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Mephenytoin-type polymorphism of drug oxidation: purification and characterization of a human liver cytochrome *P*-450 isozyme catalyzing microsomal mephenytoin hydroxylation

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A genetic polymorphism causing deficient metabolism of the anticonvulsant drug mephenytoin occurs in 5% of the Caucasian and 23% of the Japanese population. By monitoring the activities of the two major oxidative pathways of mephenytoin metabolism in the column eluates, we have purified from human livers a cytochrome *P*-450 isozyme, *P*-450 meph, which exclusively and stereoselectively catalyzes the 4-hydroxylation of (*S*)-mephenytoin, the major pathway affected by the polymorphism, whereas *P*-450 meph was virtually devoid of catalytic activity for *N*-demethylation of mephenytoin, the pathway remaining unaffected by the genetic deficiency. *P*-450 meph had an apparent M_r of 55 000 and a λ_{max} in the reduced CO-binding spectrum of 450 nm. Polyclonal rabbit antibodies against purified human *P*-450 meph almost completely inhibited the 4-hydroxylation of mephenytoin but had little effect on *N*-demethylation in human liver microsomes. In microsomes of liver biopsies of two subjects characterized in vivo as 'poor metabolizers' of mephenytoin, immunocrossreactive and immunoinhibitable material was observed with similar or identical properties to those of *P*-450 meph. There was no difference in the extent of the immunochemical reaction between microsomes of in vivo phenotyped poor metabolizers and extensive metabolizers of mephenytoin. These data suggest that *P*-450 meph is the target of the genetic deficiency and support the concept that a functionally altered variant form of *P*-450 meph causes this polymorphism.

Introduction

Genetically determined differences in oxidative drug metabolism have been reported for debrisoquine and a large number of related drugs (debrisoquine/sparteine-type polymorphism; for a review see Ref. 1), tolbutamide [2], antipyrine [3], nifedipine [4] and mephenytoin [5]. These polymorphisms give rise in the population to at least two phenotypes, so-called extensive (EM) and poor (PM) metabolizers of the particular drug. The phenotypes are defined in clinical and family studies, e.g., by urinary metabolic ratios after standard doses of the polymorphically metabolized substrate.

The genetic polymorphism of deficient metabolism of the anticonvulsant drug mephenytoin (3-methyl-5-phenyl-5-ethyl-hydantoin) is observed in

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; *P*-450, cytochrome *P*-450; EM, extensive metabolizer (phenotype); PM, poor metabolizer (phenotype)

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2–5% of Caucasian [6–8] and 23% of Japanese subjects [9]. This deficiency appears to be inherited as an autosomal recessive trait and concerns one of the two major metabolic pathways of mephenytoin, aromatic hydroxylation. The other major pathway, *N*-demethylation, remains unaffected.

Mephenytoin has a center of asymmetry at the 5-position of the hydantoin ring and its metabolism in man is highly stereoselective. Thus, in normal subjects the *S*-enantiomer is almost completely hydroxylated in the para position of the phenyl ring and rapidly excreted as (*S*)-4-hydroxymephenytoin glucuronide, whereas the *R*-enantiomer is preferentially *N*-demethylated and slowly excreted as (*R*)-5-phenyl-5-ethylhydantoin (PEH, nirvanol; Refs. 10 and 11). In hydroxylation-deficient subjects, the stereoselectivity of metabolite excretion after racemic mephenytoin is virtually absent, suggesting that the deficiency affects only the 4-hydroxylation of (*S*)-mephenytoin. *S*- and *R*-metabolites are excreted in equal amounts, predominantly as nirvanol [12]. These in vivo observations can be explained by characteristic enzymatic changes in liver microsomes of subjects phenotyped in vivo as poor metabolizers of mephenytoin. (*S*)-Mephenytoin 4-hydroxylation in these microsomes is characterized by an increased K_m, a decreased V_{max} and a loss of stereoselectivity [13]. However, the molecular mechanism responsible for the mephenytoin polymorphism has not been studied at the protein and DNA level.

We have recently provided evidence that another common polymorphism of drug metabolism which affects the oxidative metabolism of debrisoquine, sparteine, bufuralol and several other drugs (debrisoquine/sparteine-type polymorphism) is caused by the deficiency of one or possibly more than one specific hepatic cytochrome *P*-450 (*P*-450) isozyme [14,15,41]. In population and family studies, intrasubject comparison of the debrisoquine and mephenytoin hydroxylation phenotypes indicated that the two polymorphisms occur independently [6–8]. This has been supported by the lack of inhibition of sparteine oxidation by mephenytoin in human liver preparations in vitro [16]. As both major reactions of mephenytoin, hydroxylation and demethylation, are catalyzed by *P*-450-dependent monooxygenases [17,18], we have tested the hypothesis that in subjects with genetically deficient mephenytoin metabolism, a *P*-450 isozyme catalyzing the 4-hydroxylation of (*S*)-mephenytoin is wholly or partly missing or inactive, while the *P*-450 isozyme(s) for demethylation remain(s) intact. For this purpose, we have purified to electrophoretical homogeneity a human *P*-450 isozyme with high (*S*)-mephenytoin 4-hydroxylase activity, and have tentatively designated this cytochrome *P*-450 isozyme as *P*-450 meph. (Human cytochrome *P*-450 isozymes purified in our laboratory are strictly defined according to the catalytic activity on which their purification was based, i.e., *P*-450 meph, *P*-450 buf I, *P*-450 buf II. Other *P*-450 isozymes cited in this report are referred to by the designations used by the respective authors, e.g., $P450_8$ [35] and $P450_{MP}$ [39].) Polyclonal antibodies against *P*-450 meph were raised and were used to immunochemically characterize the mephenytoin 4-hydroxylase in microsomes of in vivo and in vitro phenotyped extensive and poor metabolizers of mephenytoin.

