***N*-Alkylation of the haem moiety of cytochrome *P*-450 caused by substituted dihydropyridines**

Preferential attack of different pyrrole nitrogen atoms after induction of various cytochrome *P*-450 isoenzymes

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1. 3,5-Diethoxycarbonyl-4-ethyl-1,4-dihydro-2,6-dimethylpyridine (4-ethyl-DDC) gives rise to *N*-ethylprotoporphyrin in the liver of rats by donating its 4-ethyl group to one of the pyrrole nitrogen atoms of haem. Four structural isomers are obtained, depending on which pyrrole nitrogen is alkylated. 2. When rats are pretreated with an inducer of cytochrome *P*-450, the production of *N*-ethylprotoporphyrin caused by 4-ethyl-DDC is greater, both in the whole animal and in hepatocytes incubated with the drug *in vitro*. 3. Pre-incubation of hepatocytes with 2-allyl-2-isopropylacetamide decreases the yield of *N*-ethylprotoporphyrin due to 4-ethyl-DDC, an effect largely reversed by adding exogenous haem. 4. The isomeric composition of *N*-ethylprotoporphyrin produced *in vivo* and *in vitro* depends on the cytochrome *P*-450 isoenzyme that predominates at the time of treatment, suggesting a role for the apo-cytochrome in directing alkylation on to one of the pyrrole nitrogens.

Substituted dihydropyridines, like DDC and its 4-ethyl analogue, 4-ethyl-DDC (Fig. 1*a*), give rise to *N*-alkylated porphyrins in the liver of treated animals (Tephly *et al.*, 1979; De Matteis & Gibbs, 1980; De Matteis *et al.*, 1980*a*; Ortiz de Montellano & Kunze, 1981) by donating their intact 4-alkyl group to one of the pyrrole nitrogen atoms of haem (De Matteis *et al.*, 1981; Ortiz de Montellano *et al.*, 1981; Tephly *et al.*, 1981). Both exogenous and endogenous hepatic haem can be utilized in the *N*-alkylation reaction (De Matteis *et al.*, 1982) and there is strong indirect evidence that the endogenous precursor pool(s) of haem may be cytochrome(s) *P*-450 (Wada *et al.*, 1968; Waterfield *et al.*, 1969; Abbritti & De Matteis, 1973; Ortiz de Montellano *et al.*, 1981; Coffman *et al.*, 1982).

Because of the asymmetric arrangement of the vinyl and propionate side chains in protoporphyrin IX, four structural isomers of *N*-monosubstituted protoporphyrin are possible, depending on which of the four pyrrole nitrogens has been alkylated (Fig. 1*b*). *N*-Methyl- and *N*-ethyl-protoporphyrin obtained from chemical synthesis or extracted from

liver after treatment with DDC and 4-ethyl-DDC have both been shown to be a mixture of all four structural isomers (Ortiz de Montellano & Kunze, 1981; Ortiz de Montellano *et al.*, 1981). In the case of *N*-methylprotoporphyrin produced by treatment with DDC, two different structural isomers have both been found to be optically active (De Matteis *et al.*, 1982*b*), suggesting that they are both produced on an enzymic template. However, it is not yet known whether all four isomers are produced on the same enzymic site, or whether alkylation of haem may take place on more than one site, each giving rise preferentially to one isomer of *N*-alkyl-protoporphyrin.

In this present paper we report additional evidence that the endogenous precursor pools of haem from which the *N*-alkylated protoporphyrins originate are cytochromes of the *P*-450 group. Evidence has also been obtained both *in vivo* and *in vitro* that different cytochromes *P*-450 may be involved, each giving rise preferentially to a different structural isomer of *N*-alkylprotoporphyrin.

Materials and methods

Treatment of animals

Male fed rats of the Porton (Wistar-derived) strain, ranging in body weight from 190 to 235 g,

Abbreviations used: DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine; 4-ethyl-DDC, 3,5-diethoxycarbonyl-4-ethyl-1,4-dihydro-2,6-dimethylpyridine; h.p.l.c., high-pressure liquid chromatography.

