Inhibition of Protohaem Ferro-Lyase in Experimental Porphyrria

ISOLATION AND PARTIAL CHARACTERIZATION OF A MODIFIED PORPHYRIN INHIBITOR

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1. A modified porphyrin with inhibitory activity towards protohaem ferro-lyase was purified from the livers of mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine, and partially characterized. 2. The inhibitor can be labelled by 5-amino[4-¹⁴C]-laevulinate, suggesting that it originates from pre-labelled liver haem. No radioactivity from 3,5-diethoxycarbonyl-1,4-dihydro[2,6-¹⁴C]collidine can be recovered bound to the purified abnormal porphyrin when the radioactive drug is used to induce its formation. 3. Similar modified porphyrins isolated from the livers of animals treated with 2-allyl-2-isopropylacetamide, secobarbitone or 1-ethynylcyclohexanol did not exhibit inhibitory activity toward protohaem ferro-lyase. 4. The inhibition of protohaem ferro-lyase was progressive, could be slowed down by cooling and partially prevented by preincubating the enzyme with the porphyrin substrate. Once established, inhibition could not be reversed by addition of the substrate. 5. These results suggest that the modified porphyrin irreversibly inhibits protohaem ferro-lyase and may be used as a sensitive and selective reagent to titrate the number of active centres of the enzyme.

The porphyrinogenic compound 3,5-diethoxycarbonyl-1,4-dihydrocollidine produces a very marked inhibition of protohaem ferro-lyase in the liver of rats, mice and chick embryos (Onisawa & Labbe, 1963; Tephly *et al.*, 1971; De Matteis *et al.*, 1973; Rifkind, 1979), a biochemical lesion which is thought to be responsible for the very pronounced accumulation of protoporphyrin seen in this type of experimental porphyria. We have reported (Tephly *et al.*, 1979) that a porphyrin-like inhibitor of protohaem ferro-lyase accumulates in the liver of rats and mice treated with this drug and can be isolated by differential extraction of liver homogenates and subsequent column chromatography of the extract. Experiments using isotopic precursors for haem suggested that the inhibitor might arise from haem, although separation of the abnormal porphyrin from haem was not complete and no definite conclusion could be reached on this point (Tephly *et al.*, 1979). The present work extends those observations. By using an improved isolation

technique involving combined column chromatography and t.l.c., we now confirm that the inhibitory pigment can be labelled with a precursor of haem, 5-amino[4-¹⁴C]laevulinate; in contrast we find that no radioactivity from 3,5-diethoxycarbonyl-1,4-dihydro[2,6-¹⁴C]collidine can be recovered bound to the purified abnormal porphyrin when the radioactive drug is used to induce formation of the pigment *in vivo*. Results are also presented on the partial characterization of the inhibitory pigment and on the mechanism by which it affects the activity of protohaem ferro-lyase.

Materials and Methods

Experimental

To study the possible origin of the inhibitory pigment from prelabelled liver haem, male mice (32–37 g) of the LACA strain, given free access to food and water, were injected intraperitoneally with 5-amino[4-¹⁴C]laevulinate (10 μ Ci; 0.17 μ mol/kg in 0.9% NaCl); after 2 h they were given intraperitoneally 3,5-diethoxycarbonyl-1,4-dihydrocollidine (200 mg/kg) dissolved in arachis oil (10 ml/kg) and were killed 2.5 h later. Control animals were given oil

