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Studies on the inhibition of ferrochelatase by *N*-alkylated dicarboxylic porphyrins

Steric factors involved and evidence that the inhibition is reversible

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1. The structural requirements for the inhibition of ferrochelatase by *N*-alkylated porphyrins were investigated and experiments carried out to explore the mechanism of enzyme inhibition. 2. Three dicarboxylic porphyrins, all substrates of the enzyme, are strongly inhibitory when *N*-alkylated; in contrast, uroporphyrin and coproporphyrin (which are not substrates) do not inhibit after *N*-alkylation. Free carboxylic acid functions are required for inhibition, as the methyl ester derivatives are not themselves inhibitory. 3. Porphyrins bearing the alkyl group on the pyrrole nitrogen of rings C and D are less effective inhibitors, particularly when zinc is chelated in the centre of the tetrapyrrole or the *N*-alkyl group is relatively large in size. 4. The substituents at the 2- and 4-positions of the porphyrin system may also affect the inhibitory activity, particularly for the isomers with ring C and D alkylated. 5. The zinc chelates of several *N*-alkylprotoporphyrins are inhibitory towards haem oxygenase, another haem-binding enzyme, and also in this case increasing the size of the alkyl group decreased the inhibitory activity, particularly for isomers with ring C or D alkylated. 6. The inhibition could be reversed by prolonged incubation with excess porphyrin substrate, but dealkylation of the *N*-alkylporphyrin during enzyme inhibition could not be demonstrated. 7. It is concluded (*a*) that *N*-alkylated dicarboxylic porphyrins compete reversibly with the porphyrin substrate for the enzyme active site and (*b*) that the structural and steric factors discussed above affect the inhibitory activity by modifying the affinity of the *N*-alkylporphyrin inhibitor for the enzyme.

A powerful inhibitor of ferrochelatase (protohaem ferrolyase, EC 4.99.1.1) has been isolated from the liver of mice given 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (Tephly *et al.*, 1979) and identified as *N*-methylprotoporphyrin (De Matteis *et al.*, 1980*a*; Ortiz de Montellano & Kunze, 1981; Tephly *et al.*, 1981). Synthetic *N*-methylprotoporphyrin has also been shown to inhibit ferrochelatase in crude preparations from liver (De Matteis *et al.*, 1980*a*; Ortiz de Montellano *et al.*, 1980) and micro-organisms (Houghton *et al.*, 1982; Brown *et al.*, 1982), and also the purified bovine enzyme (Dailey & Fleming, 1983).

Evidence has been obtained to suggest that in order to inhibit ferrochelatase the *N*-methylated porphyrins must bind to the porphyrin-binding site of the enzyme (De Matteis *et al.*, 1980*b*,*c*), but the detailed mechanism of the inhibitory effect has not yet been elucidated. It has been proposed that *N*-methylprotoporphyrin may act as a tight-binding inhibitor (De Matteis *et al.*, 1982*a*; Dailey & Fleming, 1983) capable of interacting with the active site of ferrochelatase with great affinity, but unable to accept the second substrate of the enzyme, a bivalent metal ion, for normal (i.e. tetra-co-ordinate) incorporation into the pyrrolic macrocycle (De Matteis *et al.*, 1982*a*). However, the alternative possibility has also been considered (De Matteis *et al.*, 1980*b*) that, once bound to the active site of ferrochelatase, *N*-methylprotoporphyrin may lead to its irreversible modification (perhaps by transferring its *N*-methyl group to the enzyme; see below).

In the present paper we provide additional evidence that the inhibitor interacts with the porphyrin-binding site of ferrochelatase. Steric factors (such as size and position of the *N*-alkyl group) that decrease the inhibitory activity of a *N*-

alkylated porphyrin probably do so by altering its affinity for the enzyme. Evidence has been obtained that the inhibition can be reversed by prolonged incubation with excess porphyrin substrate, but attempts to demonstrate that the *N*-alkylporphyrin inhibitor is dealkylated during enzyme inhibition were not successful. We conclude, in agreement with the results of a kinetic study (Dailey & Fleming, 1983), that *N*-alkylated dicarboxylic porphyrins are tight-binding inhibitors of ferrochelatase, and that they compete reversibly with the porphyrin substrate for the enzymic active centre.