Fig. 1. Structure of 4-ethyl-DDC (a) and of *N*-ethylprotoporphyrin IX (b)

Four structural isomers of the porphyrin are possible, depending on which pyrrole nitrogen is alkylated. The isomer shown is N_B, where ring B is *N*-alkylated.

were used throughout. Rats were pretreated with inducers of cytochrome *P*-450 isoenzymes by a single intraperitoneal injection of 3-methylcholanthrene (80 mg/kg body wt.), β -naphthoflavone (80 mg/kg body wt.) or DDT [1,1'-(2,2,2-trichloroethylidene)bis(4-chlorobenzene)] (75 mg/kg body wt.), the former two compounds 48 h and the latter 71 h before treating rats with 4-ethyl-DDC. Phenobarbital sodium was given (0.1% in drinking water) for 1 week before the animals were used. Control rats, which had received no pretreatment, or animals pretreated with one of the above mentioned inducers, were given 4-ethyl-DDC intraperitoneally dissolved in arachis oil (10 mg/kg body wt.) and were killed 1 h later.

Isolation and characterization of *N*-alkylated protoporphyrin

N-Ethylprotoporphyrin was extracted from liver homogenate, isolated by column chromatography on Sephadex LH-20 (De Matteis *et al.*, 1980b) and estimated in the column eluate by using an $\epsilon_{\text{mm}} = 228 \text{ mm} \cdot \text{cm}^{-1}$ for the Soret (about 415 nm) absorption of the dication derivative (De Matteis *et al.*, 1982a). The pigment was then freed from contaminating lipids by cooling at -70°C and further purified by t.l.c. on silica-gel G plates as the free carboxylic acid (De Matteis *et al.*, 1980b) and eluted from silica with 10 ml of methanol containing 0.25 ml of conc. HCl. It was transferred to chloroform (6 ml) by adding 6 ml of water and mixing; the chloroform phase was washed twice with water and taken to dryness under N₂. The methyl ester of *N*-ethylprotoporphyrin was prepared by reaction with BF₃ in methanol (Smith & Francis, 1979), transferred to chloroform, washed, dried and chro-

matographed on an h.p.l.c. column (4.6 mm $\times$ 250 mm) of Partisil 10-PAC, as described by Ortiz de Montellano *et al.* (1981), using isocratic elution with tetrahydrofuran/hexane/methanol (97:97:6, by vol.), a flow rate of 1.5 ml/min and monitoring the eluate at 417 nm. This procedure afforded almost complete separation of *N*-ethylprotoporphyrin from the 2-allyl-2-isopropylacetamide-derived pigment (see below) and also, as previously shown (Ortiz de Montellano *et al.*, 1981), resolution of *N*-ethylprotoporphyrin into its four structural isomers in the following order of elution: N_B-, N_A-, N_C- and N_D-ethyl derivatives, the suffixes A–D indicating the pyrrole ring that is *N*-ethylated in each structural isomer. The amount of each isomer was calculated from the area under the h.p.l.c. elution profile (integrated by a Shimadzu C-RIA Chromatopac instrument) and was corrected for overall recovery by comparing, in the same experiment, the amount of 417 nm-absorbing material eluted from h.p.l.c. with the total amount of pigment isolated from the Sephadex LH-20 column. Recovery from the h.p.l.c. column was 92% and 86%, with a mixture of N_A- and N_B- and a mixture of N_C- and N_D-alkylated isomers respectively; and overall recovery ranged from 25 to 55%, lower recoveries being usually observed with smaller amounts of *N*-ethylprotoporphyrin. A very similar isomeric composition was found for *N*-ethylprotoporphyrin extracted from identically treated rats, regardless of overall recovery, suggesting a similar recovery for each isomer.

Experiments with isolated hepatocytes

Hepatocytes were prepared by a collagenase perfusion technique (Paine & Legg, 1978) from rats,

some pretreated as described above with phenobarbital or β -naphthoflavone. Viability (Trypan Blue exclusion) was 88–94% at the beginning of the incubation. The methods used for incubation of hepatocytes, extraction and estimation of *N*-alkylated porphyrins and assay of their inhibitory activity *in vitro* towards protohaem ferro-lyase (ferrochelatase) have all been described previously (De Matteis *et al.*, 1982a).

Source of special chemicals

Authentic *N*-ethylprotoporphyrin dimethyl ester was prepared from protoporphyrin IX dimethyl ester as described previously (De Matteis *et al.*, 1981), except that the reaction was carried out at 100°C for 4 h. Haemin was kindly donated by Dr. S. Sassa, Rockefeller University, New York, U.S.A. 4-Ethyl-DDC was prepared by the method given in De Matteis & Prior (1962), starting with propionaldehyde. Methanol, hexane and tetrahydrofuran were all h.p.l.c. grade and were obtained from Rathburn Chemicals and Fisons Scientific Apparatus and Chromatographic Services respectively. Tetrahydrofuran was made peroxide-free and dry, immediately before use, by filtration through aluminium oxide (type UG; Koch–Light Laboratories), which had been previously activated by heating at 100°C overnight.

