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Cytochrome P-455 complex formation in the metabolism of phenylalkylamines—V.* Complex formation in human liver microsomes from various sources

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Metabolic *N*-oxidation has been established as a prerequisite for the formation of the P-455 metabolic complexes observed during the metabolism of certain primary [1–4] and secondary [4–6] aliphatic amines, with indications that nitroso species [1, 7, 8] are the actual ligands. The complexes formed are inhibitory in their nature [4] as they inhibit the hepatic mixed function oxidases, and this sequestering of the enzyme is of relevance to drug metabolism and safety.

As part of our studies on *N*-hydroxylation and cytochrome P-455 complex formation [1, 2, 5, 6], we have investigated the occurrence of complex formation in human liver microsomes during NADPH-dependent metabolism of some phenylalkylamines and their *N*-oxidized congeners (Fig. 1). The study had two objectives, namely to assess the occurrence of complex formation in human liver microsomes when using *N*-oxidized congeners of the amines as substrates, and to ascertain if human liver microsomes, like those of the rat and the rabbit [9] are capable of converting the amines to complex forming entities, i.e. whether or not *N*-oxidation was indicated.

Materials and methods. 1-Phenyl-2-propylamine (I) was supplied by ACO Läkemedel. The synthesis of *N*-hydroxy-1-phenyl-2-propylamine (III) has been previously described [10]. *N*-Benzyl-1-phenyl-2-propylamine (V) was prepared from I and benzaldehyde as described by Schaeffer *et al.* [11]. Procedures for the preparation of 3-methyl-1-phenyl-2-butylamine (II) and *N*-hydroxy-3-methyl-1-phenyl-2-butylamine (IV) will be presented elsewhere (Lindeke *et al.*, in preparation). All compounds used comprised racemic mixtures.

Samples of human livers were obtained from the human liver bank at the Huddinge hospital. The livers in the bank are well characterized and are obtained under controlled conditions shortly after circulatory arrest from cadaveric (mostly total cerebral infarction) renal transplant donors. Postmortem changes are thus minimal. For further characterization of the livers used in this study, as for the preparation of the microsomes, see von Bahr *et al.* [12]. Microsomes, prepared from frozen (−80°) liver cubes ranging from 2–5 g, were used and for comparison also rat liver microsomes obtained from control or phenobarbital-treated male Sprague–Dawley rats [6].

The formation of cytochrome P-450 product complexes was determined by the difference spectroscopy technique using an Aminco DW-2 Spectrophotometer as previously described [6]. The human liver microsomal concentration ranged from 1.8–2.5 mg protein/ml in 50 mM potassium phosphate buffer pH 7.5, while the rat liver microsomal concentrations (control and induced) were 1 mg protein/ml.

Results and discussion. The *N*-oxidized congeners (III, IV) of the amines (I, II) formed complexes during NADPH-dependent metabolism with microsomes from all the human livers tested (8 cases), with a time dependent formation of the absorbance maxima appearing at 455–457 nm. The spectra were practically identical to those obtained with these compounds and rat liver microsomes [5, 6].

To investigate the complexing activities of the phenylalkylamines, amphetamine (I) was initially studied, but as the first experiments (cases HL 1,5,7 and 8) gave negative results, the amphetamine analogue II was subsequently investigated. Compound II had namely, due to its higher lipophilicity, in a previously performed rat study [2] exhibited considerably higher complexing activities than amphetamine. It was interesting to observe that although II also appeared inactive in microsomes from the first four livers investigated, substantial activities were detected in two following cases, the results being summarized in Table 1. It is evident that while microsomes from all the eight cases formed complexes from the hydroxylamine IV, microsomes from only two out of the eight livers were capable of converting the amine II into the ultimate ligand. Moreover, with microsomes from these two livers, HL 9 and HL 13, and the hydroxylamine IV as substrate the extent of complex formation per mM P-450 reached significantly higher levels than in the other cases. This indicates that higher amounts of cytochromes capable of interacting also with the hydroxylamine (IV) were contained in these two livers. Cases HL 9 and HL 13 also exhibited rather high total amounts of the haemoprotein.

To establish if the pronounced effects seen with cases HL 9 and HL 13 are affected by storage, microsomes from these livers were prepared and reinvestigated five months later, but no decay in the activities could be noted. On this occasion also norbenzphetamine (V), previously found to be inactive with microsomes from case HL 1 and HL 5 was reinvestigated with HL 13 and found to be active (Table 1). However, as was the case with methamphetamine and rat liver microsomes [6], a lag-phase of about one minute prevailed before commencement of the time dependent increase in the absorbance, indicating that the initial oxidation of V is a rate limiting step [6].

That activity was observed with cases HL 9 and HL 13 but not with the others can have several causes, one being inborn (genetic) variations, another variations due to environmental factors, drug administration included [13]. When investigating the case histories [12] it became evident that cases HL 9 and HL 13 differed from the others inasmuch as these subjects had been repeatedly treated with phenytoin for 10 days prior to death. Using rat liver microsomes, cytochrome P-455 complex formation is a reaction that is specifically enhanced after induction with phenobarbital [14] and the effects are most pronounced when amines are used as substrates. That the cytochrome P-450 system in the liver of case HL 13 was different from the others is also supported by high rates of aminopyrine and amitriptyline demethylation as well as by carbamazepine epoxidation obtained with its microsomes [12].

