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# The molecular mechanisms of two common polymorphisms of drug oxidation—evidence for functional changes in cytochrome P-450 isozymes catalysing bufuralol and mephenytoin oxidation

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1. Using the stereospecific metabolism of (+)- and (−)-bufuralol and (+)- and (−)-metoprolol as model reactions, we have characterized the enzymic deficiency of the debrisoquine/sparteine-type polymorphism by comparing kinetic data of subjects *in vivo* with their microsomal activities *in vitro* and with reconstituted activities of cytochrome P-450 isozymes purified from human liver.

2. The metabolism of bufuralol in liver microsomes of *in vivo* phenotyped 'poor metabolizers' of debrisoquine and/or sparteine is characterized by a marked increase in $K_m$, a decrease in $V_{max}$ and a virtual loss of the stereoselectivity of the reaction. These parameters apparently allow the 'phenotyping' of microsomes *in vitro*.

3. A structural model of the active site of a cytochrome P-450 for stereospecific metabolism of bufuralol and other polymorphically metabolized substrates was constructed.

4. Two cytochrome P-450 isozymes, P-450 buf I and P-450 buf II, both with MW 50 000 Da, were purified from human liver on the basis of their ability to metabolize bufuralol to 1'-hydroxy-bufuralol. However, P-450 buf I metabolized bufuralol in a highly stereoselective fashion ((−)/(+) ratio 0·16) as compared to P-450 buf II (ratio 0·99) and had a markedly lower $K_m$ for bufuralol. Moreover, bufuralol 1'-hydroxylation by P-450 buf I was uniquely characterized by its extreme sensitivity to inhibition by quinidine.

5. Antibodies against P-450 buf I and P-450 buf II inhibited bufuralol metabolism in microsomes and with the reconstituted enzymes. Immunochemical studies with these antibodies with microsomes and translations *in vitro* of RNA from livers of extensive and poor metabolizers showed no evidence for a decrease in the recognized protein or its mRNA. Because the antibodies do not discriminate between P-450 buf I and P-450 buf II, both a decreased content of P-450 buf I or its functional alteration could explain the polymorphic metabolism in microsomes.

6. The genetically defective stereospecific metabolism of mephenytoin was determined in liver microsomes of extensive and poor metabolizers of mephenytoin phenotyped *in vivo*. Microsomes of poor metabolizers were characterized by an increased $K_m$ and a decreased $V_{max}$ for *S*-mephenytoin hydroxylation as compared to extensive metabolizers and a loss of stereospecificity for the hydroxylation of *S*-versus *R*-mephenytoin. A cytochrome P-450 with high activity for mephenytoin 4-hydroxylation was purified from human liver. Immunochemical studies with inhibitory antibodies against this isozyme suggest the presence in poor-metabolizer microsomes of a functionally altered enzyme.

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## Introduction

The nature, intensity and duration of the effects of numerous drugs depends on the activities of drug-metabolizing enzymes located primarily in the liver. These enzymes catalyse the biotransformation of drugs to inactive, active or toxic metabolites. The reactions include functionalization and conjugation, and are catalysed by a variety of different microsomal, mitochondrial and cytoplasmic enzymes. Marked differences in the rate of drug metabolism occur and many of these variations are genetically determined. Of prime importance are the microsomal polysubstrate haemoprotein mono-oxygenases (trivial name: cytochrome P-450) which incorporate one atom of molecular oxygen into the drug substrate. The majority of lipophilic drugs are oxidized by cytochrome P-450-mediated reactions (Meyer 1984, Boobis *et al.* 1985 a).

*Multiplicity and variable functional expression of cytochrome P-450 isozymes*

Over the last few years the extreme multiplicity of cytochrome P-450 isozymes has been realized. More than 10 different forms or isozymes have been isolated from livers of inbred strains of rodents. These isozymes show differences in chemical, immunological and physical properties. Most importantly, they possess different substrate specificities; although some isozymes of cytochrome P-450 exhibit broad and overlapping substrate specificity, other isozymes show a preference for one or more substrates and exhibit remarkable regio- and stereo-selectivity for the oxidation of these substrates (for review, see Nebert and Negishi 1982, Meyer 1984, Boobis *et al.* 1985 a). Several isozymes from rodent liver with different substrate specificities have been sequenced at the protein and DNA level. Moreover, these data have provided evidence that different structural genes encode the different isozymes. The number of different isozymes and their structural genes is not known and speculations on the ultimate number range from twenty to thousands. A few distinct P-450 isozymes have also been isolated from human liver (Wang *et al.* 1983, Gut *et al.* 1984), and the partial sequence of a human genomic DNA hybridizing with cDNA from a rat cytochrome P-450 has recently been published (Phillips *et al.* 1985). Cytochrome P-450 isozymes with only minimal differences in sequence but considerable differences in function are observed when individual animals of outbred strains of rabbits are analysed separately (Johnson, Finlayson and Raucy 1985). The extreme genetic heterogeneity of the human population as compared to inbred strains of laboratory animals obviously potentiates the problem of multiplicity, microheterogeneity or variability of cytochrome P-450 isozymes and makes the investigation of interindividual differences in man a formidable undertaking.