Results and discussion

Experiments *in vivo*

When control rats were given a single intraperitoneal injection of increasing amounts of 4-ethyl-DDC and were killed 1 h later, an *N*-alkylated protoporphyrin accumulated in the livers (Fig. 2a). The pigment was identified as *N*-ethylprotoporphyrin by comparison of its electronic absorption spectrum and chromatographic behaviour on h.p.l.c. with that of authentic synthetic *N*-ethylprotoporphyrin. All four structural isomers could be demonstrated but the percentage isomeric composition of the accumulating pigment was found to differ significantly according to the dose of 4-ethyl-DDC administered (Fig. 2b). When expressed as a percentage of the total *N*-ethylprotoporphyrin, the N_D isomer gradually increased with the dose of drug, whereas the N_A isomer showed a significant decline; in contrast, both the N_B and N_C isomers represented the same percentage of the total *N*-ethylprotoporphyrin (approx. 5 and 40% respectively), regardless of the dose of 4-ethyl-DDC administered. The differential behaviour of the four isomers was also confirmed by calculating the ratio of N_D to N_A isomer in the pigment extracted from each individual liver: a significant increase in this ratio was found with increasing doses of drug, but no

Fig. 2. Effect of dosing rats with increasing amounts of 4-ethyl-DDC on (a) total amount of *N*-ethylprotoporphyrin accumulating in the liver, (b) isomers N_B ($\diamond$), N_A ($\circ$), N_C ($\square$) and N_D ($\bullet$) present as a percentage of total pigment and (c) ratio of N_D/N_A ($\circ$) and N_B/N_C ($\bullet$) isomers

Rats were killed 1 h after treatment. * $P < 0.05$, compared with the corresponding value obtained after 20 mg/kg body wt. treatment; † $P < 0.01$, compared with the value after 50 mg/kg body wt. treatment.

Table 1. *Effect of treatment of rats with different inducers of cytochrome P-450 on amount and isomeric composition of N-ethylprotoporphyrin accumulating in their livers after administration of 4-ethyl-DDC*

Rats, some pretreated with inducers as indicated in the Materials and methods section, were given either 50 or 100 mg of 4-ethyl-DDC/kg body wt. and were killed 1 h later. Results given are means $\pm$ S.E.M. of the number of observations indicated or individual observations. * $P > 0.05$; ** $P < 0.02$; *** $P < 0.001$, when compared with the corresponding control value.

Pretreatment	Dose of 4-ethyl-DDC (mg/kg body wt.)	Number of observations	N-Ethylprotoporphyrin isolated from liver				
			Total (nmol/g wet wt. of liver)	Isomer composition (%)			
				N _B	N _A	N _C	N _D
None (control)	50	5	2.55 $\pm$ 0.14	5.56 $\pm$ 0.5	32.70 $\pm$ 1.25	40.18 $\pm$ 1.77	21.57 $\pm$ 0.51
	100	4	2.49 $\pm$ 0.08	4.52 $\pm$ 0.13	28.10 $\pm$ 0.66	42.35 $\pm$ 1.05	25.04 $\pm$ 0.82
DDT	50	4	2.45 $\pm$ 0.38	3.82* $\pm$ 0.2	48.07** $\pm$ 2.61	27.78*** $\pm$ 2.21	20.34 $\pm$ 0.96
	100	4	3.53** $\pm$ 0.32	3.81 $\pm$ 0.13	45.85*** $\pm$ 0.94	27.80** $\pm$ 0.85	22.55 $\pm$ 0.29
Phenobarbital	50	5	2.91 $\pm$ 0.26	5.01 $\pm$ 0.24	63.1*** $\pm$ 3.17	16.84*** $\pm$ 2.28	15.02** $\pm$ 1.13
β -Naphtho-flavone	50	4	4.12* $\pm$ 0.58	1.64*** $\pm$ 0.17	32.31 $\pm$ 2.58	14.65*** $\pm$ 1.64	51.23*** $\pm$ 3.68
3-Methyl-cholanthrene	50	2	5.43, 3.75	1.2, 1.3	37.1, 19.0	15.2, 17.0	46.5, 62.8

significant change in the ratio between the other two isomers (Fig. 2c).