In a previous study using human liver microsomes, Werringloer *et al.* [15] established the presence of multiple forms of microsomal cytochrome P-450. The results were based on differences in the complex forming activities from metyrapone and SKF 8742-A (the de-ethylated derivative of SKF-525 A). They could distinguish four types of cytochrome P-450, of which at least two were capable of complexing with products formed during NADPH-dependent metabolism of SKF 8742-A. Norbenzphetamine (V) was also included but microsomes from all their cases were inactive with this substrate. However, as indicated in a recent review by Werringloer and Estabrook [16], microsomes from a number of human livers were able to form complexes under the conditions prevailing in the metabolism of *N*-hydroxy-*p*-methoxyamphetamine, a finding that is analogous to our results with the *N*-oxidized compounds (III, IV).

* For part IV see ref. [6]. Presented in part at the 39th Int. Congr. Pharm. Sci., Brighton, Sept. 3–7, 1979.

Table 1. The extent of complex formation in NADPH-dependent metabolism of II, IV and V with human liver microsomes

Case	Liver storage (months)	P-450 (nmol · mg prot^{-1}	ΔA_{max}*		
			II†	IV‡	V‡
HL 1	6.5	0.48	—	—	nil
HL 5	11	0.41	nil	17	nil
HL 7	11	0.61	nil	22	—
HL 8	11	0.52	nil	16	—
HL 9	8	0.80	6.3	32	—
	13	0.50	6.3	38	—
HL 11	5	0.26	nil	26	—
HL 13	1	0.92	14	34	—
	6	0.86	13	36	3.4
HL 14	3	0.53	nil	24	—

* ΔA (455–490) cm^{-1} × P-450 mM^{-1} × t min^{-1}.
† [S] = 1 mM, $t = 15 \pm 0.5$.
‡ [S] = 250 μM, $t = 9 \pm 0.5$.

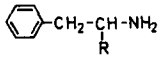
To limit the use of precious material, multiple runs, comprising multiple preparations of each liver, were generally not performed. Thus, *intra*-case statistics were not obtained. However, if the less active cases are compared on one hand, and the more active (HL 9 and HL 13) on the other, good *inter*-case statistics are obtained. This is illustrated by the mean ΔA_{max}-values, 21 and 35 respectively, for the hydroxylamine IV.

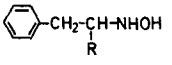
For the experimental conditions, see Materials and Methods.

Evidence that more forms of cytochromes can interact with hydroxylamines than with amines (cf. Table 1) was recently also reported by Franklin *et al.* [17]. In reconstituted monooxygenase systems using two purified rabbit lung cytochromes, they found that one of the cytochromes formed complexes from both *N*-hydroxyamphetamine (III) and norbenzphetamine (V) while the other formed the complex from III only.

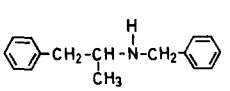
To relate the complexing activities of the human liver microsomes to those of the rat, the maximal formation of the 455 nm absorbance band (ΔA_{max} in Table 1) in the various preparations were compared, using the hydroxylamine IV (250 μM) as substrate. The average value of the two most active cases (HL 9 and HL 13) is $\Delta A_{max} = 35$ to be compared with the $\Delta A_{max} \sim 45$ and $\Delta A_{max} \sim 51$ for control and phenobarbital induced [2] rat liver microsomes, respectively.

In conclusion, the results of the present study show that human liver microsomes are qualitatively similar to those of the rat [4–8] and the rabbit [9] in forming cytochrome P-455 metabolic complexes from *N*-oxidized congeners of phenylalkylamines. When corresponding amines are used as substrates with the human liver microsomes, complex formation is not a general rule, while those of the rat and the rabbit always remain active, but less so.





I R = CH_3, II R = $CH(CH_3)_2$ III R = CH_3, IV R = $CH(CH_3)_2$



V

Fig. 1. Chemical structure of 1-phenyl-2-propylamine (amphetamine, I), 3-methyl-1-phenyl-2-butylamine (II), *N*-hydroxy-1-phenyl-2-propylamine (*N*-hydroxyamphetamine, III), *N*-hydroxy-3-methyl-1-phenyl-2-butylamine (IV), and *N*-benzyl-1-phenyl-2-propylamine (norbenzphetamine, V).

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Glutathione *S*-conjugates of phenyloxirane

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A variety of biochemical, pharmacological, and toxicological studies on glutathione *S*-conjugation of epoxides of xenobiotic olefins and arenes suggest that it plays an important role in the metabolic inactivation of toxic epoxides as active metabolites formed from parent hydrocarbons by microsomal monooxygenases. Phenyloxirane (styrene oxide), a mutagenic metabolite of the plastic monomer, styrene [1–3], has been frequently used as a standard substrate for the study of glutathione *S*-transferase [4–7]. In rat liver, the epoxide formed from styrene by microsomal monooxygenase is detoxicated through glutathione conjugation by cytosolic glutathione *S*-transferase at extremely high rate as well as through hydrolysis to phenylethanediol by microsomal epoxide hydratase at a considerably minor rate [8, 9].