Indirect studies in animals and men have suggested that differences in drug oxidation probably arise in the main from differences in the activities of hepatic cytochrome P-450 isozymes as regulated and influenced by genetic, pharmacological, environmental, physiological and disease-induced factors. The numerous influences have led to the widely held view that interindividual variation in drug oxidation is mostly under polygenic control and is multifactorial in nature. However, the recent discovery of several common polymorphisms of drug oxidation has changed this concept considerably. The occurrence of such polymorphisms indicates that oxidative metabolism of a large number of drugs is under monogenic control and that variable patterns of drug metabolism may be due to the presence of mutations of the respective genes. As is discussed in other sections of this issue, these genetic polymorphisms give rise to distinct subgroups in the population which differ

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in their ability to perform a certain drug-oxidation reaction. The considerable clinical relevance of these polymorphisms has been demonstrated, as recently summarized by Eichelbaum (1982), Idle *et al.* (1983) and Cooper and Evans (1984).

*Polymorphisms of drug metabolism involving cytochrome P-450 isozymes*

*Debrisoquine/sparteine-polymorphism.* Since the discovery of the genetically closely related polymorphisms of debrisoquine and sparteine oxidation independently in the UK and FR Germany, considerable clinical information has accumulated from many different laboratories, indicating that so-called 'poor metabolizers' (identified by urinary metabolic ratios after standard doses) represents 3–10% of the white populations of Europe and North America and possibly a higher proportion in Orientals. Family studies are consistent with an autosomal recessive inheritance of this deficiency, poor metabolizers being homozygous for a recessive gene. However, and most importantly, these poor-metabolizer subjects (as tested with debrisoquine and sparteine) have impaired oxidation of to date over 20 other drugs including phenformin, perhexiline, guanoxan, encainide, nortriptylin, other tricyclic antidepressants, the $\beta$-adrenergic receptor blocking drugs bufuralol, metoprolol and propanolol, the opioid dextromethorphan and the alkaloid *N*-propylajmalin. This suggests that the same enzyme, or enzymes under identical genetic control, may be responsible for the debrisoquine/sparteine-type polymorphism in these populations. However, the situation appears to be more complicated. A recent population study in Ghanaians indicates that the ability of Ghanaians to oxidize sparteine is independent of their capacity for debrisoquine oxidation (Eichelbaum and Woolhouse 1985). This may indicate that in Ghanaians a third allele for this locus is operative or that two different genes code for the debrisoquine- and the sparteine-oxidizing enzyme. Linkage disequilibrium would account for the dissociation of the otherwise coupled inheritance of the two polymorphisms in this latter case.

Because all reactions associated with the debrisoquine/sparteine-type polymorphism are catalysed by cytochrome P450-mono-oxygenases, over the past few years we and others have tested the hypothesis that the polymorphic metabolism of debrisoquine and other drugs may be caused by the total or partial absence, or the functional alteration, of a particular cytochrome P-450 isozyme. Using bufuralol as a model substrate, we have characterized this deficiency in human liver at the microsomal level (Meier *et al.* 1983, Dayer *et al.* 1984, Minder *et al.* 1984, Dayer *et al.* 1985 b), and in the following our present state of knowledge on the purification and characterization of the isozyme(s) involved at the protein and nucleic acid level is described.

*Mephenytoin polymorphism.* A genetic polymorphism of deficient metabolism of mephenytoin is observed in 2–5% of Caucasian and over 20% of Japanese subjects (see Kalow *et al.* 1985, this issue, pp. 379–389). This deficiency occurs independently of the debrisoquine/sparteine-type polymorphism. Also it is inherited as an autosomal recessive trait and affects one of the two major pathways of mephenytoin metabolism, namely stereoselective aromatic hydroxylation. We have characterized this deficiency in two poor metabolizers of mephenytoin at the microsomal level (Meier *et al.* 1985 a, 1985 b) as being due to the deficiency of a different cytochrome P-450 isozyme.

*Other polymorphisms.* In addition to the debrisoquine/sparteine-type and mephenytoin-type polymorphisms, other variations in drug metabolism have been discovered which may be due to variable expression or functional alterations of

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cytochrome P-450 isozymes, namely the hydroxylation of tolbutamide, the metabolism of antipyrine and the metabolism of nifedipine. Less common genetic variations of P-450 function may apply to rare families with deficiencies in the *p*-hydroxylation of phenytoin, the hydroxylation of bishydroxycoumarin and a number of other drugs (for references see other contributions in this issue).

*General considerations in regard to the molecular mechanisms responsible for genetic variations in cytochrome P-450 isozymes in humans*

The principal mechanisms causing quantitative or functional deficiencies in cytochrome P-450 isozymes in human liver are summarized in table 1. It is evident from these considerations that the elucidation of the molecular basis of these polymorphisms requires access to large quantities of human-liver tissue for isolation and purification of proteins and RNA, but also access to tissue from subjects or families phenotyped *in vivo* to establish the causal relationship between findings *in vitro* and *in vivo*. Moreover, sensitive assays for the involved metabolic reactions are necessary to monitor the purification of cytochrome P-450 isozymes with affinity for the substrates in question.

Table 1. Possible mechanisms responsible for a deficiency of cytochrome P-450 isozymes in humans.

| | |
|---|---|
| (1) | Abnormal function of enzyme |
| | (*a*) Decreased affinity for substrates |
| | (*b*) Decreased maximal velocity |
| | (*c*) Combination of (*a*) and (*b*) |
| (2) | Decreased intracellular concentration or absence of enzyme protein |
| | (*a*) Diminished rate of synthesis |
| | (i) Deletion, insertion, or rearrangement of gene |
| | (ii) Defect in transcription, RNA processing or stability |
| | (*b*) Accelerated degradation of labile enzyme variant |