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alone. In another experiment in which the covalent binding of the drug to the porphyrin nucleus of the inhibitory pigment was investigated, mice of the same sex and strain were given 25 mg of 3,5-diethoxycarbonyl-1,4-dihydro[2,6- ^{14}C]collidine/kg intraperitoneally (sp. radioactivity 1.3 $\mu\text{Ci}/\text{mg}$) and were killed 1 h later. 3,5-Diethoxycarbonyl-1,4-dihydrocollidine was prepared and purified essentially as described by De Matteis & Prior (1962), starting with ethyl [3- ^{14}C]acetoacetate for the synthesis of the drug labelled in the pyridine ring at the 2 and 6 positions. The labelled drug when run on t.l.c. on a silica gel G plate in the developing system benzene/methanol (14:1, v/v) produced a single fluorescent band (R_F 0.36), and a corresponding single peak of radioactivity was detected with a Packard radiochromatogram scanner. The spectrum of the radioactive drug was determined in methanol and, as reported by Racz & Marks (1972), it exhibited two absorption maxima at 232 and 352 nm, with a ratio between their intensities of 2.25 [Racz & Marks (1972) give a ratio of 2.23]. A small impurity was detected by capillary g.l.c., but this was estimated to be less than 5%. When the effect of 2-allyl-2-isopropylacetamide, secobarbitone [5-allyl-5-(1-methylbutyl)barbiturate] or 1-ethynylcyclohexanol was studied, male mice (28–31 g) or male rats (205–248 g) were given a single injection of one of these drugs and were killed between 1 and 4 h later. All animals were supplied by MRC Laboratory Animal Centre, Carshalton, Surrey, U.K.

Sources of special chemicals

2-Allyl-2-isopropylacetamide was a generous gift from Roche Products, Welwyn Garden City, Herts., U.K.; secobarbitone was obtained from Siegfried Ltd., CH-4800 Zofingen, Switzerland; and 1-ethynylcyclohexanol was from Aldrich Chemical Co., The Old Brickyard New Road, Gillingham, Dorset, U.K. 5-Amino[4- ^{14}C]laevulinate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Insta-gel was obtained from Packard Instruments, Caversham, Berks. RG4 7AA, U.K.

Extraction and purification of the inhibitor

Liver homogenates (50%, w/v, in 0.25 M-sucrose, equivalent to 6 g wet wt. of liver) were extracted twice with an equal volume of acetone and then twice with an equal volume of acetone containing 1.7% (v/v) conc. HCl. The acid/acetone extracts were combined and concentrated under N_2 to approx. 0.2 ml (the traces of HCl thus remaining were found to be essential for keeping the pigments in solution and also important for reproducible behaviour on chromatography); after dilution with acetone to 1.5 ml, the acid/acetone extract was applied on to a freshly packed column (30 cm $\times$ 1 cm) of Sephadex LH-20. Acetone was

used to pack the gel and to elute the fractions. Traces of HCl were detected in the first 20 fractions (see Fig. 1), including those containing the inhibitor. All fractions were analysed spectrally with a Unicam SP. 1800 spectrophotometer: although a clear Soret absorption peak (at around 400 nm) was detected in the most-coloured fractions containing haem, green pigments or protoporphyrin, this was complicated in the weakest haem and green-pigments fractions by an impurity absorbing maximally at 340–370 nm. Because of this, in some experiments the difference in absorption between the following pairs of wavelength (in nm) was used to follow the concentration of pigments in the various fractions: haem (540–610); green pigments (560–589); protoporphyrin (402–484). Fractions were evaporated to dryness under N_2 , care being taken to displace all HCl, then dissolved in dimethyl sulphoxide and various dilutions assayed for inhibition of protohaem ferro-lyase activity of mouse liver mitochondria as described by Tephly *et al.* (1979), usually with mesoporphyrin and Co^{2+} as substrates. The mitochondrial suspension, which was stable at -70°C for at least 1 month, was suitably diluted so as to adjust the control enzyme activity to 3.6 nmol of mesoporphyrin utilized/min per ml of enzyme-assay mixture; a unit of inhibitor was defined as the amount that produced 50% inhibition of enzyme activity under the standard conditions described by Tephly *et al.* (1979). In one experiment the activity of the enzyme was also assayed by using (with mouse liver mitochondria) protoporphyrin and Co^{2+} as substrates (Jones & Jones, 1969) or with rat liver homogenates by using protoporphyrin and [^{59}Fe]ferri-citrate (De Matteis & Gibbs, 1972). The ^{59}Fe -labelled haem was then isolated by the method of Bonkowsky *et al.* (1978) and counted for radioactivity in a Packard Autogamma scintillation spectrometer. When ^{14}C was the radioisotope, determination of radioactivity was by liquid-scintillation counting in a Philips PW 4510 instrument, with correction for quench by an external-standard/channels-ratio program and with Insta-gel as the scintillant.

