitochondria in vitro

In order to determine whether the enzyme inhibitions observed with *in vivo*-administration of hematoporphyrin were the result of direct action on the mitochondria, rather than of some other mechanism operative only *in vivo*, enzyme activities were

studied in mitochondria into which hematoporphyrin was incorporated *in vitro*. Other porphyrins were similarly studied. The amounts of various porphyrins in the mitochondria and the results of enzyme activity measurements are shown in Table 3 (see experimental section for details of exposure and determination). The amount of hematoporphyrin in the mitochondrial fraction is similar to that found in experiments *in vivo*. Thus, the experiments give a good comparison of the effects of administration *in vitro* and *in vivo*, and show the effects on mitochondrial enzymes by the two routes of administration to be similar. Primary succinate dehydrogenase was significantly inhibited by all compounds added, except protoporphyrin. Hematoporphyrin was as effective as Fe-hematoporphyrin. *iso*Citrate dehydrogenase was strongly inhibited by all added compounds. DPNH–cytochrome *c* reductase was inhibited less strongly than the other enzymes studied. The metal complexes were more effective inhibitors than the free porphyrins. The most effective inhibitor for all three enzymes was Fe-protoporphyrin; however, it is not clear whether this is attributable entirely to its presence in the highest amount in the mitochondria.

TABLE 3. EFFECT OF PORPHYRINS AND METALLOPORPHYRINS ON MITOCHONDRIAL ENZYMES *in vitro*

Mitochondria were obtained from six pooled mouse livers and the indicated porphyrins incubated with a one-sixth aliquot at 0°C for 1·5 hr. The porphyrin taken up by the mitochondria was obtained by the difference in absorbance of the porphyrin solutions (1 mg of porphyrin per 0·1 ml of 0·2 N KOH + 4·9 ml of 0·313 M potassium phosphate buffer, pH 7·8) before and after addition of mitochondria. Enzyme activities are expressed per total mitochondria per mouse liver (average liver weight, 1·098 g). Primary succinate dehydrogenase was assayed at 37°C; the other enzymes were assayed at 24°C.

Type of porphyrin added to mitochondria	Amount present (mg)	Primary succinate dehydrogenase (μl O_2/hr)	*iso*Citrate dehydrogenase (ΔA_{340} mμ/min)	DPNH–cytochrome *c* reductase (ΔA_{550} mμ/min)
None	—	3063	6·4	10·8
Hematoporphyrin	0·26	330	0·4	8·2
Protoporphyrin	0·96	2520	2·0	7·5
Fe-Hematoporphyrin, SSE 158-5, one Fe/mole	0·44	780	0·6	4·5
Fe-Protoporphyrin, one Fe/mole	0·79	0	0·0	2·0
Cu-Hematoporphyrin, SSE 83-2, 2 Cu/mole	0·57	510	0·0	5·4

Cytochrome oxidase of rat liver mitochondria was not appreciably affected by Cu-hematoporphyrin SSE 26-2 (concentrations of up to 25 mg/mitochondria of 10 g of liver). Porphyrin and mitochondria were pre-incubated at 30 °C for 5 min and at 38 °C for 10 min prior to assay.

Effects of porphyrins and metalloporphyrins on the activity of soluble enzymes

Mitochondrial. Two mitochondrial enzymes, *iso*citrate dehydrogenase and primary succinate dehydrogenase, were extracted from mouse liver mitochondria, in order to determine whether mitochondrial structure is necessary for inhibition. Hematoporphyrin, as well as Fe and Cu complexes of hematoporphyrin and protoporphyrin,

strongly inhibited soluble mouse liver mitochondrial *iso*citrate dehydrogenase (Table 4). The number of metal atoms per mole of porphyrin did not appear to affect the inhibition. Plots of reciprocal velocity vs. reciprocal substrate concentration, using hematoporphyrin as inhibitor, indicate that the inhibition is non-competitive with respect to *iso*citrate (Fig. 3), and is at least predominently competitive with respect to TPN, especially at the lower concentration of inhibitor (Fig. 4). The K_m was $2{\cdot}8 \times 10^{-6}$ M for TPN and $5{\cdot}8 \times 10^{-4}$ M for *iso*citrate; the K_i for TPN was $2{\cdot}8 \times 10^{-6}$ M and was $1{\cdot}4$–$4{\cdot}1 \times 10^{-5}$ M for *iso*citrate.