Materials and methods

Source of special chemicals

Bis-(*p*-nitrophenyl) phosphate was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.; uroporphyrin I and coproporphyrin III methyl esters were from Porphyrin Products, Logan, UT, U.S.A.; deuteroporphyrin IX and mesoporphyrin IX methyl esters were from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and protoporphyrin IX dimethyl ester was from Sigma Chemical Co., Poole, Dorset, U.K. Haemin was kindly given by Dr. S. Sassa, Rockefeller University, New York, NY, U.S.A. Methanol and dichloromethane (Rathburn Chemicals), hexane (Fisons Scientific Apparatus) and tetrahydrofuran (Chromatographic Services) were all h.p.l.c. grade. Tetrahydrofuran was made peroxide-free and dry, immediately before use, by filtration through aluminium oxide (type UG; Koch–Light Laboratories), previously activated by heating at 100°C overnight.

Synthesis, isolation and characterization of N-alkylated porphyrins

These were prepared by dissolving the corresponding parent porphyrin in the appropriate alkyl iodide (BDH Chemicals, Poole, Dorset, U.K.) and heating at 100°C for 4h (30h for 1-iodopropane) in tubes with polytetrafluoroethylene-lined screw caps; they were purified by repeated t.l.c. on silica gel with a developing system of chloroform/methanol (20:3, v/v) and identified by the characteristic bathochromic shifts of all absorption maxima (De Matteis *et al.*, 1982*c*; Smith & Farmer, 1982). Resolution of the various porphyrins into isomeric fractions was attained by h.p.l.c. with a Nucleosil 5 column (4.6mm × 250mm), isocratic elution with dichloromethane/methanol/conc. NH_3 (sp.gr. 0.88) (40:60:0.3, by vol.) and a flow rate of 1.5ml/min. The individual structural isomers of *N*-methyl- and *N*-ethyl-protoporphyrin IX were obtained by the h.p.l.c. method of Ortiz de Montellano & Kunze (1981) and Ortiz de Montellano *et al.* (1981*b*), which separates all structural isomers in the following order of elution: N_B-, N_A-, N_C- and N_D-alkylated pigments, the suffixes A–D indicating the pyrrole ring that is *N*-alkylated in a given isomer (see Fig. 1). The positions of the *N*-alkyl groups in the two isomeric fractions of *N*-methyl- and *N*-ethyl-mesoporphyrin IX were assigned by comparison of their h.p.l.c. properties with those of *N*-alkylmesoporphyrin derivatives obtained by subjecting known isomeric fractions of *N*-alkylprotoporphyrins to catalytic hydrogenation as follows. *N*-Alkylprotoporphyrin (approx. 200 nmol) was dissolved in 2ml of formic acid and, after being flushed with N_2 for 2min and the addition of 17mg of 5% palladium on charcoal, was allowed to react at room temperature in an atmosphere of H_2 for 5min. After adjustment of the pH of the filtrate to 7.5 with conc. NH_3, the product was transferred to chloroform, in which solvent it exhibited an aetiotype spectrum (Falk, 1964) identical with that of *N*-alkylmesoporphyrin; the methyl ester derivatives were then subjected to h.p.l.c. Catalytic hydrogenation could only be carried out successfully with the $N_C + N_D$ isomeric fractions of *N*-methyl- and *N*-ethyl-protoporphyrin. On similar treatment of the corresponding $N_A + N_B$ isomers, no pigment could be recovered in the filtrate.

Cu^{2+}-catalysed dealkylation of N-alkylporphyrin dimethyl esters

This was studied at 24°C in stoppered absorption cells with the use of a Varian 2200 spectrophotometer. To a chloroform solution of the porphyrin methyl ester (2.5ml, containing approx. 3nmol), 10μl of a methanolic solution of 0.1% (w/v) cupric acetate monohydrate was added, and the change in the spectrum of the porphyrin caused by copper addition was monitored over a period of time. After the incorporation of copper was complete (as judged from disappearance of band IV of the *N*-alkylporphyrin), the subsequent rate of dealkylation was measured by the increase in absorbance of a new Soret-band maximum characteristic of the dealkylated product.