A recent paper by Shimada et al. [40] reported the purification of two cytochrome *P*-450 isozymes, $P450_{MP\text{-}1}$ (M_r 48000) and $P450_{MP\text{-}2}$ (M_r 50000) from human liver microsomes. Both enzymes catalyzed the 4-hydroxylation of (*S*)-mephenytoin, (*S*)-nirvanol and diphenylhydantoin. Cytochrome $P450_{MP\text{-}2}$ expressed considerable (*S*)-mephenytoin *N*-demethylase activity. The 4-hydroxylase activity of purified cytochrome $P450_{MP\text{-}1}$ and $P450_{MP\text{-}2}$ could be increased over that of intact microsomes only after addition of cytochrome b_5. Cytochrome $P450_{MP\text{-}1}$ was thought to be identical to cytochrome $P450_8$ (in Ref. 35). Immunochemical studies revealed no correlation between the amount of either enzymic form present in microsomes and the in vitro determined capacity of 35 human liver microsomal preparations for (*S*)-mephenytoin 4-hydroxylation.

Materials and Methods

Purification of P-450 meph

Microsomes from kidney transplant donor livers were prepared as described previously [19]. Prior to selection of a particular liver for purification,

microsomes from this liver were characterized in vitro as extensive metabolizers of mephenytoin [13].

ω-Aminooctyl-Sepharose 4B step. The supernatant of cholate-solubilized microsomes (3300 nmol *P*-450 [14]) was applied to four ω-aminooctyl-Sepharose 4B columns (2.6 × 40 cm) operated in parallel, which had previously been equilibrated with buffer A, composed of 100 mM potassium phosphate (pH 7.25), 20% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 20 μM butylated hydroxytoluene and 0.6% (w/v) cholic acid at a flow rate of 60 ml · h^{-1}. The columns were then washed with buffer B, which was the same as buffer A except that cholic acid was lowered to 0.42% (w/v), until no further protein elution was observed. Bulk cytochrome *P*-450 could be eluted with buffer C, which contained 0.33% (w/v) cholic acid and 0.06% (w/v) Lubrol PX. When approximately 1800 ml of buffer C had passed through each column, *P*-450 and protein elution ceased. The remainder of cytochrome *P*-450 was eluted with 800 ml buffer D, containing 0.33% (w/v) cholic acid and 0.5% (w/v) Lubrol PX. As in the following chromatography steps, an aliquot of every fifth fraction of the column eluates was assayed for mephenytoin 4-hydroxylation and *N*-demethylation as described below. The same fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Those fractions with the highest mephenytoin 4-hydroxylase activity and the highest purity on SDS-polyacrylamide gel electrophoresis were pooled and arbitrarily named the AO-2 pool.

First hydroxyapatite step (Fig. 1). The AO-2 pool (66 nmol *P*-450) was brought to 18% (w/v) saturation with PEG 6000, stirred for 20 min at 4°C and centrifuged at 105 000 × *g* for 60 min. The pellet was resuspended in 5 mM potassium phosphate (pH 7.25), 20% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.2% (w/v) Lubrol PX and 0.2% (w/v) cholic acid and extensively dialyzed against the same buffer. The dialyzed material was then applied onto a hydroxyapatite column (Bio-Rad HTP, 1.6 × 16 cm) previously equilibrated with the dialysis buffer. After washing the column, cytochrome *P*-450 was eluted with the same buffer except that potassium phosphate was linearly increased from 5 to 180 mM in a total volume of 300 ml (flow rate = 30 ml · h^{-1}). Fractions enriched in mephenytoin 4-hydroxylase activity were pooled (HAP pool II).

DE-52 cellulose step (Fig. 2). The HAP pool II (10.2 nmol *P*-450) was treated with Bio-Beads SM-2 (0.1 g/ml, 2 × 1 h stirring at 4°C), concentrated over a Diaflow ultrafiltration membrane (PM 30) and extensively dialyzed against 5 mM potassium phosphate (pH 7.8), containing 20% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.2% (w/v) Lubrol PX and 0.2% (w/v) cholic acid. The dialyzed sample was applied to a DE-52 cellulose column (1.6 × 16 cm) equilibrated with the dialysis buffer. Washing of the column with the above buffer resulted in the flowthrough eluting of all the mephenytoin 4-hydroxylase activity. These fractions were concentrated and designated DE pool A.

Second hydroxyapatite step. The DE pool A (3.5 nmol *P*-450) was extensively dialyzed against 5 mM potassium phosphate (pH 7.25), 20% glycerol, 0.1 mM EDTA and 0.2% (w/v) Lubrol PX and applied onto a second hydroxyapatite column (0.9 × 2 cm) equilibrated with the dialysis buffer. The column was eluted by a stepwise gradient of 20, 40, 90, 180 and 300 mM potassium phosphate (20 ml each) and fractions of 1 ml were collected. Only with 90 and 180 mM potassium phosphate could a considerable amount of hemoprotein be eluted. Both fractions were treated repeatedly with Bio-Beads SM-2 until no further increase in activity was observed. They were then concentrated and dialyzed against 100 mM potassium phosphate (pH 7.4), 20% glycerol and stored in liquid nitrogen. Both preparations exhibited mainly mephenytoin 4-hydroxylase activity and virtually no *N*-demethylase activity. There was no indication that either activity was increased upon further detergent removal. The two fractions thus were designated *P*-450 meph, based on their main catalytic activity.

Reconstitution of mephenytoin 4-hydroxylation and demethylation activity

Screening for mephenytoin 4-hydroxylation and N-demethylation capacity of column eluates. This laboratory has described in detail the analysis by HPLC of mephenytoin metabolism by human liver microsomes [18]. Essentially the same procedure

was followed to analyze for reconstituted mephenytoin oxidase activity. Briefly 100 μl aliquots of individual fractions were freed of Lubrol PX [20] and incubated with 0.3 units NADPH–cytochrome-*P*-450 reductase, 5 mM $MgCl_2$, 5 mM isocitrate, 1 unit isocitrate dehydrogenase/ml, 1 mM $NADP^+$ and 1 mM racemic mephenytoin in 1% (v/v) propylene glycol in a final volume of 250 μl 100 mM potassium phosphate (pH 7.4). The samples were preincubated at 4°C for 30 min prior to addition of the NADPH generating system and at 37°C for 5 min before addition of the substrate. Incubation, extraction and analysis was as described, as were the detection and error limits of the assay [18]. At early stages of purification (ω-aminooctyl-Sepharose and first hydroxyapatite step) the assay was not optimized for linearity with respect to time and protein concentration.