These findings suggested that the various isomers need not be produced from a single precursor pool of haem but may originate instead from various precursor pools differing either in affinity for the alkylating drug and/or in rate at which they are utilized in the *N*-alkylation reaction. The possibility that the various structural isomers may originate from different cytochrome *P*-450 isoenzymes was investigated by pretreating rats with inducers of the cytochrome *P*-450 system and by giving them 4-ethyl-DDC. The amount and isomeric composition of the resulting *N*-ethylprotoporphyrin was then compared with that obtained in control rats (Table 1). In general the yield of *N*-ethylprotoporphyrin after 4-ethyl-DDC administration was greater in rats pretreated with inducers than in control rats. This is in agreement with previous findings obtained in intact rats and in isolated hepatocytes (Coffman *et al.*, 1982; De Matteis *et al.*, 1982a). In addition we now find that the isomeric composition of the alkylated porphyrins depends on the type of cytochrome *P*-450 predominating at the time of treatment. Thus in control animals the prevalent isomer was N_C, whereas after phenobarbital and β -naphthoflavone treatment, which mainly induce two different cytochrome isoenzymes (Lu & West, 1980), a marked increase was found in isomers N_A and N_D respectively. DDT and 3-methylcholanthrene resemble phenobarbital and β -naphthoflavone respectively in the pattern of enzyme induction produced and in the properties of the induced cytochrome *P*-450 (Conney, 1967; Vainio, 1974; Lu

& West, 1980). They also resembled phenobarbital and β -naphthoflavone in the isomeric pattern of the accumulating *N*-ethylprotoporphyrin (Table 1). These findings are consistent with the view that the various isomers are produced from more than one site and also suggest that the main phenobarbital-inducible type of cytochrome *P*-450 produces preferentially isomer N_A, whereas the β -naphthoflavone- (or 3-methylcholanthrene-) inducible type produces isomer N_D. These two cytochromes are referred to below as cytochrome *P*-450 and cytochrome *P*-448 respectively.

Experiments with isolated hepatocytes

Further evidence in favour of the involvement of the cytochromes in the production of *N*-alkylated porphyrins by 4-ethyl-DDC was obtained in isolated hepatocytes. When rats were pretreated with either phenobarbital or β -naphthoflavone *in vivo*, hepatocytes prepared from their livers produced *N*-ethylprotoporphyrin on incubation with 4-ethyl-DDC in significantly greater amounts than hepatocytes from control animals (Table 2). This confirms the conclusion of the experiments carried out *in vivo* (see above and also Coffman *et al.*, 1982) that 4-ethyl-DDC utilizes both the main types of inducible cytochromes. In contrast the production of *N*-alkylated porphyrins caused by 2-allyl-2-isopropylacetamide was markedly stimulated by pretreatment with phenobarbital, but only slightly by pretreatment with β -naphthoflavone (Table 2), in keeping with the conclusion reached previously (Baird *et al.*, 1976; Unseld & De Matteis, 1978) that with 2-allyl-2-isopropylacetamide the haem of cyto-

Table 2. Effect of pretreatment of rats with phenobarbital or β -naphthoflavone *in vivo* on the formation of N-alkylated porphyrins caused in isolated hepatocytes by either 4-ethyl-DDC or 2-allyl-2-isopropylacetamide

Rat hepatocytes were incubated with 4-ethyl-DDC or 2-allyl-2-isopropylacetamide for either 2 or 4 h. 2-Allyl-2-isopropylacetamide (16 mg) and 4-ethyl-DDC (0.6 mg) were added to each hepatocyte suspension (40 ml) both at the beginning and after 2 h incubation, each time in 0.1 ml of ethanol. Results are given as means $\pm$ S.E.M. of at least three observations (each obtained with the pooled hepatocytes of two separate incubations) or averages with individual observations in parentheses. The results obtained with phenobarbital pretreatment are from previous work (De Matteis *et al.*, 1982a) and are given for comparison.