## Results and discussion

*Establishment of a bank of human-liver tissue*

In order to investigate the molecular basis of these genetic polymorphisms we have established a bank of human-liver tissue. This bank contains 20 human livers collected from kidney transplant donors (KTD) immediately after circulatory arrest. Tissue from these livers was used to adapt and optimize fractionation procedures, storage conditions and enzyme assays (Meier *et al.* 1983, Minder *et al.* 1984). Moreover, purification procedures and RNA isolation were optimized with KTD liver tissue. In addition, over 100 wedge biopsies of 0·5 to 2 g wet wt were obtained from the livers of patients who underwent either diagnostic or therapeutic laparotomy. We have been able to study several liver biopsies of patients who have been phenotyped *in vivo* by urinary metabolite excretion or plasma levels as poor metabolizers of either debrisoquine, sparteine, bufuralol or mephenytoin. Through the collaboration of Phillippe Beaune (Department of Biochemistry, CHU Necker, Paris), Michel Eichelbaum (Department of Medicine, University of Bonn) and

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Werner Kalow and Ted Inaba (Department of Pharmacology, University of Toronto) we have received additional liver tissue of individuals either characterized *in vivo* as poor metabolizers or showing the changes *in vitro* described below which allowed their phenotypic identification.

### In vivo–in vitro *correlation of the metabolism of debrisoquine, bufuralol and metoprolol*

*Debrisoquine*. Initial studies in liver microsomes of two individuals identified as poor metabolizers by tests *in vivo* (urinary metabolic ratio) demonstrated a markedly decreased hydroxylation rate of debrisoquine (Meier *et al.* 1983), confirming and extending previous studies by Davies *et al.* (1981) who observed a lack of 4-hydroxy-debrisoquine formation in the liver biopsy of one poor metabolizer. These investigations clearly established that the deficient metabolism of debrisoquine in poor metabolizers is caused by a deficiency of a mono-oxygenase reaction in the liver. Moreover, by measuring other parameters of microsomal electron transport and the metabolism of six other substrates our data demonstrated that defective hydroxylation of debrisoquine is not related to a general impairment of microsomal oxidation reactions (Meier *et al.* 1983).

*Bufuralol*. Bufuralol is an experimental β-adrenoceptor-blocking drug of the lipophilic type which is eliminated mostly by metabolic liver clearance. Its major pathways of metabolism are aliphatic oxidation to the carbinol, 1′-hydroxy-bufuralol (alcohol metabolite), aromatic oxidation to 4-hydroxy-bufuralol (phenol metabolite) and glucuronidation of the parent drug and the phenol derivative (figure 1). In previous studies we established that the metabolism *in vivo* of racemic bufuralol to carbinol was under the same control, or under linked genetic control, as the metabolism of debrisoquine to 4-hydroxy-debrisoquine (Dayer *et al.* 1982). More recently we have observed that bufuralol 4-hydroxylation to the phenol derivative is under the same genetic control as 1′-hydroxylation (Dayer *et al.* 1985 b). However, as no stable reference material for 4-hydroxy-bufuralol is available, most of the studies dealing with polymorphic oxidation of bufuralol are restricted to the 1′-hydroxylation route which is the most conveniently used marker of the variant enzyme activity (Dayer *et al.* 1984, Minder *et al.* 1984, Boobis *et al.* 1985 b).

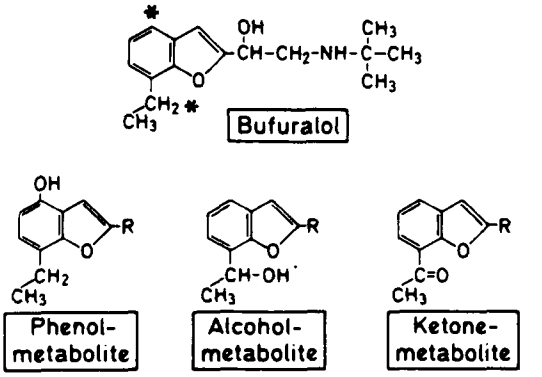


Figure 1. Structures of bufuralol and its metabolites.

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As mono-oxygenase reactions are frequently characterized by stereoselective recognition of substrates with asymmetric carbon centres, we have investigated the substrate stereoselectivity for the genetically controlled route *in vivo* (Dayer *et al.* 1985 b) and *in vitro* (Dayer *et al.* 1984).

In vivo *stereoselectivity of bufuralol 1'-hydroxylation.* Most β-adrenoceptor-blocking drugs are used in clinical medicine as racemic mixtures. To study the fate of the two isomers separately, we developed an enantioselective assay to measure each isomer in biological fluids after administration of a racemic mixture. Figure 2 shows the plasma concentrations of each bufuralol enantiomer three hours after a 30 mg oral dose of racemic bufuralol given to six extensive and to four poor metabolizers of debrisoquine (Dayer *et al.* 1985 b). In extensive metabolizers, the (−)/(+) isomer plasma ratio of bufuralol was 1·60 ± S.D. 0·25. This strongly indicates a preferential clearance of the (+)-isomer. In poor metabolizers both isomers of bufuralol were found in increased amounts in plasma, indicating that the polymorphism affects the overall metabolism of both (+)- and (−)-bufuralol. However, and contrary to the observation made with another β-blocking agent, metoprolol (see below), the (−)/(+) isomer plasma ratio increased in the poor-metabolizer subjects, most likely due to stereoselective glucuronidation of (+)-bufuralol, the sole metabolic route left intact in poor metabolizers (Dayer *et al.* 1985 b). This example emphasizes how difficult it is to interpret plasma data which of course represent the sum of multiple metabolic pathways, each one with its own selectivity. Moreover, administration of racemic mixtures may lead to competition between enantiomers for a given enzyme, resulting in an even more complex situation.