Assay of ferrochelatase and of haem oxygenase: inhibition of either enzyme by N-alkylated pigments

Mouse liver mitochondria prepared as described previously (Tephly *et al.*, 1979) were kept at −70°C and thawed immediately before use. The methods used for assay of ferrochelatase and inhibition of the enzyme by *N*-alkylporphyrins have also been given (Tephly *et al.*, 1979). When mitochondria were pre-incubated with detergent, as in the experiments of Fig. 2 and Table 4, Tween 80 was present in the incubation mixture at a concentration of 0.5% (v/v). Haem oxygenase was assayed by a slight modification of the method of

Schacter *et al.*, (1972) with haemin (33.3 μM) as substrate. A post-mitochondrial supernatant [from the liver of rats given $CoCl_2$ 16h before being killed (Maines & Kappas, 1974)] equivalent to 100 mg wet wt. of liver/ml of incubation mixture was used as a source of haem oxygenase. Dimethyl sulphoxide (25 μl/ml) was added containing the zinc complex of various *N*-alkylprotoporphyrin free carboxylates, and after a 10min pre-incubation the reaction was started by the addition of NADPH and the initial linear rate determined.

Results and discussion

Characterization of chromatographic fractions of N-alkylated porphyrins

Table 1 shows the chromatographic behaviour of several *N*-alkylated porphyrin methyl esters on an h.p.l.c. system. All dicarboxylic porphyrins could be resolved into two fractions, F_1 and F_2, each containing two structural isomers, complete resolution of the four structural isomers being only achieved in this system for the *N*-alkylated mesoporphyrins. The isomeric compositions of the two fractions of *N*-methyl- and *N*-ethyl-protoporphyrin were determined by further h.p.l.c. studies, with the system of Ortiz de Montellano & Kunze (1981), which separates all four structural isomers in a known order of elution. The composition of the *N*-methyl- and *N*-ethyl-mesoporphyrin fractions was established by converting the F_2 fractions of the *N*-alkylated protoporphyrins into the corresponding mesoporphyrins by catalytic hydrogenation. The retention times were found to be influenced more by the porphyrin moiety than by the alkyl group present on the pyrrole nitrogen, and the order of elution of the two isomeric pairs [(N_A+N_B) and (N_C+N_D)] was found to be inverted in the mesoporphyrin series compared with the protoporphyrin series. The composition of the two chromatographic fractions of *N*-n-propyl-protoporphyrin, *N*-n-propylmesoporphyrin and *N*-methyldeuteroporphyrin was not established. In the discussion that follows, the assumption is made that for the *N*-propylated porphyrins the order of elution of the isomeric pairs is the same as in the corresponding *N*-methylated and *N*-ethylated compounds.

Inhibition of ferrochelatase by N-substituted dicarboxylic porphyrins; importance of size and position of the N-alkyl group and role of the position-2 and position-4 peripheral substituents of the porphyrin ring

The inhibitory activity on ferrochelatase of mouse liver mitochondria exhibited by several *N*-alkylated porphyrins is shown in Table 2. Only porphyrins with two carboxylic acid functions were found to be markedly inhibitory. However, as the *N*-alkyl group increased in size from methyl to n-propyl, the inhibitory activity of the N_C+N_D isomeric fraction of protoporphyrin and mesoporphyrin decreased markedly. A marked difference in inhibitory activity between fractions F_1 and F_2 of *N*-methyldeuteroporphyrin and of the zinc complex of *N*-methylmesoporphyrin and *N*-methylprotoporphyrin was also found, and for the latter two pigments, the isomeric composition of which is known, again the N_C+N_D isomeric fractions

Table 1. *Retention times of several N-alkylated porphyrin methyl esters on an h.p.l.c. system and isomeric composition of the resulting chromatographic fractions*

The various porphyrins listed were obtained by alkylating the corresponding parent porphyrin methyl ester and, after purification by t.l.c., they were chromatographed on an h.p.l.c. system as indicated in the Materials and methods section. All dicarboxylic porphyrins could be separated into two chromatographic fractions, and, in the case of the alkylated mesoporphyrins, each of these was further resolved into two subfractions representing an individual structural isomer. The isomeric composition of the two fractions was established, for alkylated protoporphyrins, by their chromatographic behaviour on the h.p.l.c. system of Ortiz de Montellano & Kunze (1981), and, for mesoporphyrins, by catalytic hydrogenation of an isomeric fraction of the corresponding alkylated protoporphyrin (see the Materials and methods section).