For further purification of the green-pigment inhibitor, fractions from Sephadex LH-20 chromatography were defatted by cooling at -70°C overnight and the whitish precipitate was separated from the green-coloured supernatant by centrifugation (2000 g for 5 min) at -20°C . The supernatant was then evaporated to dryness under N_2 , and, after displacement of all traces of HCl, the pigment was dissolved in methanol and subjected to t.l.c. on silica gel G plates (0.25 mm thick; E. Merck, Darmstadt, Germany), with the developing system chloroform/methanol/acetic acid (40:10:3, by vol.). This system separates the green pigment (R_F 0.49) from both haem (R_F 0.70) and protoporphyrin

(R_F 0.80). The green pigment, which fluoresced red under u.v., was eluted with methanol; then, after evaporation of the methanol under N_2 , the pigment was dissolved in dimethyl sulphoxide and tested for inhibitory activity by the method of Tephly *et al.* (1979). A known portion of the pigment was dissolved in acetone containing 1% (v/v) of conc. HCl in order to determine its absorbance at 411 nm as the porphyrin dication.

Results

Extraction and isolation of the porphyrin inhibitor accumulating after treatment of mice with 3,5-diethoxycarbonyl-1,4-dihydrocollidine

When mice were given a porphyrogenic dose of 3,5-diethoxycarbonyl-1,4-dihydrocollidine (200 mg/kg) and were killed 2.5 h later, the acetone/HCl extract of their livers showed on Sephadex LH-20 chromatography three distinct peaks of material with a Soret absorption at around 400 nm. The first peak, representing haem, was followed by a sharp peak of a green pigment (exhibiting a porphyrin-dication spectrum) and then by a second broader peak with a typical aetio-type porphyrin spectrum, representing protoporphyrin. Control livers showed, in addition to the haem peak, a very small green pigment peak, but no protoporphyrin (Fig. 1a). In this experiment control and treated mice had been given a dose of 5-amino[4- ^{14}C]laevulinate in order to prelabel their liver haem pools, and 2 h later, at a time when precursor pools are no longer labelled, they received either oil or the drug, respectively: on Sephadex LH-20 chromatography of liver extracts from treated animals, radioactivity was recovered in both the haem and the green-pigment peaks (Fig. 1b). A powerful inhibitor of protohaem ferro-lyase activity could be detected in the green-pigment fractions from control animals and, to a much greater extent, in those from treated animals. In contrast, the haem and protoporphyrin fractions from either column were virtually inactive (Fig. 1c). No inhibitor was detected in the neutral-acetone extract of the liver homogenate, and only 7% more inhibitor could be detected in a third acetone/HCl extract, indicating that the pooled first two acetone/HCl extracts, which were applied to Sephadex LH-20, contained essentially all the inhibitor.

The crude pigment labelled with 5-amino[4- ^{14}C]laevulinate and obtained after treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine was further purified. An impurity (most probably lipid) was first separated out by lowering the temperature of the acetone solution of the green-pigment fractions obtained from the Sephadex LH-20 column and by removing the precipitate by centrifugation (2000g for 5 min) (this step did not result in any loss of

Fig. 1. *Distribution of inhibitory activity towards protohaem ferro-lyase and radioactivity in fractions eluted from Sephadex LH-20*

