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COMPARISONS OF THE FORMATION OF CYTOCHROME P-450 COMPLEXES ABSORBING AT 455 nm IN RABBIT AND RAT MICROSOMES*

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Abstract—The formation of 455 nm complexes during the metabolism of various drugs has previously been studied in the rat. The formation of 455 nm complexes during the metabolism of amphetamine congeners and other compounds has now been studied in the rabbit to determine the differences that exist between these two species. Rabbit microsomes, in contrast to those from rat, show stereochemical preference for the *l*-isomer over the *d*-isomer of amphetamine. In both species, benzyl and hydroxyl substitutions on the nitrogen increased the rate of 455 nm complex formation, while methyl substitution did not. Much lower rates of 455 nm complex formation from SKF 525-A and propoxyphene were observed in rabbit microsomes than in rat microsomes. Both rat and rabbit were incapable of forming a 455 nm complex from *p*-hydroxyamphetamine. These and other differences in 455 nm complex formation parallel, in part, the qualitative and quantitative differences observed in the metabolism of these compounds in the two species.

The complexes formed by the binding of ligands to reduced cytochrome P-450 can be identified by their characteristic absorbance spectra in the Soret region. For example, in combination with carbon monoxide, reduced cytochrome P-450 has a maximum absorbance at 450 nm, while with ethyl isocyanide reduced cytochrome P-450 forms a complex which has two maxima, one at 430 nm and another at 455 nm [1]. Recent research has centered on compounds capable of forming complexes absorbing maximally at 455 nm during oxidative metabolism in microsomal systems [2–9], since the observation that a metabolic intermediate of the methylenedioxyphenyl compound, piperonyl butoxide, forms a stable 455 nm complex that inactivates cytochrome P-450 [10]. Such complex formation caused extremely potent non-competitive inhibition of other oxidative metabolism occurring in the microsomal system. Subsequently, many other methylenedioxphenyl compounds [4], as well as compounds such as amphetamines [5, 6], SKF 525-A, SKF 26754-A [7] and Lilly 18947 [11], have been shown to be capable of forming a 455 nm complex in rat microsomes.

This study which has been presented in preliminary form [12] was undertaken to examine the 455 nm complex formation in rabbit microsomes using compounds that are known to produce such complexes in the rat. As several of the compounds examined are known to have different routes of oxidative metabolism in the two species, any differences in 455 nm complex formation may provide keys to the common reaction or mechanism responsible for the formation of this complex, from these and other compounds.

MATERIALS AND METHODS

Hepatic microsomes were prepared from mature male Sprague–Dawley rats and locally supplied male New Zealand White rabbits (1–2 kg) by the procedure described previously [13]. The animals were either left untreated or pretreated with phenobarbital (rabbits, 60 mg/kg for 4–12 days; rats 20–80 mg/kg for 1–4 days), and starved overnight before sacrifice.

Spectrophotometric measurements were performed by either dual wave-length or split-beam spectroscopy using an Aminco DW-2 UV-Vis spectrophotometer. All observations were performed with microsomes at a concentration of 2 mg protein/ml in a Tris buffer (50 mM Tris-chloride, 150 mM KCl, 10 mM $MgCl_2$; pH 7·4). Protein was determined by the biuret reaction [14]. All enzymic reactions were determined at 25°.

NADPH was obtained from Sigma Chemical Co. *d*-Benzphetamine and *d*-norbenzphetamine were gifts from The Upjohn Co.; *d*-methamphetamine was a gift from Abbott Laboratories and propoxyphene was a gift from Eli Lilly & Co. Smith, Kline & French Laboratories generously supplied *d*- and *l*-amphetamine, SKF 525-A, *p*-hydroxyamphetamine and *N*-hydroxyamphetamine.

RESULTS

Each compound examined in rabbit microsomes was initially investigated over a 1000-fold range of concentration. In rat microsomes, many of the compounds that are capable of being oxidatively metabolized to form a 455 nm complex exhibit an optimum substrate concentration, with concentrations above and below this producing slower rates of 455 nm complex formation [8, 11, 15]. The concentration for a maximum rate of 455 nm complex formation was, therefore, determined using rabbit microsomes. The substrate concentration for the maximal rate of 455 nm complex formation was found to be independent of the concentration of cytochrome P-450 in the microsomes. The optimum concentrations were 250 μM for *l*-amphetamine, *d*-amphetamine and methamphetamine, 100 μM for norbenzphetamine, 50 μM for benzpheta-