However, no conclusive evidence has been available yet for the mode of introduction of the glutathione sulfhydryl group to the oxirane carbons of phenyloxirane in the metabolic conjugation reaction. Two approaches have recently been made to this problem, besides the earlier finding by James and White [10] that both styrene and phenyloxirane yielded *N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)cysteine *in vivo* and *in vitro* as the sole sulfur-containing metabolite. One was made by Ryan and Bend [8] who claimed the conjugate formed from phenyloxirane in rat liver to be single and identical with the sole product, *S*-(1-phenyl-2-hydroxyethyl)glutathione 1 synthesized from glutathione and phenyloxirane in an aqueous ethanolic sodium hydroxide solution. The other was made by Pachecka *et al.* [11] who reported that the synthetic conjugate of Ryan and Bend as well as a biologically formed glutathione conjugate from phenyloxirane in a cytosolic fraction of rat liver contained another isomer, *S*-(2-phenyl-2-hydroxyethyl)-glutathione **2**, in higher yield. Pachecka *et al.* isolated the biologically formed conjugates as an inseparable mixture on a silica gel column, subjected it to hydrogenolysis with Raney nickel in absolute ethanol, identified two isomeric phenylethanols as desulfuration products with phenethyl alcohol and methylphenylcarbinol, and emphasized that their results were coincident with those of the previous *in vivo* work done by Seutter-Berlage *et al.* [12]. The *in vivo* work, not necessarily providing direct evidence for the glutathione conjugation problem, showed that the rats given styrene excreted two isomeric phenylhydroxyethyl-mercapturic acids in urine which were readily separable on silica gel after derivatization with diazomethane and identified with synthetic specimens prepared from phenyloxirane and *N*-acetylcysteine methyl ester in an aqueous ethanolic sodium carbonate solution.

In the present communication, we wish to provide direct evidence for the formation of two isomeric glutathione conjugates **1** and **2** from phenyloxirane in rat liver cytosol. The conjugates were isolated, separated by h.p.l.c. (high performance liquid chromatography), and identified with respective synthetic specimens through the present work. Evidence will also be provided that the Raney nickel method reported by the previous workers be inadequate for the assignment of the conjugate structures since it yields both phenethyl alcohol and methylphenylcarbinol either from **1** or from **2** alone through phenyloxirane formed as a common intermediate by their desulfuration.

A mixture of glutathione conjugates **1** and **2** was prepared from phenyloxirane and isolated as a mixture on an Amberlite XAD-2 column by the method of Ryan and Bend. An amorphous solid isolated showed a single spot at R_f 0.40 on a microcrystalline cellulose powder plate containing a fluorescent reagent in *n*-butanol–water–acetic acid (4:1:1) as a developing solvent; the spot was visualized by u.v.-rays as well as by spraying with ninhydrin followed by heating. The conjugates were separated on an octadecylsilicone column (μBondapak C_{18}, 10 μ in particle size, 3.9 mm × 30 cm) in a solvent mixture of methanol–water–acetic acid (20:180:1) by h.p.l.c. at a column temperature of 30° with monitoring at 254 nm. The u.v.-absorbing peak areas in the chromatogram indicated that the synthetic conjugate mixture contained **1** and **2** in the ratio 1:3. Evaporation of the solvent from the eluate containing each conjugate, followed by recrystallizations from methanol–water, gave colorless prisms. Structures of the separated conjugates were assigned by proton-n.m.r. spectra (Fig. 1) by the partial application of the double resonance method.

In order to obtain biologically formed glutathione conjugates of phenyloxirane, a solution of the epoxide (2 mM) in aceton (270 mM) was incubated at 37° for 10 min in the presence of glutathione (4 mM) with a 105,000 *g* supernatant fraction (0.62 mg protein/ml) from a young adult male Wistar rat liver homogenate in 0.1 M phosphate buffer, pH 7.4. The biological reaction was terminated by the immediate removal of the epoxy substrate by the extraction with *n*-pentane. The residual aqueous phase was poured onto an Amberlite XAD-2 column (1 × 30 cm for 10 ml of the phase). The column was washed with water (3 bed volumes) and eluted with 50% aqueous methanol (2 bed volumes). The residue obtained on the evaporation of the solvent from the eluate was dissolved in water containing *erythro*-1,2-dihydroxy-1-phenylpropane as an internal reference and subjected to h.p.l.c. carried out under the same conditions as mentioned above. In the chromatogram, two u.v.-absorbing peaks appeared at the same retention times as those of the synthetic conjugates **1** and **2**. They were separately eluted from the octadecylsilicone column and examined for the identity with authentic **1** and **2** by ultraviolet spectroscopy as well as by t.l.c. using a ninhydrin reagent. A quantitative study showed that the