TABLE 4. INHIBITION OF *iso*CITRATE DEHYDROGENASE BY PORPHYRINS

Enzyme was prepared from mouse liver mitochondrial acetone powder[16] essentially by the shorter method of Siebert *et al.*[17], and contained 70 units/mg. Assays with hematoporphyrin were run in tris-HCl buffer, pH 7·4, while the others were run in tris buffer, pH 9·1, to ensure solubility of the inhibitors.

Porphyrin Type	Atoms metal per mole	% Inhibition $3{\cdot}3 \times 10^{-5}$ M	% Inhibition 1×10^{-6} M
Hematoporphyrin	0	56·5	
Fe-protoporphyrin	1	95·2	56·3
Fe-hematoporphyrin BL 158-5	1	75·6	66·0
Fe-hematoporphyrin BL 159-5	2	67·1	43·1
Cu-hematoporphyrin SSE 83-2	2	93·8	22·9
Cu-protoporphyrin SSE 149-2	2	100·0	32·9
Cu-hematoporphyrin SSE 26-2	<1*	85·2	71·0
Cu-protoporphyrin	1	100·0	

* Preliminary studies indicate that this nitrated porphyrin may contain an average of less than one atom of Cu per molecule of porphyrin.[37]

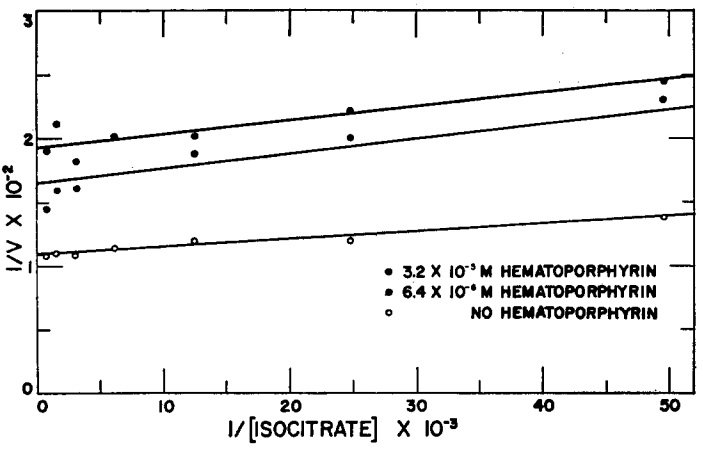

FIG. 3. Double reciprocal plot showing the effect of the addition of hematoporphyrin on the initial reaction velocity of *iso*citrate dehydrogenation using soluble mouse liver mitochondrial *iso*citrate dehydrogenase and various concentrations of *iso*citrate. Measurements were made in 0·033 M Tris-HCl buffer, pH 7·4. Concentrations are in moles/l. Reaction velocities are expressed as ΔA_{340} mμ/min. Reaction mixture contained 2 μmoles of $MnCl_2$, 500 μmoles of Tris buffer, and 0·075 μmoles of TPN in a total volume of 1·5 ml.

In preliminary observations, 5×10^{-4} M Fe-protoporphyrin, Fe-hematoporphyrin and Cu-protoporphyrin (1, 1, and 2 metal atoms/mole, respectively) inhibited soluble primary succinic dehydrogenase 90 per cent, 53 per cent and 34 per cent, respectively. Hematoporphyrin was not inhibitory in the ranges measured ($2{\cdot}5 \times 10^{-6}$–5×10^{-4} M).

Non-mitochondrial. Hematoporphyrin ($0{\cdot}93 \times 10^{-6}$–$3{\cdot}7 \times 10^{-5}$ M) had no appreciable effect on yeast enolase activity, while hemin ($1{\cdot}9$–$9{\cdot}3 \times 10^{-6}$ M) appeared to have a slight stimulatory effect. Rabbit muscle aldolase was not inhibited by hematoporphyrin ($1{\cdot}1$–$4{\cdot}2 \times 10^{-5}$ M) or hemin ($2{\cdot}0 \times 10^{-5}$–$2{\cdot}0 \times 10^{-4}$ M). An apparent inhibition by hemin was the result of hemochromagen formation with hydrazine used in the assay, since excess hydrazine completely overcame the inhibition. Beef heart lactate dehydrogenase activity was not altered by $2{\cdot}5 \times 10^{-5}$ M hemin.