Pretreatment of the rat <i>in vivo</i>	Incubation time (h)	N-alkylated porphyrins (nmol/ 100 $\times$ 10 ⁶ cells) produced by:	
		4-Ethyl-DDC	2-Allyl-2-isopropylacetamide
None	2	1.69 $\pm$ 0.22 (3)	2.13 (2.2, 2.06)
Phenobarbital	2	4.4 (4.45, 4.36)	11.8 (11.9, 11.7)
β -Naphthoflavone	2	5.28 (6.52, 4.05)	3.54 (3.52, 3.57)
None	4	2.79 $\pm$ 0.47 (3)	—
Phenobarbital	4	4.92 $\pm$ 0.31 (8)	—
β -Naphthoflavone	4	7.09 (6.96, 7.23)	—

chrome P-450 (rather than that of cytochrome P-448) is the preferential target for destructive N-alkylation.

The production of N-ethylprotoporphyrin observed in isolated hepatocytes obtained from rats pretreated with either phenobarbital or β -naphthoflavone could be further increased by addition of exogenous haematin (Table 3). The increased yield of N-ethylprotoporphyrin due to exogenous haem concerned all four structural isomers, though to a different extent. In hepatocytes from rats pretreated with phenobarbital, haematin preferentially stimulated the formation of the N_A isomer (see also Table 4), so that with supplementary haematin the isomeric profile was qualitatively similar to that seen in intact animals pretreated with phenobarbital, that is, a preponderance of isomer N_A was observed. In hepatocytes from rats pretreated with β -naphthoflavone, on the other hand, the isomeric profile was very similar to that seen *in vivo*, whether the incubation was carried out in the presence of haematin or not (as in both cases isomer N_D clearly predominated) and no preferential stimulation of N_D isomer formation by exogenous haem

Table 3. Effect of pretreatment of rats with phenobarbital or β -naphthoflavone *in vivo* and of adding exogenous haem *in vitro* on the amount and isomeric composition of N-ethylprotoporphyrin formed by isolated hepatocytes after treatment with 4-ethyl-DDC

Hepatocytes from rats pretreated with phenobarbital or β -naphthoflavone *in vivo* were incubated with 4-ethyl-DDC for 4 h in the presence or in the absence of exogenous haem (methaemalbumin). The latter was added at the start of the incubation to a final concentration of 6.25 μ M. 4-Ethyl-DDC was added as indicated in the legend to Table 2. Results are those of individual observations, each obtained with the pooled hepatocytes of two to four separate incubations.

Pretreatment of the rat <i>in vivo</i>	Exogenous haem	N-Ethylprotoporphyrin (nmol/100 $\times$ 10 ⁶ cells)				
		Total	Isomer:			
			N _B	N _A	N _C	N _D
Phenobarbital	Absent	4.31	0.52	0.72	.22	1.85
	Present	3.91	0.54	0.87	0.96	1.53
β -Naphthoflavone	Absent	8.68	1.25	3.45	1.77	2.21
	Present	10.60	1.05	4.02	2.02	3.20
	Absent	6.97	0.16	0.69	0.84	5.25
	Present	7.23	0.17	0.61	0.89	5.58
		14.10	0.68	2.96	2.44	8.02
		12.80	0.61	2.36	2.38	7.44

could be demonstrated. This suggests that exogenous haem is taken up more effectively by the apoprotein of cytochrome P-450 (there to be converted into N-ethylprotoporphyrin, predominantly isomer N_A) than by the apo-cytochrome P-448 and/or that in isolated hepatocytes, cytochrome P-450 but not cytochrome P-448 is partially in a haem-depleted state.

The effect of pre-incubating hepatocytes with 2-allyl-2-isopropylacetamide, a suicidal inhibitor of cytochrome P-450 (Ortiz de Montellano & Mico, 1981; Loosemore *et al.*, 1981), on the subsequent production of N-ethylprotoporphyrin by 4-ethyl-DDC was also investigated. It was found (Table 4) that 2-allyl-2-isopropylacetamide significantly decreased the yield of N-ethylprotoporphyrin, whether this was measured by its ability to inhibit protohaem ferro-lyase activity *in vitro* or by its Soret absorption; and the effect of 2-allyl-2-isopropylacetamide could be largely reversed by adding exogenous haematin. Both the inhibition of N-ethylprotoporphyrin formation by 2-allyl-2-isopropylacetamide and its stimulation by exogenous haem concerned preferentially isomer N_A, at least as compared with isomers N_C and N_D. Isomer N_B could not be measured accurately in hepatocytes incubated with 2-allyl-2-isopropylacetamide, as its

Table 4. *Effect of pretreatment of hepatocytes with 2-allyl-2-isopropylacetamide and of addition of exogenous haem on the amount and isomeric composition of N-ethylprotoporphyrin produced after treatment with 4-ethyl-DDC*

Isolated hepatocytes were obtained from rats pretreated with phenobarbital *in vivo*. They were first incubated for 2 h in the presence of 2-allyl-2-isopropylacetamide (3 mM) or in its absence; they were then obtained by centrifugation, resuspended in fresh medium and incubated for a further 2 h with 4-ethyl-DDC (50 μ M). Haematin was added as methaemalbumin at a final concentration of 6.25 μ M.