Reconstitution of purified cytochrome P-450 meph

Optimized reconstitution conditions with respect to the molar ratio of cytochrome *P*-450 to NADPH–cytochrome-*P*-450 reductase, lipid to *P*-450, as well as to linearity with time were maintained in the course of kinetic and functional studies of the purified enzyme. Under standard conditions, 10 pmol of purified cytochrome *P*-450 meph, 100 pmol NADPH–cytochrome-*P*-450 reductase (calculated on the basis of an M_r of 78000) and 20 μg of dilauroyl-L-α-phosphatidylcholine/ml were incubated in the presence of the NADPH generating system cited above and the indicated substrate and propylene glycol concentrations (Tables II and III) in a final volume of 100 μl 0.1 M potassium phosphate (pH 7.4). With higher substrate concentrations and in immunoinhibition studies, the incubation volume was increased to 500 μl containing 80 pmol *P*-450 meph and 1 nmol reductase. In kinetic experiments, microsomes were incubated in a final volume of 250 μl with 25 or 200 pmol microsomal *P*-450 for (*S*)- and (*R*)-mephenytoin, respectively.

Immunochemical procedures

Production of polyclonal antibodies. Polyclonal antibodies against purified cytochrome *P*-450 meph were raised in female New Zealand White rabbits. Animals were injected intradermally with 20 μg of antigen mixed 1:1 (v/v) with complete Freund's adjuvant. After 3 weeks the animals were boosted with a subcutaneous injection of again 20 μg of antigen in incomplete Freund's adjuvant. Antisera were collected 1 week later. Every 3 weeks thereafter, rabbits were boosted with 10 μg of antigen and blood was taken 1 week later. The IgG fraction of each antiserum was prepared [21] and stored in 100 mM potassium phosphate (pH 7.4), 20% (v/v) glycerol at −20°C.

Western blotting. Microsomal protein (100 μg applied per cm slot) and purified isozymes (2 μg per cm slot) were resolved on SDS-polyacrylamide gel electrophoresis and transferred from polyacrylamide gels to nitrocellulose sheets according to Towbin et al. [22]. Nitrocellulose sheets were coated at 4°C overnight with 10% (v/v) fetal calf serum in phosphate-buffered saline and then cut into strips of 0.15 × 10 cm. All further incubations and washes were carried out in the same buffer at room temperature. Strips were successively incubated for 2 h in 500 μl final volume with 5 μg of the respective IgG preparation, 5 μg goat anti-rabbit IgG (Nordic, GAR/7S) and with peroxidase-antiperoxidase complex (Nordic, R/PAP) at a 1:20000 dilution, with extensive washes for 30 min between each step. The peroxidase stain was then developed as described [23,24].

Immunoinhibition. In immunoinhibition studies 10 or 30 pmol microsomal cytochrome *P*-450 for (*S*)- and (*R*)-mephenytoin oxidation, respectively, were preincubated with the indicated amounts of IgG fractions (previously dialyzed against the incubation buffer) for 30 min at room temperature in 200 μl 0.1 M potassium phosphate buffer (pH 7.4). Inhibition in microsomes of poor metabolizer individuals was done with 35 pmol *P*-450 for both mephenytoin enantiomers to obtain measurable quantities of 4-hydroxymephenytoin. Incubation with the NADPH generating system and 800 μM mephenytoin in 0.5% propylene glycol was then performed in a final volume of 250 μl and metabolites were analyzed as described [18].

Other methods

NADPH–cytochrome-*P*-450 reductase was purified from untreated rat liver microsomes and assayed at room temperature with cytochrome *c* as electron acceptor in 0.3 M potassium phosphate

(pH 7.7) [25,26]. One unit is defined as the amount of enzyme that reduces 1 μmol cytochrome *c* per min. Cytochrome *P*-450 was measured spectrophotometrically as the reduced heme-CO complex according to Omura and Sato [27] using an ε_{mM} of 91. Protein concentration was determined according to Lowry et al. [28] with minor modification in order to avoid interferences due to detergents [29]. SDS-polyacrylamide gel electrophoresis was performed in the Laemmli system [30]. Kinetic parameters (K_m, V_{max}) were calculated by linear regression analysis using Eadie-Hofstee plots [31]. 7-Ethoxycoumarin *O*-dealkylation and *p*-nitroanisole *O*-demethylation were measured as described [19].

Materials

Chemicals and instrumentation for HPLC analysis of mephenytoin and its metabolites have been described in detail elsewhere [18]. ω-Aminooctyl-Sepharose 4B was prepared as described [32]. CNBr-activated Sepharose 4B was purchased from Pharmacia. Sigma was the source of cholic acid, Lubrol PX, and PMSF. NADPH, isocitrate and isocitrate dehydrogenase were from Boehringer, Mannheim.

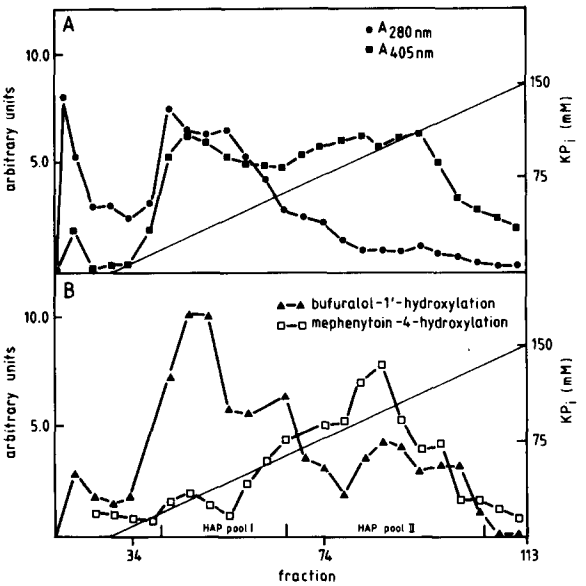


Fig. 1. Elution profile of mephenytoin 4-hydroxylation activity on the first hydroxyapatite column. (A) The protein ($A_{280\ nm}$) and heme ($A_{405\ nm}$) absorption were continuously recorded during chromatography of AO-2 material on hydroxyapatite. (B) An aliquot of every fourth fraction was reconstituted and assayed in parallel for its capacity for mephenytoin 4-hydroxylation and bufuralol 1'-hydroxylation, respectively. Samples were combined for further purification as HAP pool I and HAP pool II. The linear gradient (300 ml) from 5 to 180 mM potassium phosphate (KP_i) started at fraction 30.

