CONVERSION OF LIVER HAEM INTO N-SUBSTITUTED PORPHYRINS OR GREEN PIGMENTS

Nature of the substituent at the pyrrole nitrogen atom

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1. Introduction

Drugs can promote the conversion of liver haem into modified porphyrins (or green pigments) of two distinct classes. Pigments of the first class are obtained by treatment with unsaturated drugs containing at least one allyl, vinyl or ethynyl side chain. These drugs are metabolized by cytochrome P450 into reactive derivatives, which then become bound onto the porphyrin nucleus of the haem moiety of the cytochrome, converting it into modified porphyrins (reviewed in [1,2]). The nature of the drug metabolite responsible is still controversial: epoxides have been suggested ([2] and references therein), but there is also a proposal [3] specifically excluding epoxides and emphasizing other structural features of the metabolite, such as the presence of an 'activated' carbonyl grouping. Green pigments of this class do not inhibit liver protohaem ferro-lyase [4] (the enzyme which converts protoporphyrin to haem) and for this reason none of the unsaturated drugs responsible will cause a very marked increase in liver protoporphyrin in vivo.

In contrast 3,5-diethoxycarbonyl-1,4-dihydrocollidine, griseofulvin and isogriseofulvin produce liver accumulation of a second type of green pigment with strong inhibitory properties towards protohaem ferrolyase in vitro [4–6]. Marked inhibition of the liver enzyme is also observed in vivo after treatment with this second group of drugs [7,8] and as a consequence, the liver concentration of protoporphyrin increases markedly giving rise to the biochemical picture of hepatic protoporphyria. The 'inhibitory' pigment also appears to originate from turnover of liver haem [4], but the mechanism of its production is not understood.

Spectral studies conducted on green pigments of both classes [5,6,9] and comparison with data in [10,11] for authentic N-methylated porphyrins have suggested that both groups of green pigments, the 'inhibitory' and 'non-inhibitory' alike, are probably N-monosubstituted porphyrins. Evidence has also been obtained to suggest that the nature of the substituent at the pyrrole nitrogen atom may vary in the two groups and in the 'inhibitory' pigment may be of smaller size [6,12]. Here, the 'inhibitory' pigment obtained after feeding mice with 3,5-diethoxycarbonyl-1,4-dihydrocollidine and the 'non-inhibitory' pigment produced by treatment of rats with ethylene have both been characterized by mass spectrometry. The results suggest that the two pigments are *N*-methyl protoporphyrin and *N*-Hydroxyethyl protoporphyrin, respectively, although we cannot specify which isomer(s) are present. This interpretation is supported by a close similarity of the spectral characteristics of the 'inhibitory' pigment with those of authentic *N*-methyl protoporphyrin and, in the case of the ethylene-derived pigment, by the demonstration that its chromatographic properties can be modified by treatment with acetic anhydride, compatible with the presence of a free hydroxyl group.

2. Materials and methods

Male mice were treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine and phenobarbitone pre-treated male rats were exposed to ethylene (5%, by vol. in air

for 7 h) as in [12]. Cytochrome P450 was determined in liver homogenates [13]. The green pigments were extracted from liver homogenates and chromatographed on Sephadex LH-20 [4,5]. They were then either dissolved in dimethylsulphoxide for assay of inhibition of protohaem ferro-lyase [4], or methylated by reaction with BF_3 in methanol [14] and further purified by TLC [12]. The major band was eluted from the silica plate with methanol, evaporated to dryness and dissolved in chloroform for spectral studies. Absorption spectra of green pigments and of synthetic porphyrins were obtained by using a Unicam SP.8-100 recording spectrophotometer calibrated with a holmium filter. Zn^{2+} complexes and dications derivatives of porphyrins were obtained as in [6].

Acetylation of the pigment produced in vivo by treatment with ethylene was carried out in pyridine containing acetic anhydride (9:1, by vol.) at room temperature overnight and the pigment was then transferred to ethyl ether and, after washing the ether phase with water and evaporating it to dryness, chromatographed on silica gel G plates using chloroform/methanol (20:3, by vol.) as the developing system.

