Drug-Induced Conversion of Liver Haem into Modified Porphyrins

EVIDENCE FOR TWO CLASSES OF PRODUCTS

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A porphyrin with inhibitory activity towards protohaem ferro-lyase (EC 4.99.1.1) was isolated from the liver of mice given either griseofulvin or isogriseofulvin. This porphyrin resembles closely in chromatographic and spectral properties the inhibitory pigment isolated after treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine, but differs from other green pigments that do not inhibit protohaem ferro-lyase. A hypothesis is proposed to account for the differences in properties between the two groups of pigments and for the mechanism of inhibition of the enzyme.

Tephly *et al.* (1979) have reported that the porphyrogenic compound 3,5-diethoxycarbonyl-1,4-dihydrocollidine, previously known to cause a marked inhibition of liver protohaem ferro-lyase (EC 4.99.1.1) activity *in vivo* (Onisawa & Labbe, 1963), promotes the accumulation in the liver of a green pigment with strong inhibitory activity towards this enzyme. Labelling and spectral studies have suggested (Tephly *et al.*, 1979) that the 'inhibitory' pigment is a modified porphyrin and that it originates from liver haem. We now find that two additional porphyrogenic compounds, griseofulvin and isogriseofulvin, also known from previous work (De Matteis & Gibbs, 1975) to cause inhibition of protohaem ferro-lyase activity *in vivo*, promote accumulation in the liver of a pigment with inhibitory activity towards protohaem ferro-lyase.

Other green pigments have been described as accumulating after administration of drugs containing unsaturated (allyl or ethynyl) side chains (Ortiz de Montellano *et al.*, 1978, 1979), but these have been found not to inhibit protohaem ferro-lyase activity (De Matteis *et al.*, 1980a). The pigments obtained from the liver of animals given either 3,5-diethoxycarbonyl-1,4-dihydrocollidine, griseofulvin or isogriseofulvin have now been purified and their properties compared with those of the green pigments obtained after treatment with unsaturated drugs. There are important distinguishing features between the 'inhibitory' and the 'non-inhibitory' groups of pigments, suggesting that even though all these green pigments are modified porphyrins with an abnormal substituent at one of their pyrrole nitrogen atoms, the nature and size of this sub-

stituent may be different in the two groups. Throughout the present paper the term 'green pigments' will be used to indicate collectively this type of modified porphyrin and the term 'inhibitory' to indicate those pigments that inhibit protohaem ferro-lyase activity.

Materials and Methods

Animals

Male mice (25–33 g body wt.) of the LACA strain were allowed food (diet MRC 41B) and water *ad libitum*. 3,5-Diethoxycarbonyl-1,4-dihydrocollidine was administered intraperitoneally, dissolved in arachis oil at the dose of 100 mg/kg; sodium secobarbitone and 1-ethynylcyclohexanol were both given intraperitoneally at a dosage of 50 mg/kg in 0.9% NaCl. Mice were killed 2 h after receiving an injection. Griseofulvin and its analogues were given at a concentration of 1% (w/w) in powdered Oxoid diet containing 2% (w/w) arachis oil.

Extraction and purification of the green pigments

Green pigments were extracted from liver homogenates and chromatographed on Sephadex LH-20 by a modification (De Matteis *et al.*, 1980a) of the method of Tephly *et al.* (1979). They were either dissolved in dimethyl sulphoxide for assay of inhibition of protohaem ferro-lyase activity (De Matteis *et al.*, 1980a), or methylated by reaction with BF₃ in methanol at 70°C for 30 min (Smith & Francis, 1979) for further purification. The methylated pigments were transferred to chloroform; this was washed with 1% (w/v) NH₃ and with water,

dried over anhydrous Na_2SO_4 and applied to silica-gel G plates (0.25 mm thick; E. Merck, Darmstadt, Germany) for t.l.c. with the developing system chloroform/paraffin(kerosene)/methanol (20:5:3, by vol.). The major band was eluted from the silica plate with methanol, evaporated to dryness and dissolved in chloroform for spectral studies.

Spectral studies

Absorption spectra were obtained by using a Unicam SP.8-100 recording spectrophotometer calibrated with a holmium filter. Titration of porphyrins with acid and determination of rate of chemical incorporation of Co^{2+} into porphyrins were carried out as described by De Matteis & Cantoni (1979). The Zn^{2+} complexes of green pigments and other porphyrins were prepared by adding to 3 ml of a solution of the porphyrin methyl ester in chloroform, 0.2 ml of a 5% (w/v) methanolic solution of zinc acetate; when the reaction was complete, the chloroform phase was washed with water and dried. The spectra of the porphyrin dications were determined immediately after addition of trifluoroacetic acid (final concn. 21 mM) to a solution of the porphyrin methyl esters in chloroform.

Source of special chemicals

Formyl- and hydroxymethyl-octaethylporphyrin (both *meso*-substituted) were a gift from Professor J. Fuhrhop (Free University of Berlin, Berlin, Germany). The source of the other porphyrins and of the various drugs used in the present work was given previously (De Matteis & Gibbs, 1975; De Matteis & Cantoni, 1979; De Matteis *et al.*, 1980a).