Bufuralol disposition was also studied in urine. Chemically pure isomers and

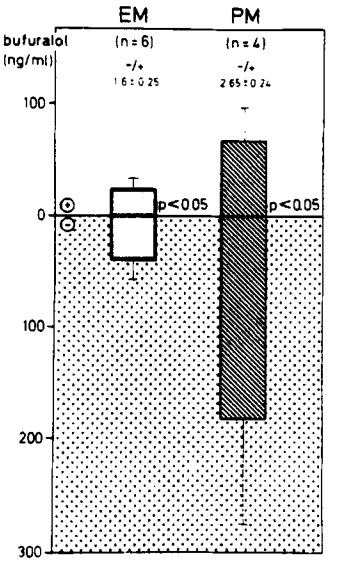


Figure 2. Plasma concentrations of (−)- and (+)-bufuralol three hours after oral administration of 30 mg of racemic bufuralol to six extensive (EM) and four poor metabolizers (PM) of debrisoquine.

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racemic bufuralol were given to extensive and poor debrisoquine hydroxylators at very low dosage (2·5 mg p.o.) for obvious ethical reasons. Figure 3 shows the urinary excretion (eight hours) of total bufuralol (after enzymic hydrolysis) and of the carbinol in two extensive metabolizers and two poor metabolizers of debrisoquine. As expected, poor metabolizers excreted less carbinol than extensive metabolizers. Extensive metabolizers excreted larger amounts of carbinol from (+)- than (−)-bufuralol, while poor metabolizers excreted about the same amount of carbinol from (+)- and (−)-bufuralol. These findings thus confirm the preferential selectivity of the (+)-substrate of the polymorphic pathway which metabolizes bufuralol in position 1′. They also show the virtual abolition of this substrate stereoselectivity in poor metabolizers.

In vitro *stereoselectivity of bufuralol 1′-hydroxylation and characteristics of liver microsomes of poor metabolizers*. In order to define the enzymic mechanisms of the polymorphic reaction, we investigated the kinetics of bufuralol 1′-hydroxylation in liver microsomes from subjects previously phenotyped *in vivo* as extensive metabolizers and poor metabolizers for debrisoquine or sparteine (Minder *et al.* 1984, Dayer *et al.* 1984). The liver samples were obtained as wedge biopsies during laparotomy for diagnostic or therapeutic purposes. Microsomes were prepared according to a micromethod which enables the processing of samples of 50–100 mg liver (wet wt). Pure (+)- and (−)-bufuralol isomers were used as substrate. Due to the fluorescence of bufuralol and its metabolites, one biopsy allows the study of numerous samples at various substrate concentrations ranging from 5 to 800 μM.

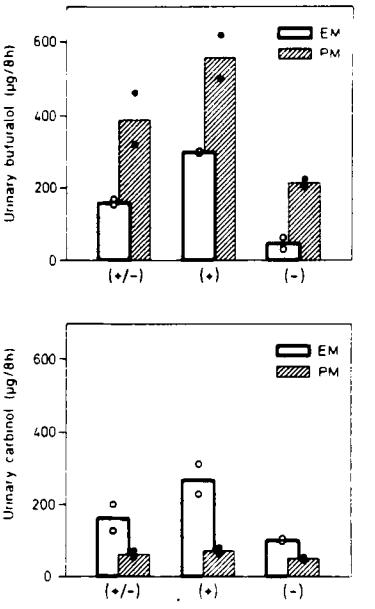


Figure 3. Urinary excretion of bufuralol (*a*) and carbinol (1′-hydroxy-bufuralol, (*b*)) in two extensive (EM) and two poor metabolizers (PM) after oral administration of racemate (+/−), or of (+)- or (−)-bufuralol.
The urine was collected for eight hours after administration of 2·5 mg of bufuralol.

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Figure 4 displays typical kinetics of bufuralol 1'-hydroxylation in microsomes from one extensive metabolizer and one poor metabolizer phenotyped *in vivo* with debrisoquine. Poor-metabolizer microsomes are characterized by a mean increase of about 5- to 15-fold in the Michaelis constant ($K_m$) (figure 4 and table 2) for both (+)- and (−)-bufuralol, a lowered maximal velocity ($V_{max}$) and a decrease of stereoselectivity as judged by the (−) to (+) ratio at $V_{max}$ (Dayer *et al.* 1984). This ratio is between 0·30 and 0·60 in extensive metabolizer and between 0·50 and 0·85 in poor-metabolizer microsomes. Detailed kinetic analysis now extends to 5 and 10 biopsies from poor metabolizers and extensive metabolizers, respectively, either characterized *in vivo* by debrisoquine or sparteine urinary metabolic ratios (Dayer *et al.* 1985 a). $V_{max}$ and isomer ratio, but not $K_m$, may show some overlap between the two