Porphyrin	Retention time (min)		Order of elution of structural isomers
	Fraction F_1	Fraction F_2	
N-Methyluroporphyrin I	5.14		–
N-Methylcoproporphyrin III	2.01		–
N-Methylprotoporphyrin IX	2.95	3.9	N_A+N_B; N_C+N_C
N-Ethylprotoporphyrin IX	2.95	4.1	N_A+N_B; N_C+N_D
N-n-Propylprotoporphyrin IX	2.88	3.9	Unknown
N-Methylmesoporphyrin IX	9.38, 9.75	14.22, 14.8	N_C+N_D, N_B+N_A
N-Ethylmesoporphyrin IX	11.28, 11.95	17.73, 18.76	N_C+N_D, N_B+N_A
N-n-Propylmesoporphyrin IX	10.69, 11.36	16.73, 17.76	Unknown
N-Methyldeuteroporphyrin IX	4.7	5.3	Unknown

Table 2. *Inhibition of ferrochelatase activity of mouse liver mitochondria produced by several N-alkylated porphyrins in vitro*
The *N*-alkylated porphyrins were first purified by t.l.c. and then chromatographed on an h.p.l.c. system by using the methyl ester derivatives, as described in the Materials and methods section. Where two main isomeric fractions were obtained (F_1 and F_2 in order of elution, see Table 1), both were collected and, after hydrolysis of the methyl esters, the *N*-substituted porphyrins were tested for inhibitory activity on ferrochelatase *in vitro* (Tephly *et al.*, 1979) as the free porphyrins (A) or, in some cases, as the zinc chelate derivatives (B). Inhibitory activity is expressed as units (De Matteis *et al.*, 1980*b*) per nmol of pigment calculated from the Soret absorption by using the published ε value (De Matteis *et al.*, 1982*b*) for *N*-methylprotoporphyrin and its zinc complex. Results given are averages ±S.E.M. for three or four observations or are individual observations each obtained with at least four concentrations of the inhibitory porphyrin (see Tephly *et al.*, 1979).

Pigment tested	Inhibitory activity (units/nmol) Fraction F_1	Fraction F_2
(*a*) Free porphyrins		
N-Methyluroporphyrin I	<0.05	
N-Methylcoproporphyrin III	0.17	
N-Methyldeuteroporphyrin IX	0.32±0.16	8.78±0.28
N-Methylmesoporphyrin IX	15.04±0.46	8.01±0.36
N-Ethylmesoporphyrin IX	0.14	8.17
N-n-Propylmesoporphyrin IX	<0.05	7.85
N-Methylprotoporphyrin IX	9.24±0.20	10.75±0.34
N-Ethylprotoporphyrin IX	6.67±0.30	5.86±0.65
N-n-Propylprotoporphyrin IX	6.06	0.11
(*b*) Zinc complexes		
N-Methylmesoporphyrin IX	0.5	6.96
N-Methylprotoporphyrin IX	15.7	2.1

were much less inhibitory than the corresponding N_A+N_B-isomer-containing fractions. These findings suggest that both size and position of the *N*-alkyl group are important for the inhibitory activity of a *N*-alkylated dicarboxylic porphyrin (see also De Matteis *et al.*, 1980*c*; Ortiz de Montellano *et al.*, 1981*a*). They are compatible with the concept (De Matteis *et al.*, 1982*b*) that when one of the propionic acid-substituted rings is alkylated (as is the case in the N_C and N_D isomers) then, because the *N*-alkylated ring is significantly tilted out of the porphyrin plane, the alignment of the two propionic acid chains with respect to each other may be altered. As a consequence, the binding of the modified porphyrin by the chelatase may be impaired, particularly when the *N*-alkyl group is large in size or a metal is present in the centre of the porphyrin macrocycle, as in both cases the distortion of the *N*-alkylated ring would be expected to be greater. An exception to this concept is, however, the finding that *N*-methylmesoporphyrin fraction F_1 (N_C+N_D isomers) is much more active than the F_2 fraction (Table 2), suggesting that other factors must also be involved.