Results

Purification of cytochrome P-450 meph

Hydrophobic chromatography on ω-aminooctyl-Sepharose 4B of solubilized liver microsomes of extensive metabolizer subjects (organ transplant donors) of both mephenytoin and bufuralol revealed that a considerable amount of mephenytoin 4-hydroxylation activity coeluted with the bulk of bufuralol 1'-hydroxylation activity [14] in the high-detergent buffer D and was almost completely separated from mephenytoin *N*-demethylase activity which eluted with the low-detergent buffer C (results not shown). This 'AO-2' pool was further chromatographed on hydroxyapatite. As illustrated in Fig. 1B, the bulk of mephenytoin 4-hydroxylation activity eluted in the HAP pool II. When analyzed on SDS-polyacrylamide gel electrophoresis, this material showed two main protein bands at 50 kDa and 55 kDa, respectively, with some minor contaminants (Fig. 3, lane 2). Chromatography of the HAP pool II material on DE-52 cellulose resulted in the separation of mephenytoin 4-hydroxylation activity, which was exclusively found in the flow-through fractions (DE-pool A, Table I), from the electrophoretically homogeneous bufuralol 1'-hydroxylation activity (*P*-450 buf II, see Fig. 2 and Fig. 3, lane 3). Rechromatography of DE-pool A on a second hydroxyapatite column resulted in the elution of two fractions of electrophoretically almost completely homogeneous mephenytoin 4-hydroxylase at 90 and at 180 mM potassium phosphate (Fig. 3, lanes 4 and 5). The two hemoprotein preparations could not be distinguished by apparent molecular weight (55 000) and functional or immunochemical properties and in this report are therefore interchangeably used and referred to as '*P*-450 meph'. The overall yield of *P*-450 meph, obtained by this procedure, was about 0.03% of total microsomal cytochrome *P*-450 (Table I). Only narrow peak fractions of mephenytoin 4-hydroxyl-

TABLE I

PURIFICATION FROM HUMAN LIVER MICROSOMES OF MEPHENYTOIN 4-HYDROXYLASE

P-450 meph eluting at 90 or 180 mM potassium phosphate could not be distinguished by spectral, electrophoretic or immunochemical techniques (see text).

Step	Protein (mg)	*P*-450 (nmol)	Specific content (nmol *P*-450/mg protein)	Yield (% *P*-450)
Microsomes	10120	3342	0.33	100
ω-Aminooctyl-Sepharose 4B (AO-2) eluate at 0.5% Lubrol PX	36.6	66	1.8	1.97
1. Hydroxyapatite column (HAP)				
HAP pool I	23.5	49.3	2.1	1.47
HAP pool II	7.46	10.2	1.5	0.3
DE-52 cellulose (DE)				
DE-pool A	2.98	3.5	4.1	0.15
2. Hydroxyapatite column (HAP)				
P-450 meph eluted at 90 mM potassium phosphate	0.042	0.3	6.8	0.009
P-450 meph eluted at 180 mM potassium phosphate	0.12	0.77	6.4	0.02

ation and bufuralol 1′-hydroxylation activities were pooled throughout the four-step purification scheme. Thus, a low yield of *P*-450 meph was accepted in order to increase the purity of the fractions for further processing. A specific content of 6.6 nmol *P*-450/mg protein was determined by CO-difference spectrum and Lowry protein determination. Although a purified *P*-450 of M_r 55 000 would be expected to have a specific content of approx. 18 nmol *P*-450/mg protein, this value is rarely achieved with minor forms of *P*-450 isozymes. Several possibilities may explain a low content of heme relative to apoprotein, namely loss of prosthetic heme during purification, insufficient reduction of oxidized heme iron [34], overestimation of the protein by the Lowry procedure [28,33] or contamination by proteins with the same molecular weight. The thus determined value is nevertheless comparable to the range of specific contents observed previously for a number of electrophoretically homogeneous purified human liver *P*-450 isozymes [35]. The CO-difference spectrum showed a maximum at 450 nm and the preparation seemed to be predominantly in the low-spin state as judged by its absorption maximum at 416 nm in the oxidized form.

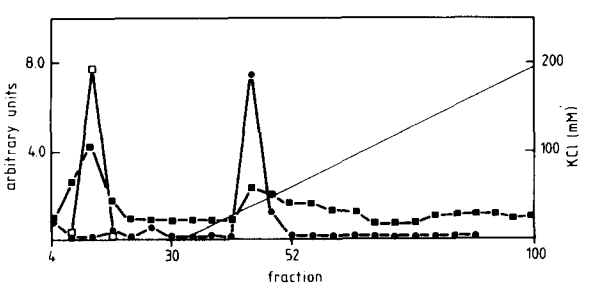


Fig. 2. Elution profile of mephenytoin 4-hydroxylation activity from DE-52 cellulose. HAP pool II material (see Fig. 1) was chromatographed on DE-52 cellulose. Protein absorption at 280 nm (■), bufuralol 1′-hydroxylation activity (●) and mephenytoin 4-hydroxylation activity (□) were monitored. The linear gradient (300 ml) of 0–250 mM potassium chloride started at fraction 30.