Treatment during:		Inhibitory activity on protohaem ferro-lyase* (units/100 mg of protein; see De Matteis <i>et al.</i> , 1982a)	N-Ethylprotoporphyrin (nmol/100 $\times$ 10 ⁶ cells)				
0–2 h	2–4 h		Isomers:				
			Total	N _B	N _A	N _C	N _D
(1) None	4-Ethyl-DDC	15.2	4.47	0.40	1.17	1.27	1.64
			4.12	0.37	0.84	1.21	1.70
(2) None	4-Ethyl-DDC + haematin	25.9	5.26	0.54	1.90	1.26	1.56
			4.91	0.46	1.79	1.24	1.42
(3) 2-Allyl-2-isopropyl- acetamide	4-Ethyl-DDC	9.5	2.56	0.23†	0.23	0.90	1.18
			1.66	0.19†	0.17	0.60	0.69
(4) 2-Allyl-2-isopropyl- acetamide	4-Ethyl-DDC + haematin	22.6	3.93	0.62†	0.48	1.13	1.69
			4.22	0.85†	0.71	1.16	1.50

* Average of three separate observations, each obtained with at least three different concentrations of the alkylated porphyrins isolated from the Sephadex LH-20 column.

† The allyl-2-isopropylacetamide-derived pigment may have contributed to these values.

peak coincided with the tail-end of elution of a very large peak of the 2-allyl-2-isopropylacetamide-derived pigment. Evidence was also obtained in these experiments that 2-allyl-2-isopropylacetamide depleted cytochrome *P*-450, whereas exogenous haem partially regenerated it (results not shown) in line with previous findings that 2-allyl-2-isopropylacetamide causes destruction of the haem moiety of cytochrome *P*-450 and that the residual apoprotein can then accept exogenous haem to reconstitute a functionally active holo-cytochrome (Sardana *et al.*, 1976; Unseld & De Matteis, 1978; Correia *et al.*, 1979; Farrell *et al.*, 1981). It can therefore be concluded from the present findings that the production of *N*-ethylprotoporphyrin by 4-ethyl-DDC utilizes a precursor pool of haem that can be depleted by 2-allyl-2-isopropylacetamide and replenished by exogenous haem, probably cytochrome *P*-450.

General discussion

The evidence produced in the present paper provides support for the view that *N*-ethylprotoporphyrin produced by treatment with 4-ethyl-DDC originates from haemoproteins of the *P*-450 group. The following lines of evidence suggest that the production of the different structural isomers of *N*-ethylprotoporphyrin is not tightly coupled but may take place on more than one site. (1) The isomeric composition of the alkylated porphyrin was found to differ slightly, but significantly, according to the dose of 4-ethyl-DDC administered. (2) In hepatocytes from phenobarbital-pretreated rats the inhibition of *N*-ethylprotoporphyrin formation by

2-allyl-2-isopropylacetamide and its stimulation by exogenous haem both concerned preferentially one of the four isomers. (3) Finally, both in the intact animal and in isolated hepatocytes the isomeric composition was found to depend on the cytochrome *P*-450 isoenzyme that predominated at the time of treatment.

It is suggested that the apoprotein of a cytochrome may specifically direct the alkyl group liberated from metabolism of a dihydropyridine on to one of the four pyrrole nitrogens, the nitrogen of rings A and D being preferentially attacked in the main phenobarbital- and β -naphthoflavone-inducible forms of the haemoprotein respectively. This selectivity may be achieved through a change in orientation of the hydrophobic binding site for a drug substrate with respect to the haem prosthetic group, so that a different pyrrole nitrogen is more accessible to alkylation; alternatively the apoprotein may cause a change in the state of haem leading to an increased reactivity of a different pyrrole nitrogen. It must be emphasized that the proposed role of the various apo-cytochromes in directing alkylation on to different pyrrole nitrogens is still largely hypothetical and more direct evidence is required.