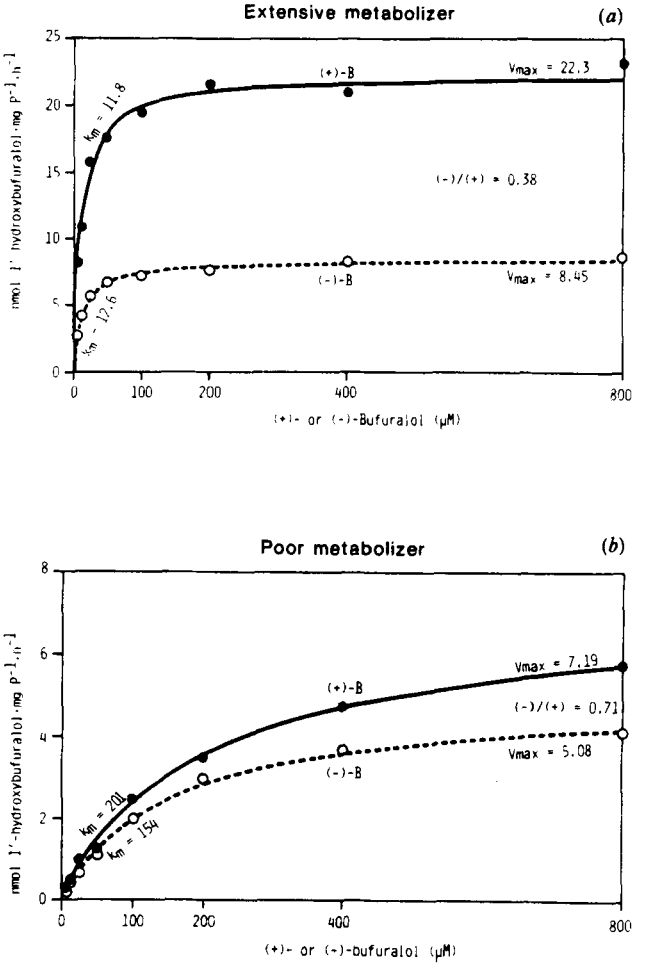


Figure 4. Bufuralol 1'-hydroxylation from (+)- or (−)-bufuralol in microsomes from one extensive metabolizer (*a*) and one poor metabolizer (*b*).

The two individuals were phenotyped by urinary metabolic ratios after administration of debrisoquine.

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phenotypes. However, we believe that the collective information from these three parameters ($K_m$, $V_{max}$ and isomer ratio) offers an approach for phenotyping of individual livers *in vitro* with a high degree of confidence. The point should be made that some preferential hydroxylation of (+)-bufuralol may still be observed in poor metabolizers, as if a small amount of the highly stereoselective and genetically controlled component of bufuralol 1′-hydroxylation was still present in poor-metabolizer microsomes.

*Metoprolol.* The polymorphic metabolism of metoprolol is discussed on pages 435–447 in this issue by Lennard (1986). For comparison with the metabolism of bufuralol *in vivo*, we administered metoprolol (100 mg p.o.) and measured the plasma concentration of (+)- and (−)-metoprolol by chiral liquid chromatography (Dayer *et al.* 1985 b). In extensive metabolizers, we observed preferential disappearance of the (+)-isomer similar to the situation with bufuralol. In contrast to bufuralol, there was loss of stereoselectivity and no increase in the (−)/(+) ratio in poor metabolizers *in vivo*. This difference may be explained by the stereoselective glucuronidation of bufuralol and the absence of such a pathway for metoprolol. The metabolism of metoprolol *in vitro* by α-hydroxylation and *O*-demethylation by a purified reconstituted human cytochrome P-450 will be described below.

In summary, our comparisons of the metabolism of bufuralol and metoprolol *in vivo* and *in vitro* in numerous extensive-metabolizer and poor-metabolizer microsomes suggest that the enzymic deficiency in poor metabolizers is characterized by a marked increase in $K_m$, a decrease in $V_{max}$ and a decrease in the stereoselectivity of the reaction. In microsomes, that is a system of multiple isozymes with overlapping substrate specificity, these findings do not allow a clear-cut differentiation between (*a*) the absence of an enzyme with low $K_m$ and consequent metabolism of bufuralol by enzymes with lower affinity or (*b*) the presence of a structural variant of the 'normal' isozyme (see also table 1).

### *A model of the active site of a cytochrome P-450 for stereospecific metabolism of bufuralol and other substrates which are subject to the debrisoquine/sparteine polymorphism*

Whether all of the more than 20 substrates presently known to be influenced in their metabolism by the debrisoquine/sparteine-type polymorphism are oxidized by the same isozyme or by different but genetically linked isozymes is unclear. If they are metabolized by the same isozyme, some common structural features for recognition by the active site would be expected. Using our data on the stereospecific metabolism of bufuralol and metoprolol and the published data on the other substrates, we have searched for common structural features defining these substrates and the oxidative reactions in question (Kronbach, Dayer and Meyer 1985). All substrates have a lipophilic domain, contain at least one basic nitrogen (N) and reactions adjacent to this N are not controlled by the polymorphic gene. Using a rigid structure, such as the one of dextromethorphan and its *O*-dealkylation pathway, we have calculated the distance between N and the reaction site. For dextromethorphan-*O*-demethylation and bufuralol 1′-hydroxylation, the distances from N to the reaction site (O) are 0·7 nm. Aromatic (lipophilic) rings are nearly coplanar in this model (figure 5). Extension of this model to other polymorphically metabolized substrates had predictive value for bufuralol-4-hydroxylation, 4-methoxyamphetamine-*O*-demethylation, and also for the metabolism of amifla-