Reconstitution of cytochrome P-450 meph

The rate of formation of mephenytoin metabolites by reconstituted *P*-450 meph was linear with incubation times of 15–60 min. The system was optimized in respect to maximal activity of mephenytoin 4-hydroxylation. A molar ratio of NADPH–cytochrome-*P*-450 reductase/*P*-450 meph of 10 : 1 and concentrations of dilauroyl-L-α-phosphatidylcholine of 20 μg/ml were found to result in the highest velocity of the reaction (data not shown). Higher protein concentrations (*P*-450 and reductase at constant ratio) led to a decrease

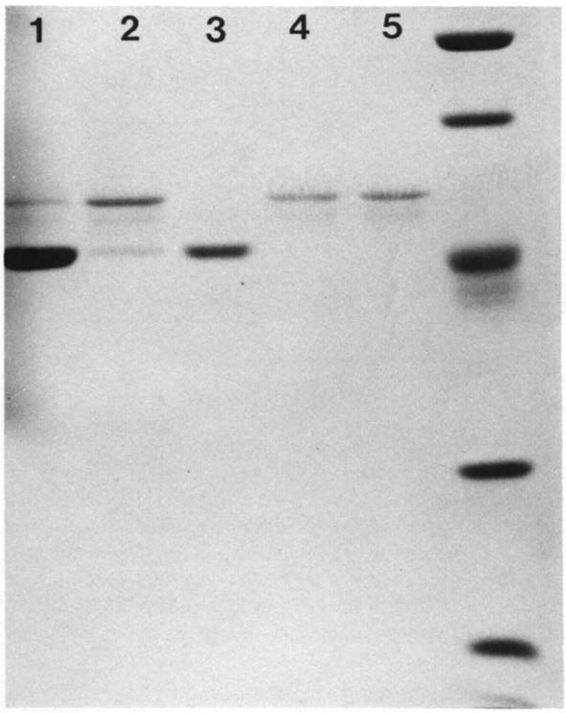


Fig. 3. SDS-polyacrylamide gel electrophoresis of purified *P*-450 meph. SDS-polyacrylamide gel electrophoresis was performed in the discontinuous Laemmli system [30] with 10% polyacrylamide separating gels. Lane 1, 2 μg of the AO-2 fraction; lane 2, 3 μg of HAP pool II; lane 3, 1.5 μg of *P*-450 buf II; lane 4, 3 μg of *P*-450 meph, eluted at 90 mM potassium phosphate from the second hydroxyapatite column; lane 5, 3 μg of *P*-450 meph, eluted at 180 mM potassium phosphate. The molecular weights of marker enzymes are (from top to bottom): 92500, 66200, 45000, 31000 and 21500.

in the specific activity as did higher incubation volumes at constant concentrations of all components. Similar effects have also been reported for reconstituted benzphetamine demethylase activity at protein concentrations higher than 0.1 μM by Guengerich and Holladay [36]. Mephenytoin 4-hydroxylation was inhibited to 40% of control by 0.05% (w/v) Emulgen 911. It has to be realized that in vitro reconstituted *P*-450 reactions and microsomal activities are not directly comparable because of the complexities of the interactions between the different components, solvent effects,

TABLE II

KINETIC PARAMETERS OF (*S*)- AND (*R*)-MEPHENYTOIN HYDROXYLATIOIN

The substrate dependence of the hydroxylation of (*S*)- and (*R*)-mephenytoin by microsomes of in vivo and in vitro phenotyped extensive (EM) and poor (PM) metabolizer individuals and of reconstituted *P*-450 meph was measured. Kinetic parameters were estimated on Eadie-Hofstee plots. Substrate concentrations were varied from 25 to 400 μM with the *S*- and from 25 to 1600 μM with the *R*-enantiomer. Values are means of two experiments with triplicate determinations.

	Propylene glycol (% (v/v))	4-Hydroxy-mephenytoin		Nirvanol	
		K_m [a]	V_{max} [b]	K_m	V_{max}
(*S*)-Mephenytoin					
EM microsomes [c]	0.25	16.5	12.29	– [d]	– [d]
	1.0	19.6	10.07	– [d]	– [d]
PM microsomes [c]	0.25	150.6	2.17	– [d]	– [d]
reconstituted *P*-450 meph	0.25	65.4	19.35	n.d. [e]	n.d.
	1.0 [f]	431.7	11.13	– [d]	– [d]
(*R*)-Mephenytoin					
EM microsomes [c]	1.0	492	1.19	609	16.85
PM microsomes	1.0	621	2.56	(1719) [g]	(66.7) [g]
reconstituted *P*-450 meph	1.0	675	0.38	n.d.	n.d.

[a] μM.
[b] nmol product formed·nmol $P\text{-}450^{-1}\cdot h^{-1}$.
[c] Data from Refs. 13 and 18.
[d] Production rate was linear within the substrate concentration range tested, no K_m and V_{max} estimation was therefore possible.
[e] n.d., not detectable.
[f] Substrate concentration was varied from 25 to 800 μM.
[g] Because of the low affinity, extrapolation of K_m and V_{max} represent only rough estimates.

residual detergents, etc. [37]. This may in part explain the only slightly increased 4-hydroxy-mephenytoin formation with *P*-450 meph as compared to microsomes (Table II).

Functional characterization of cytochrome P-450 meph

The mephenytoin 4-hydroxylase activity of purified *P*-450 meph was compared with that of microsomes of in vivo and in vitro phenotyped EM and PM subjects [13]. Using *R*- and *S*-enantiomers of mephenytoin (1% (v/v) final concentration of the solvent propylene glycol) as substrates (1 mM) a *R*/*S* ratio of 0.10 was observed for the formation of 4-hydroxy-mephenytoin in the reconstituted system, a value almost identical to that obtained in microsomes of a total of 13 EM individuals (i.e., 0.11 ± 0.04, mean $\pm$ S.D.) and quite different to the ratios of 1.10 and 0.76 observed in two PM individuals (Fig. 7; Ref. 13). The substrate-dependent formation of 4-hydroxy-mephenytoin by *P*-450 meph was investigated both with *S*- and *R*-enantiomers of mephenytoin. In experiments with (*R*)-mephenytoin, a poor substrate for 4-hydroxylation, the incubation conditions of the reconstituted system had to be modified (to the ones described for the immunoinhibition studies, see Materials and Methods) in order to obtain measurable amounts of 4-hydroxy-mephenytoin also at low substrate concentrations. A considerable problem was the poor solubility of mephenytoin [16,18]. At high substrate concentrations increased addition (i.e., up to 1% (v/v)) of the organic solvent propylene glycol was required, but this resulted in decreased production rates of 4-hydroxy-mephenytoin (Table II; Ref. 18). To maintain comparability, kinetic parameters of (*S*)-mephenytoin oxidation therefore were determined at 0.25% and 1.0% (v/v) propylene glycol (Table II). Purified and reconstituted *P*-450 meph exhibited Michaelis-Menten kinetics for mephenytoin 4-hydroxylation (Fig. 4), whereas the trace of mephenytoin *N*-demethylation activity associated with *P*-450 meph showed linearly increasing production rates within the substrate concentration range tested (Table II; Ref. 18). As opposed to incubations of microsomes, the propylene glycol concentration had a marked effect on the affinity of purified *P*-450 meph for (*S*)-