Results and Discussion

Liver accumulation of an 'inhibitory' green pigment in griseofulvin-induced porphyria

Griseofulvin {(2*S*-*trans*)-7-chloro-2',4,6-trimethoxy-6'-methylspiro[benzofuran-2(3*H*),1'-[2]cyclohexene]-3,4'-dione} and isogriseofulvin {(2*S*-*trans*)-7-chloro-4,4',6-trimethoxy-6'-methyl-spiro[benzofuran-2(3*H*),1'-[3]cyclohexene]-2',3-dione} both induce hepatic porphyria and inhibit protohaem ferro-lyase *in vivo*, whereas the 2'- β -hydroxyethyl thioether analogue of griseofulvin is inactive in both respects (De Matteis & Gibbs, 1975). We now find that griseofulvin and isogriseofulvin both cause accumulation in the liver of a green pigment with inhibitory activity towards protohaem ferro-lyase, but the griseofulvin thioether analogue fails to do so. The units of inhibitor (per g wet wt. of liver) recovered after various treatments are given below as the average for two observations, each obtained with pooled livers of four mice, with individual observations in parentheses. Control: 2.72 (1.85, 3.6); isogriseofulvin for 1 day: 20.6 (17.5,

23.7); griseofulvin for 4 days: 30.7 (25.2, 36.3); griseofulvin thioether analogue for 4 days: 2.65 (1.9, 3.4).

Evidence that the 'inhibitory' green pigments are N-substituted porphyrins

The green pigments isolated from the liver of animals given drugs containing either allyl or ethynyl substituents closely resemble authentic *N*-monomethylated porphyrins in the following respects (De Matteis & Cantoni, 1979; De Matteis *et al.*, 1980b). (1) In both groups of porphyrins the absorption maxima of the porphyrin neutral spectrum show bathochromic shifts (as compared with the maxima of their respective parent porphyrins), which are very similar in order of intensity and also in absolute values. (2) Both green pigments and *N*-methylated porphyrins, on titration with acid, give rise readily to a porphyrin monocation, which then requires relatively large amounts of acid for conversion into the dication. (3) Both groups of modified porphyrins incorporate metal ions chemically more readily than do their parent porphyrins. (4) The following additional similarity between green pigments and a *N*-methylated porphyrin has now been established. On incorporation of zinc into any of the green pigments produced by treatment with unsaturated drugs (see also Ortiz de Montellano *et al.*, 1978; 1979), the Soret band increases in intensity only slightly and undergoes a bathochromic shift, as compared with the corresponding free porphyrin (from about 417 nm to about 430 nm), whereas the visible spectrum shows, in addition to the α - and β -bands typical of a metalloporphyrin, a minor band approx. 200 nm towards the red from the Soret maximum (band α' in Table 1). This is unusual as, on incorporation of zinc, most porphyrins, including porphyrins bearing strong electron-withdrawing substituents at the β -positions of the pyrrole rings or substituted at their bridge *meso* carbon atom (results not shown), exhibit marked enhancement of the Soret absorption (with no significant shift of the Soret maximum) and only the α and β visible bands are present. The unusual spectral characteristics of the zinc complex of the green pigments are now found to be shared by authentic *N*-methylated octaethylporphyrin and can probably be ascribed to the steric strain and incomplete co-ordination of the metal due to the presence of a substituent at one of the pyrrole nitrogen atoms; thus the Soret maximum increases from 412 nm (free porphyrin) to 422 nm (zinc complex), and an additional minor band (α') is present after incorporation of zinc (Table 1).

The 'inhibitory' green pigments produced by treatment of mice with either 3,5-diethoxycarbonyl-1,4-dihydrocollidine, griseofulvin or isogriseofulvin have now been purified as the methyl esters and

Table 1. Absorption spectra of the green pigments obtained after treatment with either secobarbitone [5-(1-methylbutyl)-5-(2-propenyl)pyrimidine-2,4,6(1*H*,2*H*,5*H*)-trione], 1-ethynylcyclohexanol, 3,5-diethoxycarbonyl-1,4-dihydrocollidine or griseofulvin: comparison with protoporphyrin and with other model porphyrins

The neutral spectrum of all porphyrins listed here is of an aetio type, with the following absorption maxima for the griseofulvin- and 3,5-diethoxycarbonyl-1,4-dihydrocollidine-induced pigments: Soret (419 nm), band IV (513), III (546), II (595), I (625), I (652). These maxima are similar to those reported for the neutral spectrum of other green pigments (De Matteis *et al.*, 1980*b* and references therein). The dications and zinc complexes of the various porphyrins were prepared as indicated in the Materials and Methods section and their spectra determined in chloroform; the absorption maxima of the Soret and visible bands are given below, together with the bathochromic shifts (from the maxima of the parent porphyrins). Note that pigments (B) and (C) below do not inhibit protohaem ferro-lyase activity, whereas pigments (D) and (E) are strongly inhibitory.