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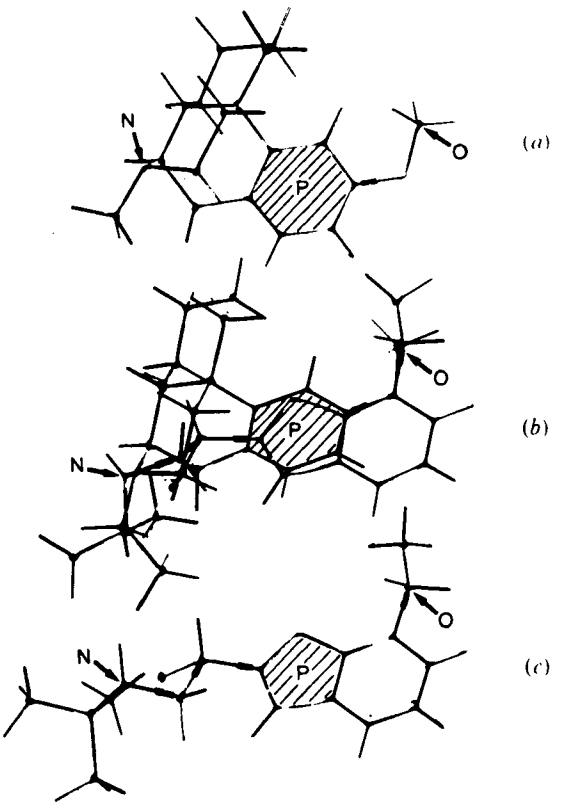


Figure 5. Model of the common structural features of substrates of the debrisoquine/sparteine-type polymorphism.

The three-dimensional structures of dextrometorphan (*a*), bufuralol (*c*), and the juxtaposition of dextrometorphan and bufuralol (*b*) are shown. N, Basic nitrogen atom; P, lipophilic plane; O, oxidation site.

mine, amitriptyline, encainide, guanoxane, imipramine and perhexiline. But to our surprise the polymorphic pathways of the classical substrates, debrisoquine and sparteine, cannot be predicted with this model. For debrisoquine, the distance of $C_4$ to N is only 0·5 nm and the model would only fit the hydroxylation at $C_7$. For sparteine, the initial site of oxidation is still debated (Guengerich 1984).

With regard to bufuralol 1′-hydroxylation, the model is consistent with the concept that the presumed binding site (N) is not enantioselective per se. This would explain that in extensive-metabolizer microsomes and in the purified P-450 isozyme P-450 buf I (see below) the $K_m$ values for (+)- and (−)-bufuralol are virtually identical, and that in poor-metabolizer microsomes an increased $K_m$ is observed for both (+)- and (−)-bufuralol (Dayer *et al.* 1984, table 2). However, as mentioned previously, this increase in $K_m$ could also be due to the absence of an enzyme with low $K_m$ and metabolism of bufuralol by isozymes with higher $K_m$ values. The regioselectivity, that is 1′- vs 4-hydroxylation of bufuralol observed *in vivo* (Dayer *et al.* 1985 b), indicates that the site of oxidative attack is determined by the interaction between the hydroxy group adjacent to the asymmetric carbon in the side-chain with another polar group (namely oxygen) in the lipophilic plane, favouring 1′-hydroxyl-

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Table 2. Kinetic parameters of (−)- and (+)-bufuralol 1′-hydroxylation to carbinol in microsomes of extensive and poor metabolizers and in reconstituted P-450 buf I and P-450 buf II.

| Bufuralol hydroxylation to carbinol measured in | Kinetic parameters | | | | | |
|---|---|---|---|---|---|---|
| | $K_m$ (μM) | | $V_{max}$ (nmol CA/nmol P-450 per 15 min) | | Ratio (−)/(+)† | $K_i$ (μM) quinidine |
| | (−) | (+) | (−) | (+) | | |
| EM microsomes‡ | 35·3 | 47·3 | 4·1 | 8·6 | 0·48 | 0·010 |
| PM microsomes‡ | 233·5 | 182·5 | 1·5 | 1·9 | 0·77 | ‖ |
| P-450 buf I§ | 50·8 | 60·3 | 8·9 | 53·5 | 0·16 | 0·050 |
| P-450 buf II§ | 304·0 | 245·0 | 36·11 | 36·39 | 0·99 | 100·0 |

†(−)- And (+)-bufuralol were used as substrates and the ratio of CA formed from each isomer was determined at $V_{max}$.

‡Mean values, as in Dayer *et al.* (1984).

§Results of a typical experiment with the reconstituted subforms of cytochrome P-450 buf.

‖With poor-metabolizer microsomes a major decrease in the $V_{max}$ was observed only at quinidine concentrations above 10 μM.

EM, Extensive metabolizer; PM, poor metabolizer; CA, carbinol.

ation of (+)-bufuralol and 4-hydroxylation of (−)-bufuralol. Finally, antibodies raised against purified cytochrome P-450 buf I (see below) inhibited both (+)- and (−)-bufuralol hydroxylation, again supporting the above-mentioned characterization of the substrate binding site. Thus, the proposed model (figure 5) explains many of the features of stereo- and regio-selective bufuralol metabolism and its alteration in poor metabolizers through the absence or functional alternation of cytochrome P-450 buf I (see below). Most importantly, the model has predictive value for a number of other polymorphically metabolized substrates, but as mentioned does not fit the 4-hydroxylation of debrisoquine. For the debrisoquine 4-hydroxylation, this could point either to a different but co-regulated isozyme or a different binding site within the same enzyme.