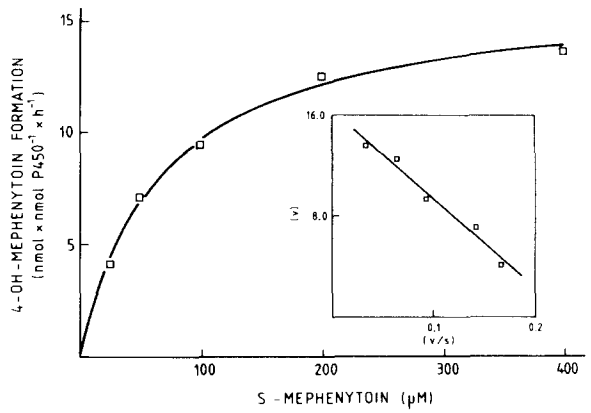


Fig. 4. Substrate-dependent formation of 4-hydroxy-mephenytoin by purified *P*-450 meph. Purified *P*-450 meph (10 pmol) was reconstituted with reductase (100 pmol) and dilauroyl-L-α-phosphatidylcholine (20 μg/ml) in 100 μl final volume as described. (*S*)-mephenytoin concentration was varied from 25 to 400 μM in 0.25% (v/v) final concentration of propylene glycol. Values represent means of triplicate determinations. Kinetic parameters were calculated by linear regression analysis of Eadie-Hofstee plots (inset).

mephenytoin, possibly due to solvent interactions with the purified isozyme (Table II; Ref. 18). The kinetic parameters determined for substrate-dependent (*R*)-mephenytoin 4-hydroxylation by PM microsomes are in accordance with previous findings in vitro [13] and in vivo [38] and demonstrate that only (*S*)-mephenytoin hydroxylation is affected in individuals of the poor metabolizer phenotype.

Immunochemical characterization of cytochrome P-450 meph crossreactivity

Polyclonal antibodies against *P*-450 meph were raised in rabbits and IgG fractions were prepared as described [21]. The specificity of these IgG preparations was examined on Western blots. Anti-*P*-450 meph IgG recognized the homologous antigen on Western blots (Fig. 5, lane A) and mainly one single band of corresponding molecular weight in human liver microsomes (Fig. 5, lane C). Interestingly, antibodies raised in rabbits against a human liver *P*-450 isozyme purified by Wang et al. and named $P450_8$ [35] kindly provided by Philippe Beaune, Paris, recognized purified *P*-450 meph (Fig. 5, lane B)) and a major protein band corresponding in molecular weight

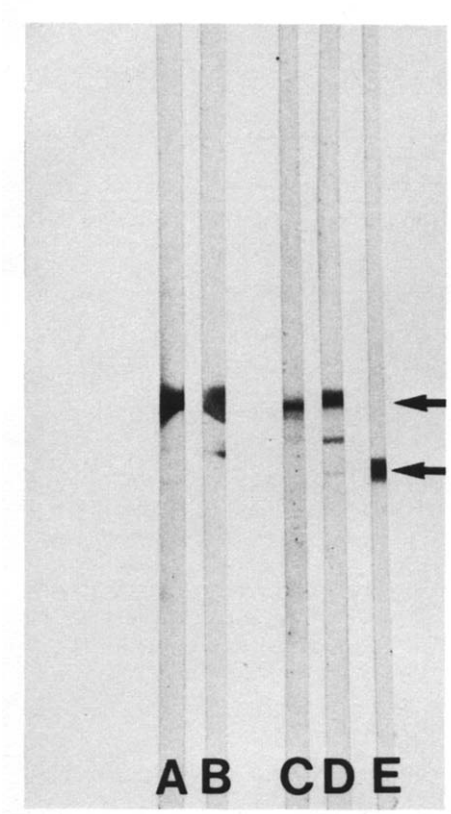


Fig. 5. Immunochemical relatedness of *P*-450 meph to $P450_8$. Purified *P*-450 meph was transferred from polyacrylamide gels to nitrocellulose sheets and strips were incubated with anti-*P*-450 meph IgG (lane A) or anti-$P450_8$ sera (lane B) and developed as described in Materials and Methods. The cross-reactivity of anti-*P*-450 meph IgG (lane C), anti-$P450_8$ serum (lane D) and anti-*P*-450 buf I IgG (lane E) with polypeptides of other molecular weights than that of the homologous antigen was examined on Western blots of human liver microsomes. Arrows indicate molecular weights of 55000 (upper) and 50000 (lower).

to *P*-450 meph (M_r 55000) in human liver microsomes (Fig. 5, lane D) besides a protein of lower molecular weight. On the other hand, as exemplified with anti-*P*-450 buf I (Fig. 5, lane E), antibodies against *P*-450 buf I and *P*-450 buf II, two functionally distinct *P*-450 isozymes which are purified concomitantly to *P*-450 meph from the same human livers [41], did not recognize *P*-450 meph or a protein band corresponding in molecular weight to *P*-450 meph in human liver microsomes.