Porphyrin (with name of inducing drug in parentheses)	Absorption maxima or bathochromic shifts (nm)						
	Dication			Zinc complex			
	Soret	II	I	Soret	β	α	α'
(A) Protoporphyrin IX methyl ester	411	556	600	410	539	578	—
(B) Green pigment methyl ester (secobarbitone)*	419	565.5	610	431	547	589	633
Bathochromic shifts (B-A) ...	8	9.5	10	21	8	11	—
(C) Green pigment methyl ester (1-ethynylcyclohexanol)	419	566	613	431	548	589	631
Bathochromic shifts (C-A) ...	8	10	13	21	9	11	—
(D) Green pigment methyl ester (3,5-diethoxycarbonyl-1,4-dihydrocollidine)	412	560	604	431	547	596	633
Bathochromic shifts (D-A) ...	1	4	4	21	8	18	—
(E) Green pigment methyl ester (griseofulvin)†	412	561	605	431	547	596	634
Bathochromic shifts (E-A) ...	1	5	5	21	8	18	—
(F) Octaethylporphyrin	404	549	592	401	531	568	—
(G) <i>N</i> -Methylated octaethylporphyrin	405	551	596	422	538	585	625
Bathochromic shifts (G-F) ...	1	2	4	21	7	17	—

* Very similar absorption maxima were obtained for both the dication and zinc-complex spectra with the pigment produced by 2-allyl-2-isopropylacetamide.

† Very similar absorption maxima were obtained for both the dication and zinc-complex spectra with the pigment produced by isogriseofulvin.

analysed spectrally. Their identification as inhibitors was confirmed by showing that, after hydrolysis of the esters either by aqueous HCl or by methanolic KOH (Falk, 1964), they were strongly inhibitory towards protohaem ferro-lyase activity. These pigments all resembled closely the green pigments obtained from the livers of rats treated with unsaturated drugs in their neutral spectrum (Table 1; see also Tephly *et al.*, 1979), in their titration curve with acid and in their increased reactivity with Co^{2+} *in vitro*. They also gave rise to a zinc complex (Table 1) with the unusual spectral properties discussed above. It is therefore concluded that both groups of pigments, the 'inhibitory' and the 'non-inhibitory' alike, are probably *N*-monosubstituted porphyrins.

Spectral and chromatographic differences between the 'inhibitory' and 'non-inhibitory' green pigments

In spite of the above spectral similarities between

the two groups of pigments, only one, the 'inhibitory' group, affects the activity of protohaem ferro-lyase (De Matteis *et al.*, 1980*a*). The following additional differences between the two groups of pigments are now reported. First, on t.l.c. of the methyl esters, the R_F values for the 'inhibitory' pigments were quite similar regardless of the drug used to produce them *in vivo* (they ranged between 0.09 and 0.13 according to the experiment), whereas the R_F values for the 'non-inhibitory' green pigments varied according to the drug (White, 1978; De Matteis *et al.*, 1980*b*). Secondly, the two groups of pigments showed significant differences in the spectra of the dications and zinc complexes (Table 1), with the 'inhibitory' group resembling much more closely *N*-methyl octaethyl porphyrin in their bathochromic shifts from the respective parent porphyrins. An attractive interpretation for these chromatographic and spectral differences is that the nature of the substituent at the pyrrole nitrogen atom

may vary in the two groups, and in the 'inhibitory' pigments may be of smaller size. This would be compatible with the latter pigments resembling closely (see Table 1) *N*-methylated octaethylporphyrin (where the substituent at the nitrogen atom is small), and also with the observation that whereas drugs that give rise to 'non-inhibitory' green pigments become bound on to their porphyrin nucleus (Ortiz de Montellano, 1978; 1979), 3,5-diethoxycarbonyl-1,4-dihydrocollidine, which produces an inhibitory pigment, apparently does not (De Matteis *et al.*, 1980a). Perhaps with the latter drug, and with griseofulvin, an endogenous reactive species of small molecular size (or a small fragment of the drug itself) becomes bound on to the pyrrole nitrogen atom.

As discussed above, the presence of a substituent at one of the porphyrin nitrogen atoms will interfere with a normal incorporation of a bivalent metal, and this may explain the inhibition of protohaem ferro-lyase if the modified porphyrin were to be accepted as a substrate by the enzyme. The latter may then be blocked in an intermediary stage of the enzymic reaction, unable to release the 'completed' metalloporphyrin product. There is, in fact, evidence that inhibition of the enzyme is achieved through interaction of the inhibitor with its active centre (De Matteis *et al.*, 1980a), so it is likely that when the substituent at the pyrrole nitrogen atom is very large (as is the case with the 'non-inhibitory' green pigments), the binding of the modified porphyrin on to the enzyme will be sterically hindered. We now find (F. De Matteis, A. H. Gibbs & A. G.

Smith, unpublished work) that *N*-methylated meso-porphyrin (where the *N*-substituent is of a small size) is a powerful inhibitor of protohaem ferro-lyase. This is compatible with the hypothesis considered above.

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