*Purification and characterization of human cytochrome P-450 isozymes catalysing the oxidation of bufuralol*

Using tissue from kidney transplant donor livers we have purified several cytochrome P-450 isoenzymes. By application of the extremely sensitive assay for bufuralol-1′-hydroxylation we have developed a procedure for the reproducible purification of a cytochrome P-450 isozyme (P-450 buf) with a high turnover number for this reaction (Gut *et al.* 1984, 1985). This was achieved by monitoring the activity of bufuralol-1′-hydroxylation in column eluates of sequential hydrophobic and ion-exchange chromatographies after removal of detergents and addition of purified NADPH cytochrome P-450 reductase and a NADPH-regenerating system. Through the first two chromatographic steps, P-450 buf was apparently tightly associated with a non-haem protein of equal molecular weight but could to a large extent be separated from this protein by ion-exchange chromatography on DE-52 cellulose. The preparation was homogeneous on SDS–PAGE and had an apparent molecular weight of 50 000 ± 1000 Da. Its specific content was estimated at 6·8 nmol P-450 haemoprotein/mg protein as based on Lowry protein and CO-binding spectrum. In a soluble non-membraneous reconstituted system, P-450 buf had a high turnover number for bufuralol to 1′-hydroxy-bufuralol hydroxylation,

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but had little activity for benzphetamine *N*-demethylation and was devoid of ethoxycoumarin-*O*-deethylase activity. Because of the marked stereoselectivity of microsomal bufuralol hydroxylation, the activity of the purified isozyme was determined with both enantiomers of bufuralol. Indeed, the isolated reconstituted P-450 buf had a markedly increased selectivity for (+)-bufuralol, resulting in a (−)/(+) ratio of around 0·1, which was strikingly different from that in extensive-metabolizer microsomes (0·4–0·6).

Moreover, P-450 buf also catalysed metoprolol metabolism in a highly stereoselective fashion providing further evidence that this human isozyme selectively metabolizes substrates subjected to the debrisoquine/sparteine-type polymorphism.

More recently, we have been able to isolate and purify two functionally different 'subforms' of P-450 buf from human liver—P-450 buf I and P-450 buf II (table 2, Gut *et al.* 1985). Both isozymes have molecular weights of ~50 000 Da and are homogeneous on SDS–PAGE. Some of their catalytic properties with bufuralol and other substrates are summarized in tables 2 and 3. P-450 buf I metabolizes (+)-bufuralol in a highly stereoselective fashion with a high turnover number of 55 nmol carbinol × nmol P-450$^{-1}$ × 15 min$^{-1}$, while P-450 buf II shows no selectivity for either isomer. Moreover, under identical experimental conditions the apparent $K_m$ for bufuralol is four to five times higher with P-450 buf II than with P-450 buf I, and metabolism by P-450 buf II results in the generation of other metabolites in addition to 1′-hydroxy-bufuralol. Quinidine, an extremely potent inhibitor of the metabolism of sparteine in human-liver microsomes (Otton, Inaba and Kalow 1984) and of metoprolol metabolism *in vivo* (Leeman, Dayer and Meyer 1985), inhibited the 1′-hydroxylation of bufuralol with an apparent $K_i$ for P-450 buf I which was almost 1000 times lower than the $K_i$ for P-450 buf II. A comparison of the stereochemical and kinetic characteristics of these two isozymes with the activities of extensive-metabolizer and poor-metabolizer microsomes (table 2) is consistent with the concept that the bufuralol hydroxylation activity in poor-metabolizer microsomes could be due largely to the presence of P-450 buf II, while most if not all of the activity of P-450 buf I is missing.

Table 3. Catalytic activities of human-liver microsomes and reconstituted cytochrome P-450 buf I and P-450 buf II.

| Monitored activity | Catalytic activity measured (nmol product formed × nmol P-450$^{-1}$ × 15 min$^{-1}$)† | | |
|---|---|---|---|
| | Microsomes | Reconstituted P-450 buf I | Reconstituted P-450 buf II |
| (+)-Bufuralol 1′-hydroxylation | 8·6 | 55·1 | 37·3 |
| (−)-Bufuralol 1′-hydroxylation | 4·1 | 9·3 | 38·6 |
| (+)-Metoprolol α-hydroxylation | | 0·65‡ | |
| *O*-demethylation | | 6·1‡ | |
| (−)-Metoprolol α-hydroxlation | | 1·76‡ | |
| *O*-demethylation | | 4·2‡ | |
| d-Benzphetamine-*N*-demethylation | 12·1 | 4·1 | n.m. |
| 7-Ethoxycoumarin-*O*-deethylation | 1·75 | n.d. | n.d. |

† Values represent means of at least two determinations.
‡ Only determined with reconstituted P-450 buf (see Gut *et al.* 1984).
n.d., Not detected; n.m., not measured.

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*Immunochemical studies with antibodies against P-450 buf*

Polyclonal antibodies against P-450 buf I and P-450 buf II were raised in rabbits. These antibodies inhibited microsomal bufuralol-1′-hydroxylation and reconstituted P-450 buf activity by 80% and >95%, respectively, whereas a number of other activities, among them mephenytoin 4-hydroxylase, were not affected. Immunochemical analysis by Western blots on nitrocellulose showed that in microsomes the antibodies recognized only one protein band in the molecular weight 50 000 Da region. However, immunoblots of the purified proteins revealed that these antibodies did not discriminate between either P-450 buf and the non-haem protein of identical molecular weight already mentioned, or between P-450 buf I and P-450 buf II, even after solid-phase immunoabsorption of the anti-P-450 buf antiserum on the heterologous antigen. There was no correlation between bufuralol hydroxylation activity *in vitro* in microsomes of extensive-metabolizer and poor-metabolizer subjects phenotyped *in vivo* and the extent of the immunochemical reaction. Moreover, microsomes of poor metabolizers phenotyped with sparteine also exhibited the same extent of immunostaining as microsomes of extensive metabolizers. However, because of the crossreactivity of the antibodies between P-450 buf I and P-450 buf II a quantification of the relative contents of these two catalytically different subforms of P-450 buf is not yet possible. A major effort is presently being made in our laboratory to develop more specific probes.