Immunoinhibition

Anti-*P*-450 meph inhibited the 4-hydroxylation of both (*S*)- and (*R*)-mephenytoin in human liver microsomes and with reconstituted *P*-450 meph in a concentration-dependent manner (Fig. 6A and B). A residual activity of 14% and 19% for EM microsomes and reconstituted *P*-450 meph was observed at a ratio of 16 mg IgG/nmol *P*-450 with (*S*)-mephenytoin as substrate. The corresponding values with (*R*)-mephenytoin were 46% and 72%, respectively (Table II). The nirvanol production from both enantiomers was only slightly inhibited in microsomes of either phenotype, suggesting that isozymes other than *P*-450 meph catalyze the *N*-demethylation of mephenytoin (Table III and Fig. 6B). Interestingly, residual mephenytoin 4-hydroxylation activity, exhibited by microsomes of an in vivo and in vitro phenotyped PM of mephenytoin, also was inhibited by anti-*P*-450 meph to about 70% for the *S*-enantiomer and to about 50% for the *R*-enantiomer. Again, a possible immunochemical relatedness of *P*-450 meph and human cytochrome $P450_8$

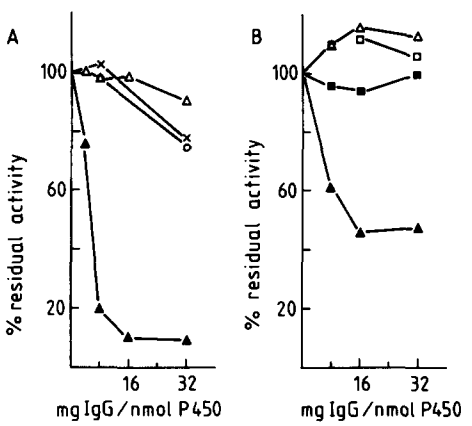


Fig. 6. Immunoinhibition by polyclonal anti-*P*-450 meph antibodies of microsomal mephenytoin 4-hydroxylase activity. (A) Concentration-dependent inhibition of microsomal (*S*)-mephenytoin 4-hydroxylase activity by anti-*P*-450 meph IgG (▲), anti-*P*-450 buf I IgG (○), anti-*P*-450 buf II IgG (×) and preimmune IgG (△). The concentration of (*S*)-mephenytoin was 800 μM (propylene glycol 1% (v/v)). (B) Concentration-dependent inhibition of (*R*)-mephenytoin 4-hydroxylation by anti-*P*-450 meph IgG (▲) and preimmune IgG (△) and inhibition of (*R*)-mephenytoin *N*-demethylation by anti-*P*-450 meph IgG (■) and preimmune IgG (□). The concentration of (*R*)-mephenytoin was 1600 μM (propylene glycol 1% (v/v)). Incubation conditions were as described in the text.

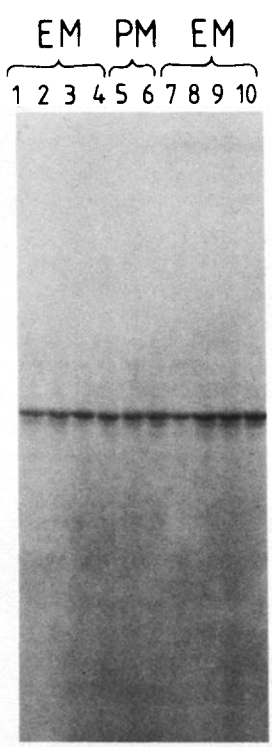
TABLE III

IMMUNOINHIBITION BY ANTI-*P*-450 MEPH OF MEPHENYTOIN 4-HYDROXYLATION AND MEPHENYTOIN *N*-DEMETHYLATION

16 mg IgG/nmol *P*-450 were used in microsomes of in vivo and in vitro phenotyped extensive (EM) and poor (PM) metabolizer subjects of mephenytoin and in reconstituted *P*-450 meph.

	4-Hydroxy-mephenytoin production (nmol·nmol *P*-450^{-1}·h^{-1})				Nirvanol production (nmol·nmol *P*-450^{-1}·h^{-1})			
	control	preimmune IgG	anti-*P*-450 meph	residual activity (%)	control	preimmune IgG	anti-*P*-450 meph	residual activity (%)
(*S*)-Mephenytoin								
EM microsomes	14.05	14.54	1.90	14 (2) [a]	39.75	40.91	30.78	77 (111) [a]
PM microsomes	1.80	1.92	0.57	32 (30) [a]	47.59	49.41	33.79	72 (82) [a]
reconstituted *P*-450 meph [b]	7.45	5.51	1.43	19	0.73	0.51	0.51	0.2636
(*R*)-Mephenytoin								
EM microsomes	0.94	1.10	0.43	46	14.66	16.36	13.77	94
PM microsomes	1.59	1.56	0.79	50	26.65	25.95	21.25	80
reconstituted *P*-450 meph [c]	0.25	0.30	0.18	72	n.d. [d]	n.d.	n.d.	

[a] Data in parentheses were obtained with anti-$P450_8$.
[b] Substrate concentration was 800 μM (*S*)-mephenytoin in 1% propylene glycol.
[c] Substrate concentration was 1600 μM (*R*)-mephenytoin in 1% propylene glycol.
[d] n.d., not detectable.



	Subject	HI	R/S-Ratio	Phenotype
1	P.J.	1.9	0.11	EM
2	M.C	6.4	0.08	
3	L.P.	8.0	0.07	
4	T.S	1.8	0.12	
5	V.A.	127.3	0.76	PM
6	R.E.	53.4	1.10	
7	S.J	1.8	0.14	EM
8	M.M.	1.7	0.07	
9	G.L.	1.6	0.12	
10	G.M	2.0	0.21	

Fig. 7. Western blotting using anti-*P*-450 meph serum of human liver microsomes of in vivo and in vitro phenotyped extensive (EM) and poor (PM) metabolizer subjects of mephenytoin. 17 μg microsomal protein was separated on SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose sheet [22] which was incubated with anti-*P*-450 meph serum (dilution 1 : 100) and developed as described in Material and Methods. Microsomes from in vivo and in vitro phenotyped poor and extensive metabolizer subjects were of the same individuals as previously described [13]. HI, mephenytoin hydroxylation index (micromoles of (*S*)-mephenytoin administered/micromoles of 4-hydroxy-mephenytoin in urine collected for 8–12 h); *R*/*S* ratio, ratio of (*R*)- and (*S*)-mephenytoin 4-hydroxylation in microsomes [13].

[35] was observed, since anti-$P450_8$ sera caused about the same extent of immunoinhibition as did anti-*P*-450 meph, and this in both EM and PM microsomes (Table III). Virtually no inhibition of mephenytoin 4-hydroxylation activity by antisera against two other human *P*-450s, *P*-450 buf I and *P*-450 buf II, was observed (Fig. 6A). On the other hand, anti-*P*-450 meph had no influence on bufuralol 1′-hydroxylase activity and did not inhibit ethoxycoumarin *O*-deethylation and *p*-nitroanisole *O*-demethylation in human liver microsomes (data not shown).