The findings given in Table 2 also indicate that the nature of the substituent at the 2 and 4 positions at the periphery of a dicarboxylic porphyrin (Fig. 1) may also be important for the inhibition of ferrochelatase by *N*-alkylated porphyrins, and, in particular, by their N_C and N_D isomers. In deuteroporphyrin two hydrogen atoms are present

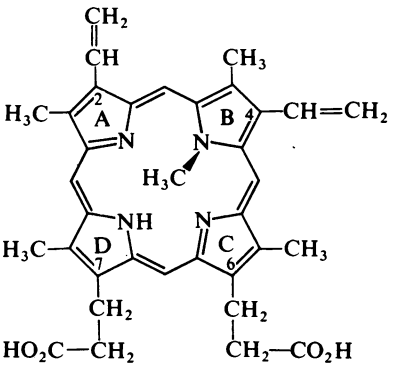

Fig. 1. *Structure of N-methylprotoporphyrin IX*
Four structural isomers of this porphyrin are possible, depending on which pyrrole nitrogen is alkylated. The isomer shown is N_B, where ring B is *N*-methylated. In deuteroporphyrin IX and mesoporphyrin IX the two vinyl groups in position 2 and 4 of protoporphyrin are both substituted by two hydrogen atoms and two ethyl groups respectively.

at the 2- and 4-positions of the porphyrin system. If the F_1 fraction of *N*-methyldeuteroporphyrin contains the N_C+N_D isomers (this has not yet been established), then a loss of inhibitory activity is already observed when a methyl substituent is present on the nitrogen of rings C and D of this porphyrin, whereas with mesoporphyrin and proto-

porphyrin (where two ethyl/vinyl groups are present at the 2- and 4-positions) loss of activity was only seen when the *N*-substituent increased in size to an ethyl and n-propyl respectively. These findings suggest that the vinyl groups of protoporphyrin and to a lesser extent the ethyl groups of mesoporphyrin may be involved in the binding of the porphyrin at the active site, as they apparently counteract the negative influence of ring distortion and loss of alignment of the two propionate side chains, which have been discussed above. It is significant to point out in this connection that, although the unmodified protoporphyrin, mesoporphyrin and deuteroporphyrin are all substrates of ferrochelatase, they are bound by the enzyme with decreasing affinity, as measured by their respective K_m values (Honeybourne *et al.*, 1979).

Experiments with haem oxygenase

Haem oxygenase, the rate-limiting enzyme of haem degradation, can also accept dicarboxylic porphyrins (as metal complexes) at its active site, and in this case also the two propionic acid side chains in positions 6 and 7 (Fig. 1) appear to be involved in binding (Frydman *et al.*, 1981). The iron complexes (or haems) are substrates of the enzyme and undergo conversion into bile pigments (Frydman *et al.*, 1981); in contrast, the zinc complexes cannot be degraded, but will inhibit the enzymic reaction by competing for binding with the normal substrate, haem (Maines, 1981; Yoshinaga *et al.*, 1982). Table 3 shows that the zinc complexes of several *N*-alkylated protoporphyrins all inhibit haem oxygenase, and here again a large *N*-alkyl group will decrease the inhibitory activity, especially when it is present on the C and D rings. Similar ranking of inhibitory activity was found with the corresponding *N*-alkylmesoporphyrin–zinc complexes (results not shown). The findings are also compatible with size and position of the *N*-alkyl group affecting the affinity of the alkylated porphyrin for a haem-like binding site, as has been discussed above.

N-Methylprotoporphyrin methyl ester is not itself an inhibitor of ferrochelatase

An apparent exception to the concept that, in order to inhibit ferrochelatase, an *N*-alkylated porphyrin must first bind the porphyrin-binding site of the enzyme is the finding from two laboratories (Houghton *et al.*, 1982; Brown *et al.*, 1982) that the methyl ester of *N*-methylprotoporphyrin is also markedly inhibitory. Previous work (Porra & Ross, 1965) had shown that esterification renders dicarboxylic porphyrins inactive as substrates of ferrochelatase; so, if binding of *N*-methylprotoporphyrin to ferrochelatase were involved in the inhibition of the enzyme, its dimethyl ester would be expected to be non-inhibitory.

We have now confirmed that, when the methyl ester of *N*-methyl protoporphyrin is added to a crude ferrochelatase preparation (in our case, liver mitochondria), the enzyme becomes inhibited. However, with the methyl ester there is far less inhibitory activity than with the free acid, and the inhibition develops much more slowly (Fig. 2) and can be almost completely prevented by an inhibitor of carboxylesterases, bis-(*p*-nitrophenyl) phosphate (Table 4). We conclude that the methyl ester is not itself inhibitory and that the inhibition found after its addition is probably due to its hydrolysis by carboxylesterases present in the enzyme preparations.