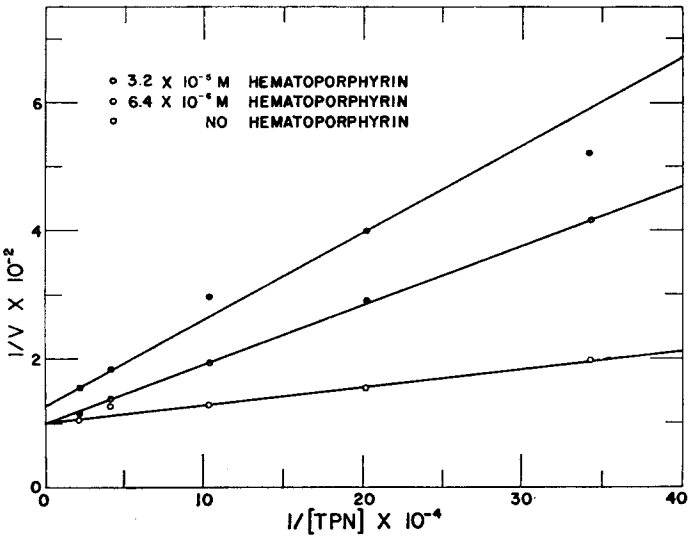

FIG. 4. Double reciprocal plot showing the effect of the addition of hematoporphyrin on the initial reaction velocity of *iso*citrate dehydrogenation, using soluble mouse liver mitochondrial *iso*citrate dehydrogenase and various concentrations of TPN. Measurements were made in Tris-HCl buffer. 0·033 M, pH 7·4. Concentrations are in moles/l. Reaction velocities are expressed as ΔA_{340} mμ/min, The reaction mixture contained 2 μmoles of $MnCl_2$, 500 μmoles of Tris buffer, and 1 μmole of *iso*-citrate in a total volume of 1·5 ml.

Absorption spectrum of hemin in the presence of TPN and DPN

The absorption spectrum of $2{\cdot}5 \times 10^{-5}$ M hemin from 350–700 mμ was not altered by $1{\cdot}2 \times 10^{-4}$ M TPN $\pm$ $1{\cdot}2 \times 10^{-3}$ M $MnCl_2$ or by $6{\cdot}7 \times 10^{-4}$ M DPN. Accordingly, there was no spectral evidence for the possibility that the inhibitory effects of porphyrins on those enzymes utilizing DPN or TPN is attributable to complex formation between the nucleotides and porphyrins.

DISCUSSION

These data indicate that a portion of parenterally administered hematoporphyrin localizes in liver mitochondria, in which about 15–20 per cent is apparently converted

to metalloporphyrin, probably by means of the iron-inserting enzyme recently described in mitochondria.[36] This localization is associated with striking inhibition of mitochondrial dehydrogenases, most notably of the two Krebs cycle enzymes, primary succinate dehydrogenase and *iso*citrate dehydrogenase. These enzyme inhibitions, as well as others which may be present, may account, at least in part, for the marked toxicity and sharp fall in oxygen consumption noted in these mice.[1]

The activities of mitochondrial dehydrogenases were inhibited while the non-mitochondrial dehydrogenase (lactate dehydrogenase) and the mitochondrial terminal oxidase (cytochrome oxidase) were not affected. The non-oxidative enzyme, serum cholinesterase, has been reported to be inhibited.[9] In the case of primary succinate dehydrogenase and *iso*citrate dehydrogenase, the mitochondrial structure was not shown to be essential for the inhibition, since the enzymes extracted from the mitochondria were also inhibited. No consistent correlations are noted between the structure of the porphyrin and the presence or absence of complexed metal and the enzyme inhibitions observed. A variety of metalloporphyrins, and in most cases free porphyrins, were effective inhibitors. All the enzymes inhibited contain metal whose function is not clear. Since porphyrin-metal complexes, as well as free porphyrins, exert, inhibitions on the enzymes studied, it is unlikely that the mechanism of inhibition depends on coordination of the central porphyrin nitrogen atoms with enzyme metal. In the case of *iso*citrate dehydrogenase, inhibition with hematoporphyrin was competitive with respect to TPN and non-competitive with respect to *iso*citrate, a finding which suggests reversible binding at the TPN-binding site.