Authentic N-monomethylated protoporphyrin was prepared from protoporphyrin IX dimethyl ester by reaction with methyl fluorosulphonate at 20°C for 1 day or alternatively with methyl iodide at 100°C in a sealed tube for 1 day. It was separated from *N,N*-dimethyl protoporphyrin, *N,N,N*-trimethyl protoporphyrin and from the unreacted parent porphyrin by column chromatography and further purified by HPLC. Two different fractions, probably representing two different N-substituted isomers, were obtained in sufficient amounts to allow further characterization by mass and NMR spectrometry, and visible spectroscopy; studies of the in vitro inhibitory activity of the two fractions on protohaem ferro-lyase [4] were also performed, after hydrolysis of the methyl esters by aqueous 6.8 M HCl for 4.5 h [15]. Field desorption mass spectra of porphyrin methyl esters were obtained with a Varian CH5D mass spectrometer and Varian SS-100 data system, as in [16].

3. Results and discussion

When phenobarbitone pre-treated rats were exposed to ethylene in air there was a significant loss of liver cytochrome P450 and, as in [12], a green pigment which did not inhibit protohaem ferro-lyase activity in vitro could be demonstrated in their livers. Values of cytochrome P450 found after treatment were 77.3 nmol/g liver ± 5.5 (SEM of 3 obs.), whereas values of 136.3 ± 7.7 nmol/g were found in appropriate controls given no ethylene. These findings are similar to those reported [1,2] with other unsaturated compounds of more complex structure and larger molecular size. The pigment produced by ethylene was chosen for further characterization by mass spectrometry and a comparison with the 'inhibitory' pigment produced by treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine because:

(i) The yield of green pigment was considerably greater after ethylene treatment than after a single dose of either 2-allyl-2-isopropylacetamide, secobarbitone sodium or 1-ethynylcyclohexanol [4].

(ii) As the simplest olefin, ethylene does not possess any of the additional structural features (e.g., *hetero* atom substitution) which are present in most unsaturated drugs studied so far and which could lead to secondary modification of structure of the green pigments, thereby complicating the interpretation of the results.

A field desorption mass spectrum was obtained for the ethylene green pigment and exhibited a molecular ion (M^+) at *m/e* 634 and a protonated ion ($M^+ + H$) at *m/e* 635. This is compatible with a structure of *N*-hydroxyethyl protoporphyrin dimethyl ester as the peak at *m/e* 634 would result from the sum: Protoporphyrin dimethyl ester (590) – one pyrrole proton (1) + the hydroxyethyl grouping (45). Although we have not yet synthesized *N*-hydroxyethyl protoporphyrin so that a direct comparison with the authentic synthetic porphyrin has not yet been possible, nevertheless the presence of a free hydroxyl group is suggested by the finding that the TLC mobility of both the ethylene pigment and of its Zn^{2+} complex can be increased on treatment with acetic anhydride. R_F values found for the ethylene pigments were: before acetylation 0.12; after acetylation 0.39; and for its Zn^{2+} complex 0.68 and 0.80, before and after acetylation, respectively. These findings are in line with the conclusion [1–3,9,17,18] that unsaturated drugs become bound onto the prosthetic group of cytochrome P450 via the formation of a reactive intermediate. Since the simple olefin, ethylene (and in [19], acetylene itself) have been found to be active, we conclude that the essential structural requirement for the effect is an unsaturated bond between two carbon atoms and that the additional structural fea-

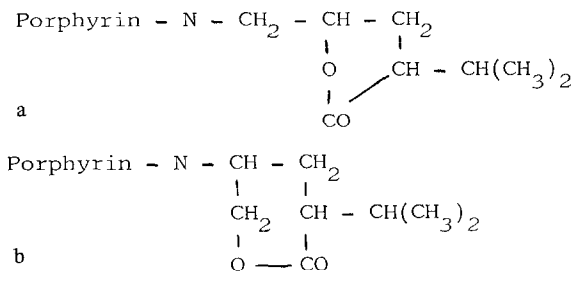

Fig.1. Possible structures for the green pigment isolated after treatment with 2-allyl-2-isopropylacetamide, with either (a) the γ lactone or (b) the δ lactone derivative of the drug bound onto one of the porphyrin nitrogens.