Dealkylation of N-alkylporphyrins caused by metal incorporation: possible relevance to inhibition of ferrochelatase

When copper acetate was added to a solution of

Table 3. *Inhibition of haem oxygenase from rat liver caused by the zinc chelates of N-methyl- and N-n-propylprotoporphyrins*
Rat liver haem oxygenase from animals pretreated with $CoCl_2$ was assayed *in vitro* as described in the Materials and methods section, and the inhibition caused by the zinc complex of *N*-alkylated protoporphyrins was determined. Results given are the averages ± S.E.M. for three observations. In the assay of haem oxygenase the biliverdin produced is further converted into bilirubin by biliverdin reductase present in excess in the enzyme preparation: no inhibition of the reductase by *N*-methylprotoporphyrin–zinc complex was found (results not given).

Pigment tested (and isomeric type)	Final concn. of inhibitor ...	Inhibition of haem oxygenase (%) 0.25 μM	0.47 μM	1.5 μM
N-Methylprotoporphyrin–zinc complex (N_A)		56.4 ± 1.6	80.6 ± 0.6	–
N-Methylprotoporphyrin–zinc complex (N_D)		25.1 ± 1.6	41.8 ± 1.1	–
N-n-Propylprotoporphyrin–zinc complex ($N_A + N_B$)		30.6 ± 3.2	66.3 ± 4.8	–
N-n-Propylprotoporphyrin–zinc complex ($N_C + N_D$)		–	6.2 ± 1.4	42.1 ± 4.5

Table 4. *Effect of bis-(p-nitrophenyl) phosphate on the inhibition of ferrochelatase caused by N-methylprotoporphyrin dimethyl ester in mouse liver mitochondria*

Mitochondria were incubated in the presence of detergent with and without bis-(*p*-nitrophenyl) phosphate [BNPP, an inhibitor of carboxylesterase (Heymann *et al.*, 1969)] for 30min at 37°C. *N*-Methylprotoporphyrin (N_C+N_D isomeric fraction) was then added, either as the free carboxylate porphyrin (78 pmol) or as the methyl ester (280pmol), and the incubation was continued for a further 15min, when the activity of ferrochelatase was determined. Values given are rates of ferrochelatase activity (in nmol of mesoporphyrin utilized/min per ml), with percentage inhibition caused by *N*-methylprotoporphyrin given in parentheses, and are the averages for triplicate observations of a typical experiment, where the S.E.M. was <4% of the mean value.

Final concn. of BNPP added during pre-incubation (μM)	*N*-Methylprotoporphyrin added ...	Ferrochelatase activity [nmol/min per ml (% inhibition)]		
		None	Free carboxylate	Dimethyl ester
0		3.27	1.93 (41%)	1.68 (49%)
100		3.40	2.11 (38%)	2.91 (14.4%)
500		3.47	2.00 (42.2%)	3.30 (5%)

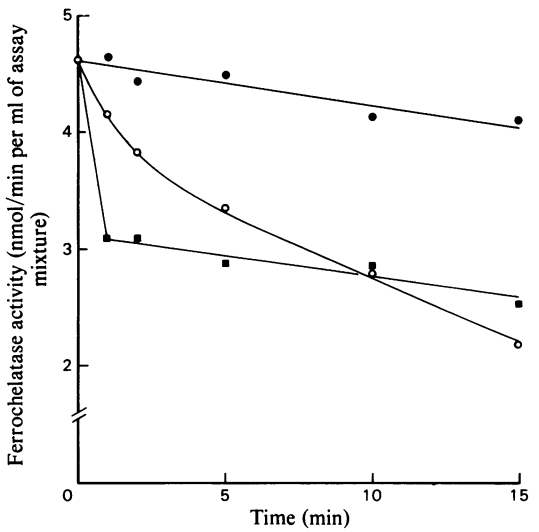

Fig. 2. *Time course of the inhibition of ferrochelatase caused by N-methylprotoporphyrin IX: comparison between the free carboxylic acid and the dimethyl ester*

Mitochondria were incubated for 2 min in pre-warmed incubation mixtures containing Tween 80. Dimethyl sulphoxide was then added either on its own (●) or containing *N*-methylprotoporphyrin (N_D isomer) as the free acid (■, 78pmol) or as the dimethyl ester (○, 280pmol). The incubation was carried out for various times thereafter, and the substrates were then added and the ferrochelatase activity was assayed immediately.