Immunoreactivity of P-450 meph in liver microsomes of EM and PM phenotype subjects

Polyclonal antibodies against cytochrome *P*-450 meph were used to immunoquantitate this enzyme on Western blots of microsomes of the EM or PM phenotype. There was no visible difference in the extent of the immunochemical reaction between microsomes of in vivo and in vitro phenotyped [13] PM and EM individuals (Fig. 7). In addition no difference was observed using anti-$P450_8$ sera to detect $P450_8$ on Western blots of microsomes of both phenotypes (results not shown). There was no correlation between the extent of immunochemical reaction and in vivo hydroxylation index nor the in vitro R/S ratio of the two phenotypes (Fig. 7).

Discussion

A *P*-450 isozyme which catalyzes the 4-hydroxylation of mephenytoin, *P*-450 meph, was purified to electrophoretical homogeneity from microsomes of different human livers. The following findings suggest that this isozyme is the target of the genetic deficiency known as mephenytoin polymorphism. (1) The purified enzyme catalyzes almost exclusively the 4-hydroxylation and not the *N*-demethylation of mephenytoin. The polymorphism indeed affects only the 4-hydroxylation pathway of mephenytoin metabolism [13,38]. (2) The purified enzyme metabolizes mephenytoin in a highly stereoselective fashion, preferring the *S*-enantiomer as substrate for 4-hydroxylation. The selectivity of the aromatic hydroxylation pathway for (*S*)-mephenytoin and its specific involvement in the polymorphism has been documented in pharmacokinetic studies in vivo [38] and in human liver microsomes in vitro (Table II; Ref. 13). (3) Polyclonal antibodies against purified *P*-450 meph almost completely inhibited the 4-hydroxylation of mephenytoin but had little effect on *N*-demethylation. The collective evidence from these data suggests that *P*-450 meph accounts for most of the normal microsomal 4-hydroxylation activity and that its deficiency may cause the mephenytoin polymorphism. This hypothesis is supported by the drastically decreased V_{max} of 4-hydroxylation of (*S*)-mephenytoin in liver microsomes of two subjects characterized in vivo as poor metabolizers (PM) of mephenytoin (Table II; Ref. 13). As a consequence of a loss or functional deficiency of this highly stereoselective enzyme one would expect a preferential loss of (*S*)-mephenytoin metabolism in poor metabolizers. This was indeed found in the micrososomes of two carriers of this genetic deficiency [13].

In order to understand the mechanism of the mephenytoin polymorphism, the inhibitory polyclonal anti-*P*-450 meph antibodies were used to test for the presence or absence of immunoreactive material in PM microsomes (Fig. 7). Immunoreactive material of the appropriate M_r was clearly present in all microsomes of both phenotypes and there was no correlation between microsomal mephenytoin 4-hydroxylation activity and the extent of the immunochemical reaction. Moreover, the residual hydroxylation activity in PM microsomes was still inhibited by anti-*P*-450 meph. These data are indicative of the presence in PM microsomes of immunocrossreactive and immunoinhibitable material with similar or identical properties to those of *P*-450 meph. Possible analogy to the polymorphism affecting bufuralol hydroxylation described in previous reports from this laboratory [14,15], these data provide a strong argument against the total absence of *P*-450 meph as the cause of the mephenytoin polymorphism. They rather support the presence of a functionally altered variant form of *P*-450 meph in PM microsomes. Unfortunately, evaluation of this hypothesis requires access to sufficient quantities of liver tissue of an in vivo characterized PM subject to analyze functional and structural characteristics of the crossreactive enzyme(s). For ethical and other reasons, this material has not been available to

date. It cannot be completely ruled out that another non-*P*-450 protein of identical molecular weight is recognized in both EM and PM microsomes in Western blots. As the antigen against which the polyclonal serum was raised had a low heme content, this possibility has to be taken into consideration. However, the anti-$P450_8$ sera raised against a *P*-450 preparation with a higher heme content [35] yielded the same results.

P-450 meph could clearly be separated by our chromatographic procedures from two other *P*-450 isozymes, *P*-450 buf I and *P*-450 buf II, which are involved in the debrisoquine/sparteine type polymorphism of drug oxidation [14,15,41] and also from the fractions which catalyze the *N*-demethylation of mephenytoin. In other terms, the approach of monitoring specific catalytic activities in column eluates throughout the procedure allows the isolation of several different *P*-450 isozymes from the same human liver preparation.

Moreover, monitoring the prototype reactions of a number of independently occurring genetic polymorphisms of drug oxidation, the purification from the same human liver of the respective, presumably distinct *P*-450 isozymes becomes feasible. *P*-450 meph (apparent M_r 55000) appears to be related to or identical with $P450_8$, one of six *P*-450 isozymes purified from human liver in another laboratory [35]. However, the apparent M_r of $P450_8$ was reported as 49600. Anti-$P450_8$ serum recognized purified *P*-450 meph as well as one major band of similar molecular weight in human liver microsomes and inhibited (*S*)-mephenytoin 4-hydroxylation both in EM and PM microsomes, indicating a strong immunochemical relatedness if not identity of *P*-450 meph and $P450_8$. We have no explanation for the discrepancy in the apparent M_r values observed. The same laboratory recently reported that purified $P450_8$ may correspond functionally and immunochemically to a *P*-450 isozyme ($P450_{MP}$, M_r 48000) with mephenytoin 4-hydroxylation activity purified to homogeneity from human liver [39]. Moreover, in some individuals a variant form of $P450_{MP}$, immunochemically similar but of a higher M_r of 50000, was found. In microsomes of ten individuals tested in this laboratory with anti-*P*-450 meph an additional faint protein band was recognized in all samples (Fig. 7). This protein had an M_r of 53000 and appears to be a contaminant, as anti-$P450_8$ sera exclusively recognized a single band of M_r 55000 (results not shown). In conclusion, by monitoring the catalytic activity of a polymorphically affected *P*-450 function, we have isolated a human *P*-450 isozyme, the deficiency of which could explain the mephenytoin-type polymorphism of drug oxidation.

Acknowledgements

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