Male mice were injected with 5-amino[4- ^{14}C]laevulinate (10 μ Ci/kg, intraperitoneally); 2 h later they were given either 3,5-diethoxycarbonyl-1,4-dihydrocollidine (200 mg/kg in arachis oil) or oil alone and were killed after a further 2.5 h. Acid/acetone extracts were prepared from the pooled liver homogenates of 12 control (●) and 12 treated (○) mice (each equivalent to 6 g wet wt. of liver) and chromatographed on Sephadex LH-20 as described in the Materials and Methods section; 40 3 ml fractions were collected. The approximate amount of material absorbing at approx. 400 nm was determined by using the wavelength pairs given in the Materials and Methods section and is given in arbitrary units (a), together with the radioactivity (b) and inhibitory activity (c) recovered in each fraction.

inhibitory activity). The crude pigment was then subjected to t.l.c., and the green band (R_F 0.49), which fluoresced red under u.v., was eluted and its inhibitory activity on protohaem ferro-lyase related to both radioactivity and Soret absorbance at 411 nm. The latter was expressed as nmol of protoporphyrin equivalents, by using the published ϵ values (Smith, 1975) for protoporphyrin dication. After t.l.c. a slight loss of inhibitory activity was seen (when related to radioactivity) and a similar loss of Soret absorbance (with respect to radioactivity) was also seen, so that the inhibitory activity per unit of Soret absorbance did not change. In two experiments units of inhibitor per nmol of protoporphyrin equivalents were as follows (averages with individual observations in paren-

theses): (a) applied on plate, 29.75 (29.7, 29.8); (b) recovered after t.l.c., 30.5 (34.0, 27.1). This strongly suggests that the inhibitor of protohaem ferro-lyase is the green pigment and that a small amount of the pigment undergoes degradation and loss of inhibitory activity during the purification. The crude pigment was also found to be labile when stored at +4°C in the dark, with losses of up to half its original inhibitory activity overnight. It could be stored without loss of activity for at least 1 month at -70°C dissolved in either acid/acetone or dimethyl sulphoxide.

In contrast with the labelling of the inhibitory pigment by a precursor of haem, when the ¹⁴C-labelled drug was used to stimulate production of the inhibitory green pigment *in vivo*, no significant radioactivity could be found associated with the purified inhibitory pigment (Table 1). Most of the radioactivity present in the liver homogenate could be recovered in the acetone extracts, and only 0.6% of the total radioactivity was found in the green-pigment fractions from Sephadex LH-20 column chromatography: this radioactivity was some 5-fold less than expected if the drug were bound to the pigment in a 1:1 stoichiometry, and could be further decreased markedly by t.l.c. of the green pigment. It can therefore be concluded that during the induced formation of the green pigment from liver haem the drug (or at least its pyridine ring) does not become irreversibly bound to the porphyrin nucleus of the inhibitor.

The inhibitory pigment produced by treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine has

been shown to exhibit (as the free base) an aetio-type spectrum, with all absorption maxima shifted towards the red, as compared with those of protoporphyrin (Tephly *et al.*, 1979). Green porphyrin pigments with similar spectral characteristics have also been described after treatment with several drugs containing unsaturated substituents, such as 2-allyl-2-isopropylacetamide (McDonagh *et al.*, 1976; Unseld & De Matteis, 1976), secobarbitone (De Matteis & Cantoni, 1979) and ethynyl-substituted compounds (Ortiz de Montellano *et al.*, 1979). The green pigments produced by treatment with 2-allyl-2-isopropylacetamide, secobarbitone or ethynylcyclohexanol could also be obtained by the same technique of acetone/HCl extraction followed by Sephadex LH-20 column chromatography, but, in contrast with those obtained after treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine, they did not inhibit significantly protohaem ferro-lyase activity of mouse liver mitochondria (Table 2).

Studies on the inhibition of protohaem ferro-lyase activity

The green pigment obtained from the livers of mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine was found to inhibit the activity of protohaem ferro-lyase not only when mesoporphyrin and Co²⁺ were substrates, but also when the enzyme was assayed by measuring the incorporation of either ⁵⁹Fe or Co²⁺ into protoporphyrin (Table 3).