N-methylprotoporphyrin IX dimethyl ester in chloroform, there was a rapid disappearance of the neutral spectrum of the porphyrin (suggesting incorporation of the metal), followed by the gradual appearance of a new spectral species characteristic of the copper chelate of protoporphyrin IX. Similar copper-dependent *N*-dealkylation could be demonstrated with *N*-methyldeuteroporphyrin and *N*-methylmesoporphyrin. The incorporation of copper was apparently necessary for subsequent *N*-demethylation, as, when zinc was first incorporated in the centre of the tetrapyrrole, copper was no longer effective. Although both zinc and copper have been reported to promote demethylation of *N*-methylated model porphyrins in chemical systems (Shears & Hambright, 1970; Lavallee, 1976), zinc required much more vigorous conditions than did copper.

We wondered whether a similar *N*-dealkylation reaction might occur during the attempted metabolism of the *N*-alkylated porphyrin by ferrochelatase, and result in loss of enzyme activity through transfer of the alkyl group to a suitable nucleophile at the enzyme active site. No correlation could be found between copper-catalysed dealkylation of the various *N*-alkylporphyrins in this chemical system and their inhibitory activity on ferrochelatase. For example, both fractions of *N*-n-propylprotoporphyrin were poorly dealkylated, whereas both isomeric fractions of *N*-methylmesoporphyrin and of *N*-methyldeuteroporphyrin were rapidly dealkylated; yet marked differences in inhibitory activity were found between different isomers in all three cases (see Table 2).

Attempts to demonstrate production of the dealkylated porphyrin during inhibition of ferrochelatase by *N*-methylmesoporphyrin (F_2 fraction) were not successful. In this experiment, 1 inhibitory unit of the zinc complex of fraction F_2 above was incubated for 15min with liver mitochondria, and then, after enzyme inhibition had taken place, the mitochondria were extracted with methanol/$HClO_4$ and the fluorescence of the extract was

determined. The zinc complex was used to prevent any ferrochelatase-dependent incorporation of endogenous iron into the tetrapyrrolic system, giving rise to an acid-stable non-fluorescent derivative (mesohaem); the zinc complex was, on the contrary, found to be labile to acid, so that the porphyrin fluorescence could be determined. Mesoporphyrin fluoresces 40 times more intensely than *N*-methylmesoporphyrin, but no increase in fluorescence could be demonstrated after enzyme inhibition, even though the increase expected [as calculated on the basis of a 1:1 porphyrin/enzyme stoichiometry, with the use of M_r and turnover number given by Dailey & Fleming (1983) for the purified liver enzyme] should have been measurable. Therefore there is as yet no evidence to support the hypothesis of alkyl transfer from the porphyrin to the enzyme during the inhibition of ferrochelatase by *N*-alkylated porphyrins. Also, the kinetic data of Dailey & Fleming (1983) do not favour an irreversible inactivation of the enzyme, nor does the finding that the inhibition can be reversed by excess porphyrin substrate, as described below.

Reversal of the inhibition of ferrochelatase by prolonged incubation with excess substrate

When liver mitochondria were first treated with a *N*-alkylated porphyrin so as to inhibit their ferrochelatase activity by 30–40%, a reversal of the inhibition could then be obtained by a further incubation in presence of excess mesoporphyrin. Only a slight recovery of enzymic activity, if any, was found with either isomeric fraction of *N*-methylprotoporphyrin or with the N_A+N_B isomeric fraction of *N*-ethylprotoporphyrin. In contrast, the inhibition caused by the N_C+N_D mixture of either *N*-ethylprotoporphyrin (Fig. 3*b*) or of *N*-n-propylprotoporphyrin (results not shown) were very markedly reversed by the substrate. The inhibition caused by *N*-methyldeuteroporphyrin (F_2 fraction) was also more readily reversed than that of *N*-methylprotoporphyrin (Fig. 3*a*), although in this case the effect was less marked and not so clear-cut. Therefore a reversal of enzyme inhibition was seen after incubation with mesoporphyrin, particularly with those porphyrin inhibitors that either have a large-size alkyl group on the nitrogen of ring C and D or that lack side chains at the 2 and 4 positions of the porphyrin system. This provides more direct support from the concept discussed above that these steric and structural features decrease the affinity of a *N*-alkylporphyrin for the binding site of ferrochelatase. Porphyrins with very low affinity (for example *N*-n-propylprotoporphyrin, isomers N_C and N_D) will not be inhibitory, even when initial rates of enzyme activity are measured and the interval between adding substrate and measuring enzymic rate is kept as short as possible. Others with intermediate affinity will inhibit markedly immediately after the substrate is added, but will then be