Two lines of evidence fail to indicate significant inhibition of cytochrome activity by these structural analogues (identical in the case of protohemin) of the normal prosthetic groups: cytochrome oxidase activity is not diminished, and the inhibition of DPNH–cytochrome *c* reductase is not striking, as compared to the inhibition of primary succinate dehydrogenase, although there is evidence that these enzymes share a common pathway in the electron transport chain.[38–40] Failure to find conclusive evidence for alteration in cytochrome activity does not completely rule out the possibility of an interchange of an abnormal for a normal heme group, or of incorporation of an abnormal heme group into newly synthesized cytochromes, since there is evidence in reconstitution experiments for the *in vitro*-exchange of abnormal for normal heme groups, with resultant activity, which in some cases was greater than that for the normal heme protein.[41, 42] That newly formed hemin may actually be incorporated into cytochromes at a time later than the 2-hr period in these experiments is suggested by the data of Schulz, who found an almost two-fold increase in cytochrome oxidase activity in rat liver 5 days after the intraperitoneal injection of hematoporphyrin.[43]

These data corroborate the previous findings of the inhibition by porphyrins[10–12] or by hemins, but not porphyrins[13], of succinate-cytochrome *c* reductase and succinoxidase in particulate preparations. While marked inhibition of succinate dehydrogenase usually was found with both porphyrins and metalloporphyrins, protoporphyrin with mitochondria, and hematoporphyrin with soluble primary succinate dehydrogenase, were essentially non-inhibitory. Conversion of porphyrin to Fe-porphyrin may account for the inhibition observed with hematoporphyrin in mitochondria, but does not explain why protoporphyrin was not similarly effective. The finding that succinate dehydrogenase inhibition is attributable largely or completely

to inhibition of the primary succinate dehydrogenase, rather than to a block in the electron transport chain, confirms previous suggestive evidence.[11, 13] The pronounced inhibition of succinate–cytochrome *c* reductase appears largely or entirely the result of inhibition of the primary dehydrogenase, since DPNH–cytochrome *c* reductase was much less inhibited.

Similar oxidative enzyme inhibitions conceivably may play a role in the biochemical pathogenesis of the porphyrias which are usually associated with increased amounts of porphyrins in tissues.[11, 44, 45] The fact that nervous tissue can least well withstand the effects of anoxia, and the marked neurological impairments, usually fatal when involving the medullary respiratory center, which are found in the hepatic porphyrias, are compatible with this possibility. If the porphyrin accumulation in a particular tissue (e.g. nervous) were confined to vital cells, perhaps from over-production of porphyrin in them, profound functional effects could be observed without a massive accumulation of porphyrin in the tissue generally. It would also be of interest to see if porphobilinogen, δ-aminolevulinic acid and other intermediates of porphyrin biosynthesis which are increased in some of the porphyrias may exert effects similar to the porphyrins. These inhibitions do not explain the increased amounts of porphyrins found in porphyria, suggestions for which have been reviewed,[45] but rather offer a possible mechanism for the functional impairments noted (e.g. hepatic, neurologic, probable early loss of abnormal porphyrin-laden normoblasts). Other possibilities for these impairments, such as failure of cytochrome synthesis or multiple effects, certainly are not excluded. The enzyme inhibitions noted with the various porphyrins and metalloporphyrins in this study do not correlate with their effects on radiosensitivity, and therefore offer no clue at to the mechanism of the latter.

It is of some interest that steroid hormones which are thought to act physiologically as agents of metabolic control have recently been found to inhibit some oxidative enzyme systems, among which are DPNH–cytochrome *c* reductase and L-glutamate dehydrogenase.[46] It is conceivable that change in the concentration of naturally occurring porphyrins and metalloporphyrins may act as a control mechanism for some of the mitochondrial oxidative enzymes, although there is insufficient evidence from the data at hand to show that this could occur physiologically. Such a mechanism would be a feed-back system, since block of the citric acid cycle would inhibit the production of succinyl-coenzyme A, which is a necessary starting material in the pathway of porphyrin biosynthesis.[45] One may also speculate that cells producing excess porphyrin may be relatively insensitive to this possible inhibitory control (and to others responsive to lower levels of porphyrins?) of porphyrin synthesis, whereas release of porphyrin to sensitive cells may result in marked inhibition of cellular oxidation and function.

In other studies with hematoporphyrin, heating in alkali produced an inhibitor of succinate dehydrogenase at least 100 times more potent than the parent hematoporphyrin.[12] The visible absorption spectrum of alkali-altered hematoporphyrin is different from that of the hematoporphyrin from which it was derived.[43] Minor porphyrin components have recently been observed in various hematoporphyrin preparations.[8] The contribution, if any, of these porphyrins to the enzymic effects noted in the present study is not known. It is quite possible that they are similar to hematoporphyrin in this regard, since a number of highly purified

metalloporphyrins and protoporphyrin generally were similarly effective as inhibitors in these studies.

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