Further experiments were carried out in order to investigate the mechanism by which the green

Table 1. Recovery of radioactivity from intraperitoneally injected 3,5-diethoxycarbonyl-1,4-dihydro[2,6-¹⁴C]collidine in the liver homogenate of treated mice and in green pigments isolated and purified from their livers

Mice were treated and their liver extract was chromatographed on Sephadex LH-20 columns as indicated in the Materials and Methods section. The green-pigment fraction was further purified by separating out lipids at low temperature and subsequently by t.l.c. The radioactivity of each fraction was determined and expressed as pmol of drug equivalents. The Soret (411 nm) absorbance of the green pigments was also determined and expressed as pmol of protoporphyrin equivalents by using the published ϵ value (Smith, 1975) for the Soret absorbance of protoporphyrin dication. All values given refer to the pooled fractions obtained from 6 g original wt. of liver. Results given were obtained with the pooled livers of 12 mice and were confirmed once.

Fraction	Radioactivity (pmol of drug equivalents) (A)	Soret absorbance of green pigment (pmol of proto- porphyrin equivalents) (B)	A/B ratios
Total homogenate	414 000	—	—
Acetone extracts	296 000	—	—
Acetone/HCl extracts (applied on LH-20)	27 000	—	—
Green-pigment fractions from LH-20	2340	11 900	0.20
Same, defatted (applied to t.l.c. plate)	1820	10 140	0.18
Green pigment eluted from t.l.c. plate*	54	7800	0.007

* This fraction was also assayed for inhibition of protohaem ferro-lyase and found to be markedly active (25.4 units of inhibitor/nmol of protoporphyrin equivalent).

Table 2. *Effect of treating rats or mice with either secobarbitone [5-allyl-5-(1-methylbutyl)barbituric acid], 2-allyl-2-isopropylacetamide or 1-ethynylcyclohexanol on the concentration of green porphyrin pigments and of inhibitor of protohaem ferro-lyase activity in their liver: comparison with the effects of 3,5-diethoxycarbonyl-1,4-dihydrocollidine*

Fed rats or mice were given a single injection of drug either intraperitoneally (i.p.) or subcutaneously (s.c.) and were killed at the times indicated. Acetone/HCl extracts of their livers were prepared and chromatographed on Sephadex LH-20 as indicated in the Materials and Methods section, and the green-pigment fractions were assayed for inhibitory activity on protohaem ferro-lyase. The Soret absorbance of the green-pigment fractions was also determined and the amount of green pigments expressed as nmol of protoporphyrin equivalents, as indicated in the legend to Table 1: because of an impurity absorbing in the Soret region, these values can only be taken as approximate, especially when small amounts of green pigments were present. Results given are those of an individual observation obtained with pooled livers of five mice and of at least two rats.

Species	Treatment and dose (ml or mg/kg)	Time of killing (after treatment)	Green pigments (nmol of protoporphyrin equivalents/g of liver)	Inhibitor (units)	
				(per g of liver)	(per nmol)
Mouse	Arachis oil, 10 ml i.p.	1 h	0.3	3.6	12
	3,5-Diethoxycarbonyl-1,4-dihydrocollidine, 10 mg i.p.	1 h	1.62	37.0	22.8
	Secobarbitone (sodium salt), 50 mg i.p.	2.5 h	2.89	4.0	1.38
	Secobarbitone (sodium salt), 100 mg i.p.	1.5 h	2.59	1.5	0.58
Rat	Arachis oil, 10 ml i.p.	1 h	Traces	0.42	—
	3,5-Diethoxycarbonyl-1,4-dihydrocollidine, 25 mg i.p.	1 h	0.46	7.2	15.6
	*1-Ethynylcyclohexanol, 50 mg i.p.	4 h	3.84	1.27	0.33
	*2-Allyl-2-isopropylacetamide, 400 mg, s.c.	2 h	13.30	1.09	0.08

* In order to increase the yield of green pigments these rats had been given phenobarbitone sodium (1 mg/ml) in their drinking water for a week before further treatment.