tures implicated in [3] are not essential. The presence of a free hydroxyl group on the ethylene pigment would be compatible with the reactive intermediate being an epoxide, as reaction of an epoxide with a nucleophilic centre is expected to generate an hydroxyl group [20,21]. An epoxide intermediate would be at variance with the conclusions in [3], although their mass spectral data for the porphyrin adduct of a metabolite of 2-allyl-2-isopropylacetamide may be still compatible with an epoxide intermediate. Thus the molecular ion in [3] with a *m/e* value of 730 could be accounted for by the sum: Protoporphyrin dimethyl ester (590) – one pyrrole proton (1) + either the γ lactone (3-isopropyl-5-methyltetrahydro-2-furanone) or δ lactone grouping (141) attached onto the pyrrole nitrogen. This grouping could result from an intermediary epoxide (4,5-epoxy-2-isopropyl pentenamide) generating on reaction with the pyrrole nitrogen a free hydroxyl group, which could subsequently react at the terminal amide carboxyl group to produce the corresponding γ lactone or δ lactone (fig.1).

A field desorption mass spectrum was also obtained for the pigment isolated from the liver of mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine. This pigment exhibited a molecular ion (M^+) at *m/e* 604 and a monoprotonated ion ($M^+ + H$) at *m/e* 605. This is compatible with a structure of *N*-methyl protoporphyrin [protoporphyrin dimethyl ester (590) – one pyrrole proton (1) + the CH_3 grouping (15)], a conclusion supported by the findings:

(1) The synthetic *N*-methylated protoporphyrin which also gave molecular ions at *m/e* 604 and 605 by field desorption mass spectrometry, exhibited UV-visible absorption spectra very similar to those of the pigment obtained after treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine, whether the spectra were obtained with the porphyrin free base or with their Zn^{2+} chelate and dication derivatives (tables 1,2).

(2) Both fractions of synthetic *N*-methyl protoporphyrin were strongly inhibitory on protohaem ferro-lyase activity in vitro, fraction 1 exhibiting 17.3 units/nmol pigment (calculated in terms of

Table 1
Neutral absorption spectrum of the green pigment isolated from the liver of mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine – Comparison with protoporphyrin and with authentic *N*-methyl protoporphyrin

Porphyrin (inducing drug in parentheses)	Absorption maxima (nm)					
	Soret	IV	III	II	Ia	I
Protoporphyrin IX dimethyl ester[a]	407	505	541	575	603	630
Green pigment methyl ester (3,5-diethoxycarbonyl-1,4--dihydrocollidine)[b]	419	513	546	595	625	652
N-Methylated Protoporphyrin IX dimethyl ester: fraction 1	419	514	547.6	594		653
fraction 2	418.5	513.8	546.5	594.5	625	652.4

[a] From [15]; [b] from [6]

All porphyrins listed here exhibit an *aetio*-type spectrum: the absorption maxima of the modified porphyrins are shifted towards the red as compared with those of protoporphyrin, the extent of these bathochromic shifts being very similar to those reported for other N-monosubstituted porphyrins [9]. Two separate fractions were obtained from synthetic *N*-methyl protoporphyrin and these may represent different isomers

Table 2
Absorption spectra of the dication and zinc complex derivatives of the green pigment isolated from the liver of mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine – Comparison with *N*-methyl protoporphyrin

Porphyrin (inducing drug in parentheses)	Absorption maxima (nm)						
	Dication			Zinc complex			
	Soret	II	I	Soret	β	α	α′
Green pigment methyl ester (3,5-diethoxycarbonyl-1,4-dihydrocollidine)[a]	412	560	604	431	547	596	633
N-Methyl protoporphyrin IX dimethyl ester: fraction 1	411.8	559.6	604	431	546.7	596	634[b]
fraction 2	412.6	561	603.5	431	544.3	593	631.6

[a] From [6]; [b] this absorption maximum was not clearly defined

The dications and zinc complexes of the porphyrins were prepared as in [6] and their spectra determined in chloroform

protoporphyrin equivalents) and fraction 2, 13.0 units/nmol. These fractions were therefore 86% and 64% as active as the natural pigment [12]. These data are compatible with the findings that in the case of the 'inhibitory' pigment the drug responsible for its production does not itself become bound onto the porphyrin nucleus [4] and that the pyrrole nitrogen atom bears instead a substituent of sufficiently small size to allow interaction of the modified porphyrin with the active centre of the enzyme protohaem ferro-lyase [6,12]. The source of the methyl group in the naturally occurring pigment is not yet known, neither is the isomeric type of the synthetic porphyrins and of the naturally occurring pigment (i.e., which one of the pyrrole nitrogen has become methylated).

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