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Inhibition of protohaem ferro-lyase by *N*-substituted porphyrins

Structural requirements for the inhibitory effect

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N-Methyl mesoporphyrin was a powerful inhibitor of protohaem ferro-lyase *in vitro*, whereas *N*-ethyl mesoporphyrin and *N*-methyl coproporphyrin were not and neither was the newly described green pigment produced by giving rats ethylene. This suggests that the size of the substituent at a pyrrole nitrogen and also the number of carboxylic acid side chains of the substituted porphyrin are important for the inhibitory effect. Evidence that *N*-methyl mesoporphyrin inhibited the enzyme, whereas the ethylene-derived pigment did not, was also obtained *in vivo*.

Tephly *et al.* (1979) and De Matteis & Gibbs (1980) have reported that the porphyrogenic compounds 3,5-diethoxycarbonyl-1,4-dihydrocollidine, griseofulvin and isogriseofulvin, previously known to cause a marked inhibition of liver protohaem ferro-lyase (EC 4.99.1.1) activity *in vivo* (Onisawa & Labbe, 1963; Lockhead *et al.*, 1967; De Matteis & Gibbs, 1975) all promote the accumulation in the liver of a modified porphyrin with strong inhibitory activity towards this enzyme. This porphyrin (henceforth referred to as 'inhibitory') appears to originate from liver haem and to inhibit the enzyme irreversibly, probably by combining with the porphyrin binding site at the active centre (De Matteis *et al.*, 1980).

Spectral studies have been conducted on the 'inhibitory' pigment and a comparison made with similar pigments which do not inhibit protohaem ferro-lyase and which are obtained after treatment with unsaturated drugs. The results suggest that both groups of pigments, the 'inhibitory' and the 'non-inhibitory' alike, are *N*-monosubstituted porphyrins, but that the nature of the substituent at a pyrrole nitrogen atom may vary in the two groups, and in the 'inhibitory' pigment may be of small size (De Matteis & Gibbs, 1980).

In agreement with this interpretation, we now find that, when added to normal mouse mitochondria *in vitro*, *N*-methylated mesoporphyrin (where the *N*-substitutent is of a small size) is a powerful inhibitor of protohaem ferro-lyase, whereas the *N*-ethyl analogue is not; neither is the newly described porphyrin obtained by reaction of ethylene with cytochrome *P*-450 haem *in vivo* (and therefore expected to bear a bound two-carbon substituent at one of its pyrrole nitrogens). *N*-methyl mesoporphyrin also causes inhibition of protohaem ferro-lyase *in vivo*, as judged by reduced activity of the enzyme in mitochondria isolated from mice injected with this porphyrin, and also by liver accumulation of protoporphyrin (the substrate of the inhibited enzyme). Under these conditions enhanced activity of liver 5-aminolaevulinate synthase (EC 2.3.1.37) is also found, the adaptive response expected to follow a partial block in liver haem biosynthesis.

Materials and methods

Animals

Male mice (33–38g body wt.) of the LACA strain were allowed food (diet MRC 41B) and water *ad libitum*. 3,5-Diethoxycarbonyl-1,4-dihydrocollidine was given intraperitoneally, dissolved in arachis oil at the dose of 100mg/kg and the mice were killed after 2h. When the effect of *N*-methylated mesoporphyrin was investigated *in vivo*, mice were first given an intraperitoneal injection of 50mg of pentobarbitone sodium/kg and, approx. 10min later, while under anaesthesia, their tail vein was cannulated and 80nmol of *N*-methylated mesoporphyrin [calculated by using the published ε value (Smith, 1975) for the Soret absorbance of mesoporphyrin monocation], equivalent to 730 units of inhibitor (De Matteis *et al.*, 1980), was injected into each mouse dissolved in 50μl of either dimethyl sulphoxide/0.9% (w/v) NaCl (saline) mixture (1:1, v/v) or in 50μl of saline adjusted to pH9.5 with dilute NaOH. Control mice received the solvent alone.

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Male Porton rats were given phenobarbitone first and then exposed to ethylene (5% in air for 7h) in glass-fibre chambers, as described by White (1978) for similar experiments involving acetylene. Ethylene (99.9%, CP grade) was obtained from BOC Special Gases, Deer Park Road, London, U.K.

Enzyme assays and analytical procedures

Protohaem ferro-lyase activity was determined with liver mitochondria by a modification (De Matteis *et al.*, 1973) of the method of Jones & Jones (1969). 5-Aminolaevulinate synthase was assayed in liver homogenates by the radiochemical method described by Condie & Tephly (1978), including the purification step involving partition into ethyl acetate, with a final concentration of [2,3-^{14}C]-succinate of 0.37mM. Porphyrins were extracted from total liver homogenates into 0.9M-$HClO_4$/ethanol (1:1, v/v) and measured fluorimetrically (Granick *et al.*, 1975). Protein was determined by the method of Aldridge (1962).