Table 3. *Effect of green pigments isolated from the liver of mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine on the incorporation of ^{59}Fe into protoporphyrin and of Co^{2+} into either protoporphyrin or mesoporphyrin*

For this experiment, 12 male LACA mice were injected with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (200 mg/kg intraperitoneally), killed 2.5 h later and their livers pooled. The green pigments were obtained by Sephadex LH-20 chromatography, evaporated to dryness and dissolved in dimethyl sulphoxide. To study their effect on the incorporation of ^{59}Fe into protoporphyrin, 4 ml of 10% (w/v) rat liver homogenate was preincubated at 37°C for 15 min with 0.1 ml of dimethyl sulphoxide with or without 50 units of inhibitor, then protoporphyrin was added and, 2 min later, [^{59}Fe]ferric citrate: the incubation was stopped after further 15 min incubation and the radioactivity of the isolated haemin determined. The incorporation of Co^{2+} into either protoporphyrin or mesoporphyrin was determined by dual-wavelength spectrophotometry (Jones & Jones, 1969) by using mouse liver mitochondria preincubated for 15 min with 5 units of inhibitor, under the standard conditions described in the Materials and Methods section. With protoporphyrin, care was taken to subtract from the rate obtained after addition of Co^{2+} a cobalt-independent rate (approx. 20% of total rate), which proved resistant to the addition of the inhibitor.

Addition	Incorporation of ^{59}Fe into protoporphyrin (c.p.m. recovered in haem)	Incorporation of Co^{2+} (nmol of porphyrin/min per ml of assay mixture) into:	
		Mesoporphyrin	Protoporphyrin
Dimethyl sulphoxide	106 500	3.37	0.34
Green pigment inhibitor in dimethyl sulphoxide	47 000	0.46	0.11
Inhibition (%)	55.9	86.4	67.7

pigment inhibits protohaem ferro-lyase activity. When the green pigment was preincubated with mouse liver mitochondria before assay of the protohaem ferro-lyase activity, the degree of inhibition of the enzyme was found to depend on the temperature of preincubation (less inhibition being observed on preincubation at 0°C than at 37°C) and also, with large amounts of inhibitor, on the time

of preincubation (Fig. 2). When a first period of preincubation at 0°C was followed by a second at 37°C, the inhibition finally attained was the same as on incubation at 37°C from the start: so the inhibitor was still potentially active at 0°C, but did not inhibit the enzyme as effectively as at 37°C. These findings suggest that either the access of the inhibitor to the enzyme or the inhibition process

Fig. 2. Effect of preincubating mouse liver mitochondria with the green-pigment inhibitor on their protohaem ferro-lyase activity

Mitochondria were preincubated at 0°C or at 37°C with the green-pigment inhibitor obtained by Sephadex LH-20 chromatography from the liver of mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine. After different times of preincubation, mitochondria were transferred into a spectrophotometric cell containing mesoporphyrin and their protohaem ferro-lyase activity was determined after addition of Co^{2+} . Preincubations were with 1 unit of inhibitor at 0°C (Δ) or at 37°C ($\blacktriangle$), with 3 units of inhibitor at 0°C ($\square$) or at 37°C ($\blacksquare$), or with dimethyl sulphoxide alone at 37°C ($\bullet$). Samples indicated thus (O) were first preincubated for 10 min at 0°C, then for a further 10 min at 37°C.

itself is temperature-dependent. The majority of the enzyme activity is inhibited rapidly, requiring relatively small amounts of inhibitor; but there may be a small amount of enzyme activity, which is inhibited more slowly and requires larger amounts of inhibitor.