The isolation of two subforms of P-450 buf, P-450 buf I and P-450 buf II allows the formulation of more refined hypotheses on the molecular mechanism causing the poor-metabolizer status. The microsomal data suggest that P-450 buf I and P-450 buf II both contribute to overall bufuralol-1′-hydroxylation activity in extensive-metabolizers' liver. A first possibility is that P-450 buf I and P-450 buf II are two structurally similar, but functionally distinct and independently controlled, P-450 isozymes. Either a decrease in the content of P-450 buf I or a structural alteration of its active site could then explain the enzymic changes in poor-metabolizer microsomes. A second possibility is that P-450 buf I and P-450 buf II are two functional forms of the same P-450 isozyme. In this case the genetic deficiency could result in a minor structural change at the active site of P-450 buf I and cause its functional transformation into P-450 buf II, which would then be responsible for most of the metabolism of bufuralol to the carbinol in poor-metabolizer microsomes. The fact that P-450 buf I and P-450 buf II have identical molecular weights, electrophoretic behaviour and immunochemical properties is consistent with the presence of structurally very similar proteins which nevertheless differ markedly in their stereochemical and catalytic properties.

*Isolation and translation of RNA directing the synthesis of P-450 buf*

A complete understanding of the genetic deficiency causing these polymorphisms of cytochrome P-450 function will require studies at the DNA level. In preparation for this we have isolated RNA from livers of extensive-metabolizer and poor-metabolizer subjects. Translation of this RNA in nuclease-treated rabbit reticulocyte lysates directed the synthesis of protein(s) immunoprecipitable by the anti-P-450 buf antibodies (figure 6; C. Skoda unpublished). As expected from the microsomal immunochemical studies, RNA from poor metabolizers directed the synthesis of equal amounts of immunoreactive protein as RNA from extensive metabolizers. As discussed earlier, the interpretation of this finding depends on the specificity of the anti-P-450 buf antibodies. A λgt 11 human-liver cDNA library was

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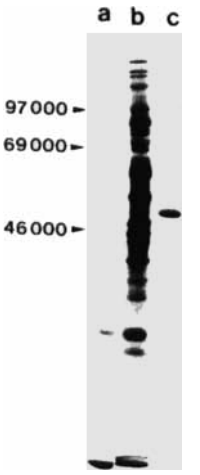


Figure 6. Cell-free translation of poly (A)-rich RNA from human liver.
Rabbit reticulocyte lysates were incubated with $^{35}$S-methionine and either no exogenous RNA (a) or poly(A)-rich RNA isolated from human liver (b, c). In (c) the translation mixture was subjected to immunoprecipitation with rabbit antiserum against cytochrome P-450 buf. Shown are the autoradiographs of SDS–PAGE of the translation mixtures or the immunoprecipitate.

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constructed on the basis of this RNA from extensive-metabolizer livers. It is presently being used to isolate cDNA inserts coding the expressed lacZ gene fusion proteins which are recognized by antibodies against P-450 buf. These cDNA probes will be used to compare the gene structures of poor and extensive metabolizers and to develop tests to genotype individuals.

*Characterization of the enzymic deficiency of the mephenytoin polymorphism*

The genetically deficient metabolism of the anticonvulsant mephenytoin involves the stereospecific aromatic 4-hydroxylation of *S*-mephenytoin. The other major pathway of mephenytoin metabolism, *N*-demethylation, remains unaffected (see Kalow *et al.* 1985, pp. 379–389 in this issue). To characterize this enzymic deficiency, the rate of 4-hydroxylation and of *N*-demethylation of *S*- and *R*-mephenytoin was determined in liver microsomes of 13 extensive and two poor metabolizers of mephenytoin phenotyped *in vivo* (Meier *et al.* 1985 a, 1985 b). Detailed kinetic studies revealed that microsomes of poor metabolizers were characterized by a high $K_m$ and a low $V_{max}$ for *S*-mephenytoin hydroxylation and a loss of stereoselectivity for the hydroxylation of *S*-mephenytoin vs *R*-mephenytoin. The microsomal formation of 4-hydroxy-mephenytoin from *R*-mephenytoin and the rate of mephenytoin demethylation remained unaffected by the polymorphism. We have purified a cytochrome P-450 isozyme from human liver with a high activity for mephenytoin 4-hydroxylation (P-450 meph, unpublished data). Rabbit antibodies raised against this isozyme inhibited microsomal mephenytoin hydroxylation by over 80%. On Western blots there was no correlation between the hydroxylation index *in vivo*, the microsomal activity of mephenytoin hydroxylation and the extent of the immunochemical reaction. These findings are of course very similar to those characteristic for the enzymic deficiency in livers of subjects with the

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bufuralol-type polymorphism, namely increased $K_m$ and loss of stereospecificity of the reaction, and indicate the presence of a functionally altered enzyme rather than a quantitative decrease in enzyme protein as the cause of the mephenytoin polymorphism.

## Conclusions

The presented data on two common genetic polymorphisms of drug oxidation, using the stereospecific metabolism of bufuralol and of mephenytoin as model reactions in microsomes and cytochrome P-450 isozymes purified from human liver, indicate that the decreased content or structural alteration of specific P-450 isozymes are causing the observed functional changes in microsomes of poor metabolizers. We believe that because of their frequency of occurrence and clinical significance, the elucidation of these and other genetic polymorphisms of human P-450 function deserves a major effort. Similar polymorphisms may explain interindividual differences in the metabolism of endogenous substrates of cytochrome P-450 isozymes. An understanding of these mechanisms of variation may ultimately explain the extreme multiplicity of cytochrome P-450 isozymes.

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