Synthesis of N-alkylated porphyrins and purification of green pigments

Coproporphyrin III methyl ester (0.5mg) (a gift from Professor C. Rimington, Department of Chemical Pathology, University College Hospital Medical School, London, U.K.) or 2.5mg of mesoporphyrin IX methyl ester (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) were heated with 0.5ml of either methyl iodide or ethyl iodide (BDH Chemicals, Poole, Dorset, U.K.) at 120°C for 18h under vacuum (a modification of the method of McEwen, 1946); the resulting *N*-alkylated porphyrins were separated from the parent porphyrins and purified by repeated t.l.c. on silica-gel by using a developing system of chloroform/methanol (10:1, v/v). Under these conditions all *N*-alkylated porphyrins exhibited considerably lower R_F values than their respective parent porphyrins. The identity of the products was confirmed by the characteristic bathochromic shifts of all absorption maxima of the porphyrin neutral spectrum (see below) and also by recording their mass spectra with a VG Micromass 70/70 mass spectrometer. The molecular ion was the predominant peak in all cases, but evidence of pyrolysis with partial regeneration of the parent porphyrin was also obtained in agreement with previous findings (Jackson & Dearden, 1973). The mass spectrum of *N*-ethyl mesoporphyrin suggested the presence of the *N*-methylated analogue (and traces of methyl iodide were found in ethyl iodide). Details of the chemical syntheses and of the mass spectrometry data will be given elsewhere (A. G. Smith & P. Farmer, unpublished work). The methyl esters of the *N*-alkylated porphyrins were hydrolysed (Falk, 1964) before testing their inhibitory activity on protohaem ferro-lyase *in vitro* or *in vivo*. Green pigments were extracted from liver homogenates and isolated by column chromatography for assay of inhibition of protohaem ferro-lyase activity (De Matteis *et al.*, 1980) or further purified as the methyl esters before recording their absorption spectra. Spectral studies were carried out as described by De Matteis & Gibbs (1980).

Results and discussion

Spectral studies

The green pigment produced by giving rats ethylene and the various synthetic *N*-alkylated porphyrins all exhibited a neutral spectrum of the aetio-type with the absorption maxima shifted towards the red, as compared with those of their parent unsubstituted porphyrins, the bathochromic shifts being very similar to those previously reported for *N*-methyl octaethyl-porphyrin and for other green pigments (De Matteis & Cantoni, 1979). In addition, all the modified porphyrins studied here were found to be very basic, as judged from the very low concentration of acid required to generate the porphyrin monocation. These findings confirm and extend data previously reported (Neuberger & Scott, 1952; Jackson & Dearden, 1973; De Matteis & Cantoni, 1979) in line with the concept that the various green pigments are *N*-monosubstituted porphyrins.

Significant differences exist in the spectrum of the porphyrin dication between 'inhibitory' and 'non-inhibitory' groups of green pigments (De Matteis & Gibbs, 1980), the absorption maxima being shifted towards the red (as compared with those of the parent porphyrins) to a different extent in the two groups. The dication spectra of the various pigments showed the following bathochromic shifts [calculated from the absorption maxima of the parent porphyrins (nm)]. Ethylene green pigment: Soret (7), band II (9.5), I (10); *N*-methyl mesoporphyrin: Soret (2), II (3), I (4); *N*-ethyl mesoporphyrin: Soret (5), II (7.5), I (7). Therefore, by comparison with previous data (De Matteis & Gibbs, 1980), *N*-methyl mesoporphyrin behaved spectrally as an 'inhibitory' pigment, the porphyrin obtained with ethylene as a 'non-inhibitory' pigment and *N*-ethyl mesoporphyrin somewhere in between.

Enzymic studies in vitro

The various porphyrins listed above were tested *in vitro* for their ability to inhibit protohaem ferro-lyase of mouse liver mitochondria (Table 1). *N*-Methyl mesoporphyrin was found to be a very powerful inhibitor of the enzyme, approx. 55% as active as the inhibitor isolated after giving 3,5-diethoxycarbonyl-1,4-dihydrocollidine. The ethylene pigment, where a

two-carbon substituent may be expected to be bound onto one of the pyrrole nitrogens (Ortiz de Montellano *et al.*, 1978; De Matteis & Cantoni, 1979), was on the other hand virtually inactive. Some inhibitory activity was exhibited by *N*-ethyl mesoporphyrin, but this was only about 8% of that shown by *N*-methyl mesoporphyrin. Mass spectrometry indicated that the *N*-ethyl mesoporphyrin contained small amounts of the *N*-methylated analogue and some at least of the inhibitory activity may be due to this contaminant. We conclude that the size of the substituent at the pyrrole nitrogen is important for the inhibitory effect and that in the inhibitor extracted from porphyric livers the substituent may be small, less bulky, that is, than a two-carbon unit.