This interpretation is supported by the results of a dose-response experiment (Fig. 3a), which illustrates the effect of preincubation for 15 min of mouse liver mitochondria with increasing amounts of inhibitor at two different stages of purification. With either preparation of the inhibitor, the decline of enzymic activity was related linearly to the amount of inhibitor added, up to approx. 60% inhibition: thereafter larger amounts of inhibitor were required to produce equivalent losses of activity. This finding indicates that, as suggested by Tephly *et al.* (1979), the loss of enzymic activity can be used to estimate the amount of inhibitor present provided that 50% inhibition is not exceeded and that the initial enzymic activity is kept constant. A similar linear relationship was also found between amount of inhibitor added and loss of enzymic activity (up to at least 50% inhibition) with a range of different concentrations of mitochondria (results not shown), and, over the whole range examined, the dose of inhibitor required for 50% inhibition of the enzyme was proportional to the initial enzymic activity (Fig. 3b). This suggests strongly that under these conditions the inhibitor can be used to titrate the enzyme, 1 unit of inhibitor being responsible for the loss of

Fig. 3. Inhibition of mitochondrial protohaem ferro-lyase caused *in vitro* by the green pigment obtained by treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine

Mitochondria were preincubated at 37°C for 15 min with dimethyl sulphoxide containing increasing amounts of the green-pigment inhibitor: the latter was either obtained directly by Sephadex LH-20 chromatography (O) or had been further purified by t.l.c. ($\bullet$). The dose-response obtained with an initial enzyme activity of 3.6 nmol of mesoporphyrin utilized/min per ml of assay incubation mixture is shown in panel (a). Similar linear declines of enzymic activity were obtained when four additional concentrations of mitochondria were tested. In panel (b) the amount of inhibitor required for 50% inhibition of the enzyme is plotted against the initial enzymic activity (i.e. the activity before inhibition).

the same amount of enzymic activity (corresponding to 1.8 nmol of mesoporphyrin utilized/min per ml of assay incubation mixture) regardless of the

Fig. 4. Lineweaver-Burk plots of $1/v$ versus $1/[S]$ for the control and for the inhibited enzyme

Mouse liver mitochondria were preincubated with dimethyl sulphoxide (control enzyme, O) or with 0.51 unit of inhibitor (inhibited enzyme, ●) at 37°C for 15 min; they were then transferred into a spectrophotometric cell and protohaem ferro-lyase activity was determined. In (a) the concentration of mesoporphyrin was varied from 4 to 36 μM and that of Co²⁺ was kept constant at 36 μM. In (b) the concentration of Co²⁺ was varied between 4 and 36 μM, and that of mesoporphyrin was kept constant at 36 μM. The $1/v$ values are expressed as (μM-mesoporphyrin utilized/min)⁻¹.

concentration of mitochondrial protein and of the total amount of enzyme present.

Once the inhibition was established, addition of the porphyrin substrate necessary to assay protohaem ferro-lyase activity could not reverse it, even though the concentration of substrate exceeded that of the inhibitor by over 1000-fold on a molar basis. Thus the concentration of mesoporphyrin in the assay mixture was 36 μM, whereas the concentration of green pigment necessary to inhibit the enzyme by 50% under standard conditions was calculated to be approx. 8.5 nM [in these calculations the Soret (412 nm) ε of protoporphyrin dication (Smith, 1975) was used and also the specific-activity value of the green pigment (inhibitor units/Soret absorbance) obtained after purification by t.l.c. (see Table 1)]. The inhibited enzyme did not display any change in K_m value for either mesoporphyrin or Co²⁺ from those seen with the uninhibited enzyme (Fig. 4: 2.3 μM and 6.7 μM respectively). K_m values previously reported for liver mitochondria protohaem ferro-lyase are as follows: mesoporphyrin, 1.9 μM (Honeybourne *et al.*, 1979); Co²⁺, 8.0 μM (Bugany *et al.*, 1971).

When the porphyrin substrate was added before the inhibitor, significant protection of the enzyme was obtained (Table 4), suggesting that the inhibitor and the porphyrin substrate may compete with each other for the active centre of the enzyme. No protection was afforded by preincubating the enzyme with its metal substrate, Co²⁺ (results not shown).