There are, as yet, no results to link the third major isomer (N_C) of *N*-ethylprotoporphyrin to a specific haemoprotein. This isomer behaved differently from the other two major isomers in the dose-response experiment described in Fig. 2 and also after administration of inducers. So if a specific cytochrome is responsible for its production, a different cytochrome *P*-450 isoenzyme is likely to be involved.

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References

- Abbritti, G. & De Matteis, F. (1973) *Enzyme* **16**, 196–202
- Baird, M. B., Birnbaum, L. S., Samis, H. V., Massie, H. R. & Zimmerman, J. A. (1976) *Biochem. Pharmacol.* **25**, 2415–2417
- Coffman, B. L., Ingall, G. & Tephly, T. R. (1982) *Arch. Biochem. Biophys.* **218**, 220–224
- Conney, A. H. (1967) *Pharmacol. Rev.* **19**, 317–366
- Correia, M. A., Farrell, G. C., Schmid, R., Ortiz de Montellano, P. R., Yost, G. S. & Mico, B. A. (1979) *J. Biol. Chem.* **254**, 15–17
- De Matteis, F. & Gibbs, A. H. (1980) *Biochem. J.* **187**, 285–288
- De Matteis, F. & Prior, B. (1962) *Biochem. J.* **83**, 1–8
- De Matteis, F., Gibbs, A. H., Jackson, A. H. & Weerasinghe, S. (1980a) *FEBS Lett.* **119**, 109–112
- De Matteis, F., Gibbs, A. H. & Tephly, T. R. (1980b) *Biochem. J.* **188**, 145–152
- De Matteis, F., Gibbs, A. H., Farmer, P. B. & Lamb, J. H. (1981) *FEBS Lett.* **129**, 328–331
- De Matteis, F., Hollands, C., Gibbs, A. H., De Sa, N. & Rizzardini, M. (1982a) *FEBS Lett.* **145**, 87–92
- De Matteis, F., Jackson, A. H., Gibbs, A. H., Rao, K. R. N., Atton, J., Weerasinghe, S. & Hollands, C. (1982b) *FEBS Lett.* **142**, 44–48
- Farrell, G. C., Gollan, J. L., Correia, M. A. & Schmid, R. (1981) *J. Pharmacol. Exp. Ther.* **218**, 363–367
- Loosemore, M. J., Wogan, G. N. & Walsh, C. (1981) *J. Biol. Chem.* **256**, 8705–8712
- Lu, A. Y. H. & West, S. B. (1980) *Pharmacol. Rev.* **31**, 277–295
- Ortiz de Montellano, P. R. & Kunze, K. L. (1981) *J. Am. Chem. Soc.* **103**, 4225–4230
- Ortiz de Montellano, P. R. & Mico, B. A. (1981) *Arch. Biochem. Biophys.* **206**, 43–50
- Ortiz de Montellano, P. R., Beilan, M. S. & Kunze, K. L. (1981) *J. Biol. Chem.* **256**, 6708–6713
- Paine, A. J. & Legg, R. F. (1978) *Biochem. Biophys. Res. Commun.* **81**, 672–679
- Sardana, M. K., Rajamanickam, C. & Padmanaban, G. (1976) in *Porphyryns in Human Disease* (Doss, M., ed.), pp. 62–70, S. Karger, Basel
- Smith, A. G. & Francis, J. E. (1979) *Biochem. J.* **183**, 455–458
- Tephly, T. R., Gibbs, A. H. & De Matteis, F. (1979) *Biochem. J.* **180**, 241–244
- Tephly, T. R., Coffman, B. L., Ingall, G., Abou-Zeit-Har, M. S., Goff, H. M., Tabb, H. D. & Smith, K. M. (1981) *Arch. Biochem. Biophys.* **212**, 120–126
- Unsold, A. & De Matteis, F. (1978) *Int. J. Biochem.* **9**, 865–869
- Vainio, H. (1974) *Chem.-Biol. Interact.* **9**, 7–14
- Wada, O., Yano, Y., Urata, G. & Nakao, K. (1968) *Biochem. Pharmacol.* **17**, 595–603
- Waterfield, M. D., Del Favero, A. & Gray, C. H. (1969) *Biochim. Biophys. Acta* **184**, 470–473