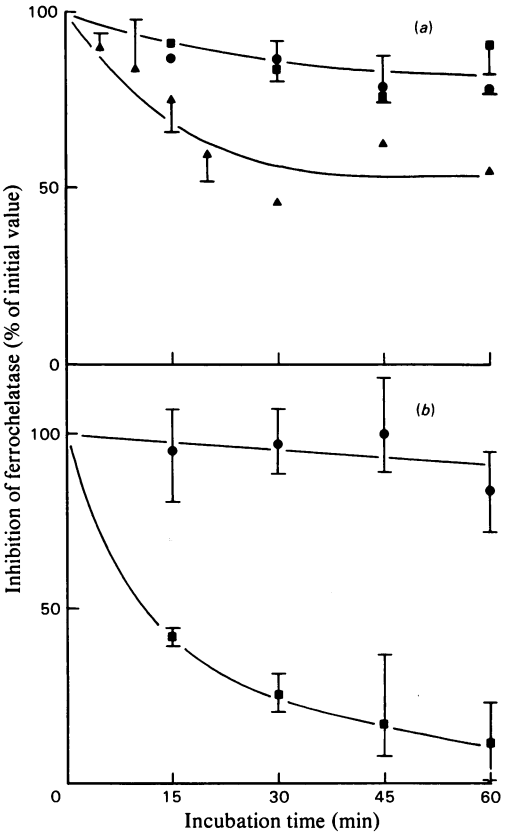

Fig. 3. *Recovery of ferrochelatase activity on incubation of 'inhibited' mitochondria with mesoporphyrin*

Mitochondria were pre-incubated for 15min under standard conditions with either dimethyl sulphoxide (controls) or with an *N*-alkylated porphyrin inhibitor of ferrochelatase (treated). Mesoporphyrin (100nmol) was then added and the incubation continued for the times indicated, when cobalt was added and the ferrochelatase assayed in both the control and the treated series of samples. The inhibition seen at each time point in the treated samples (as compared with the corresponding controls) is expressed as a percentage of the initial value of inhibition found at zero time, that is, before incubation with mesoporphyrin. Porphyrin inhibitors added were: in (*a*): ●, *N*-methylprotoporphyrin (N_A+N_B), 70 pmol; ■, *N*-methylprotoporphyrin (N_C+N_D), 70pmol; ▲, *N*-methyldeuteroporphyrin (fraction F_2), 66 pmol; in (*b*): ●, *N*-ethylprotoporphyrin (N_A+N_B), 117pmol; ■, *N*-ethylprotoporphyrin (N_C+N_D), 117pmol. Values given are those of an individual experiment or averages with range of two or three experiments.

gradually displaced by the porphyrin substrate, resulting in enzyme recovery.

This may explain in part the apparent discrepancy between our own findings that, without preincubation with substrate, *N*-ethylprotoporphyrin, isomers N_C and N_D are strongly inhibitory (Table 2), and those of Ortiz de Montellano *et al.* (1981*a*), who found the same isomers not to be inhibitory. As their enzymic assay involved a set-time incubation, the inhibitor may have been displaced by the porphyrin substrate during the assay. Slight discrepancies between the present findings and previously reported data should also be noted. Thus the modest difference in inhibitory activity between the two isomeric fractions of *N*-methylprotoporphyrin reported previously (De Matteis *et al.*, 1982*b*) could not be reproduced. Since in this case the assay technique was identical, we can only assume that there are variations between mitochondrial preparations in their susceptibility to *N*-alkylporphyrins.

In conclusion, of the two mechanisms considered for the inhibition of ferrochelatase by *N*-alkylporphyrins (i.e. irreversible inactivation by alkyl transfer to the enzyme and tight-binding competive inhibition), the latter appears much more likely.

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