Discussion

The present work has confirmed the previous finding (Tephly *et al.*, 1979) that a porphyrin-like

Table 4. Effect of preincubating mouse liver mitochondria with mesoporphyrin on the incubation of protohaem ferro-lyase caused by the green pigment *in vitro*

Mouse liver mitochondria were preincubated at 37°C for 3 min with 0.167 M-Tris/HCl buffer, pH 8.2, containing mesoporphyrin (141 μM) and Tween 80 (0.29%) in a total volume of 0.7 ml. After 3 min, 50 μl of dimethyl sulphoxide alone or with 1 unit of inhibitor was added and the incubation was continued for further 15 min; a 0.5 ml sample was then transferred to a spectrophotometric cell for assay of the enzyme in the presence of mesoporphyrin (44 μM) and Co²⁺ (36 μM). In control samples only Tween was added at the start of the preincubation and mesoporphyrin was added 15 min after the inhibitor. Results given were the averages of two observations (individual values in parentheses) and were confirmed in a separate experiment.

Preincubation		Protohaem ferro-lyase activity	
First addition	Second addition	(nmol/min per ml)	(% inhibition)
Tween 80	Dimethyl sulphoxide	2.8	57.0
		(2.86, 2.74)	
		1.20	
Tween 80	Inhibitor + dimethyl sulphoxide	(1.17, 1.24)	21.3
		2.89	
		(2.78, 3.00)	
Mesoporphyrin + Tween	Dimethyl sulphoxide	2.27	21.3
		(2.18, 2.37)	
		2.27	
Mesoporphyrin + Tween	Inhibitor + dimethyl sulphoxide	(2.18, 2.37)	21.3
		2.27	
		(2.18, 2.37)	

pigment with strong inhibitory properties towards protohaem ferro-lyase can be isolated from the liver of mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine. Labelling experiments with 5-amino[4- ^{14}C]laevulinate suggest strongly that the abnormal porphyrin inhibitor originates from turnover of liver haem.

No labelling of the inhibitory pigment could be demonstrated from ^{14}C -labelled drug, in contrast with the findings obtained with the green porphyrins produced by 2-allyl-2-isopropylacetamide or by ethynyl-substituted drugs, where covalent binding of the respective drug has been reported (Ortiz de Montellano *et al.*, 1978, 1979). The green pigments produced by treatment with 2-allyl-2-isopropylacetamide, secobarbitone or 1-ethynylcyclohexanol have now been isolated in a partially purified form, but none of them has been found to inhibit significantly protohaem ferro-lyase activity (Table 2). This suggests that even though all these abnormal green pigments originate from liver haem and share some spectral characteristics, the detailed mechanism of their production and their structure are not necessarily the same. These aspects require further study.

The inhibition of protohaem ferro-lyase by the green pigments *in vitro* was progressive, could be slowed down by cooling and partially prevented by preincubating the enzyme with the porphyrin substrate, before addition of the inhibitor. Once established, the inhibition could not be reversed by the addition of the porphyrin substrate, and the inhibited enzyme did not show any change in K_m values for either mesoporphyrin or Co^{2+} . The following tentative interpretation can be put forward for these findings: the inhibition process may be a suicidal type of inactivation reaction, involving irreversible interaction of the inhibitor with the active centre of the enzyme and requiring enzymic turnover.

An attempt was made to estimate the amount of the porphyrin inhibitor from the Soret absorption of its porphyrin-dication spectrum, by using the published value for protoporphyrin. On this basis the inhibitor is very active, a 50% inhibition of the enzyme being obtained under standard conditions with an approx. 8 nM concentration of the pigment.

An interesting finding was that 1 pmol of green-pigment inhibitor was responsible for the loss of the same amount of enzymic activity (corresponding to approx. 60 pmol of mesoporphyrin utilized/min per ml of assay incubation mixture), regardless of the

concentration of mitochondrial protein and of total amount of enzyme present. This is compatible with a mechanism of irreversible inactivation and suggests that the inhibitor is a sensitive and selective reagent for the enzyme and may be used to titrate the number of its active centres.

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