N-Methylated coproporphyrin was also virtually devoid of inhibitory activity (Table 1); since coproporphyrin III is not a substrate for the enzyme, whereas mesoporphyrin IX is a good substrate (Porra & Jones, 1963), this finding suggests that inhibition of the enzyme is mediated through the interaction of the *N*-methylated porphyrin with the active centre, an interpretation already put forward for the effect of the naturally occurring inhibitor (De Matteis *et al.*, 1980).

Table 1. *Inhibitory activity towards protohaem ferro-lyase of various N-alkylated porphyrins and of green pigments isolated from the liver after giving 3,5-diethoxycarbonyl-1,4-dihydrocollidine or ethylene*

Results given are averages of at least two individual observations obtained with different batches of natural or synthetic porphyrins. The inhibitory activity was assayed *in vitro* as described (De Matteis *et al.*, 1980) and is expressed as units of inhibitor per nmol of porphyrin calculated from the Soret absorption by using the published ε value (Smith, 1975) for the mesoporphyrin monocation.

Porphyrin	Inhibitory activity (units/nmol)
Green pigment (with 3,5-diethoxycarbonyl-1,4-dihydrocollidine)	20.2
N-Methyl mesoporphyrin	11.4
N-Ethyl mesoporphyrin	0.89
Green pigment (with ethylene)	0.15
N-Methyl coproporphyrin	0.15

Studies in vivo

In animals given ethylene a very marked hepatic accumulation of a green pigment could be demonstrated but, in spite of this, the mitochondria obtained from their livers showed normal protohaem ferro-lyase activity (results not shown), indicating that this green pigment is not inhibitory *in vivo*.

In contrast, when *N*-methyl mesoporphyrin was injected intravenously and the liver mitochondria were then isolated from the treated animals, a marked and long-lasting inhibition of the enzyme was found (Table 2). A significant accumulation of porphyrin was also noted: the fluorescence emission spectrum of the liver extract from treated mice was quite distinct from that of *N*-methyl mesoporphyrin and compatible with the accumulating porphyrin being mostly protoporphyrin. In addition, when *N*-methyl mesoporphyrin was injected dissolved in saline (rather than in dimethyl sulphoxide) a

Table 2. *Effect of giving N-methylated mesoporphyrin to mice on the protohaem ferro-lyase and 5-aminolaevulinate synthase activities and on the concentration of liver porphyrins*

Mice were injected with *N*-methyl mesoporphyrin dissolved either in dimethyl sulphoxide (DMSO) or in saline as described in the Materials and methods section and were killed at different times after treatment. Results are means ± S.E.M. of the number of observations in parentheses. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, when compared with the corresponding control values; †$P<0.05$, ‡$P<0.001$, when compared with the corresponding 4h control values when saline was given. N.D., Not determined.

Treatment (and solvent)	Time of killing (h after treatment)	Protohaem ferro-lyase activity (nmol of mesoporphyrin utilized min^{-1} mg of $protein^{-1}$)	Total porphyrins (nmol/g wet wt. of liver)	5-Aminolaevulinate synthase activity (nmol min^{-1} g wet wt. of $liver^{-1}$)
Control (DMSO)	2	1.46 ± 0.13 (4)	N.D.	N.D.
N-Methyl mesoporphyrin (DMSO)	2	0.44 ± 0.03 (4)***	N.D.	N.D.
Control (DMSO)	4	1.36 ± 0.15 (4)	0.28 ± 0.02 (5)‡	0.69 ± 0.03 (5)†
N-Methyl mesoporphyrin (DMSO)	4	0.47 ± 0.04 (4)**	0.55 ± 0.02 (5)***	0.61 ± 0.06 (5)
Control (DMSO)	22	1.38 ± 0.19 (3)	N.D.	N.D.
N-Methyl mesoporphyrin (DMSO)	22	0.77 ± 0.09 (3)*	N.D.	N.D.
Control (saline)	4	1.58 ± 0.09 (4)	0.80 ± 0.06 (4)	0.94 ± 0.09 (5)
N-Methyl mesoporphyrin	4	0.48 ± 0.02 (4)***	6.66 ± 1.79 (5)*	1.97 ± 0.37 (5)*

significant stimulation of 5-aminolaevulinate synthase was also seen and the accumulation of liver porphyrin was considerably greater. Dimethyl sulphoxide also caused a significant decrease in liver 5-aminolaevulinate synthase activity and porphyrin concentration in the control animals (Table 2).

In agreement with previous findings (De Matteis *et al.*, 1973) we conclude that the accumulation of protoporphyrin depends both on the degree of inhibition of protohaem ferro-lyase and also on the extent of secondary stimulation of 5-aminolaevulinate synthase. The present findings support the hypothesis that inhibition of protohaem ferrolyase is the primary mechanism by which griseofulvin and 3,5-diethoxycarbonyl-1,4-dihydrocollidine induce hepatic porphyria.

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