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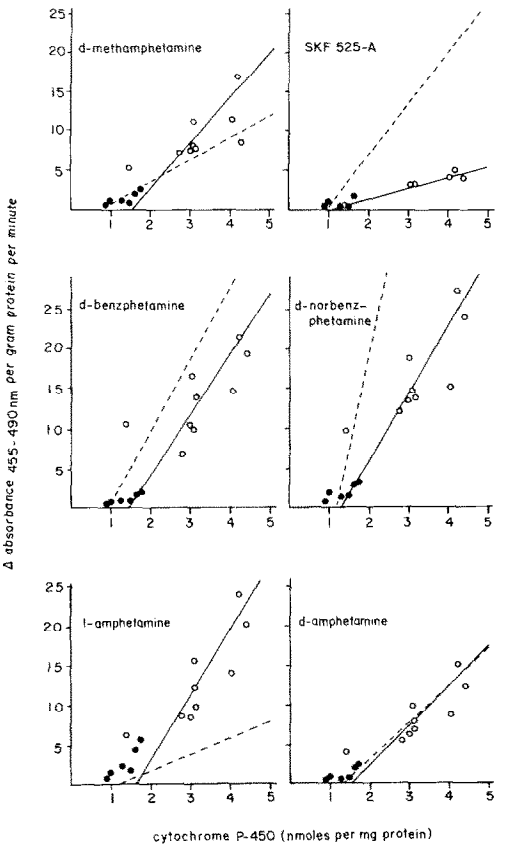

Fig. 1. Rate of 455 nm complex formation from amphetamines and related compounds by microsomes from untreated and phenobarbital-pretreated rabbits. The rate of 455 nm complex formation was examined at optimum substrate concentrations using microsomes from untreated (●) and phenobarbital-pretreated (60 mg/kg/day for 4–12 days) rabbits (○). Unweighted least squares-linear regression lines for the rates of complex formation from phenobarbital-pretreated rabbits were calculated (solid line). Similar lines from phenobarbital-pretreated rats are also shown for comparison (dashed line).

mine, 330 μM for *N*-hydroxyamphetamine and 33 μM for SKF 525-A and propoxyphene. *p*-Hydroxyamphetamine did not form 455 nm complexes at any concentration in rabbit microsomes. This is similar to the results using rat microsomes where *p*-hydroxyamphetamine was the only one of these nine substrates which was incapable of forming a complex. All of the compounds were checked by split-beam spectroscopy to establish that the increase in absorbance measured in the dual wavelength mode was indeed due to a peak at 455 nm and not to an artifact of an absorbance with a maximum at another wavelength. All the compounds which formed complexes showed the same absorbance maximum (455 nm) in both rats and rabbits.

Figures 1 and 2 show the rate of 455 nm complex formation in control and phenobarbital-pretreated rabbit microsomes. The solid lines represent regression curves for the results obtained using microsomes from phenobarbital-pretreated rabbits. The dashed lines show a similar line derived from experiments performed using rat microsomes [8, 15]. It is readily apparent that in rabbits the rate of 455 nm complex formation is highest for *N*-hydroxyamphetamine (Fig. 2) and lowest for SKF 525-A (Fig. 1). The other five compounds (Fig. 1) fall between these two extremes for phenobarbital-pretreated rabbits, with rates in the following order: *d*-amphetamine < methamphetamine < *l*-amphetamine < benzphetamine < norbenzphetamine. It should perhaps be noted that, with the possible exception of *l*-amphetamine, the regression lines calculated for the results from microsomes from phenobarbital-pretreated rabbits pass through or very close to the values from microsomes of untreated animals. Since these regression lines intersect the abscissa at a cytochrome P-450 value close to that of the control animals, it suggests that, as was found in the rat [15], the 455 nm complex formation is predominantly a property of induced microsomes. However, the small activity which is present in control animals does raise the question of whether these animals have undergone a small amount of induction due to unknown environmental factors, or whether they normally contain small amounts of the cytochrome P-450 enzyme activity responsible for 455 nm complex formation.

The mean maximum rates of 455 nm complex formation for control and phenobarbital-pretreated rabbits from Figs. 1 and 2 are tabulated in Table 1 and compared with the values obtained with rat microsomes. In addition, two other compounds which were investigated are listed: propoxyphene and *p*-hydroxyamphetamine. It should be noted that the values in this table are expressed per μmole cytochrome P-450, not per g protein. The higher specific activity of phenobarbital-pretreated microsomes with all substrates reinforces the argument that 455 nm complex formation is predominantly a property of induced microsomes. Examination of these results also shows many interesting differences between the two species. The *l*-isomer of amphetamine is preferred to the *d*-isomer in both untreated and phenobarbital-pretreated rabbits; the opposite is true in the rat. Both species show similar control values for *d*-amphetamine and methamphetamine, although upon phenobarbital pretreatment the

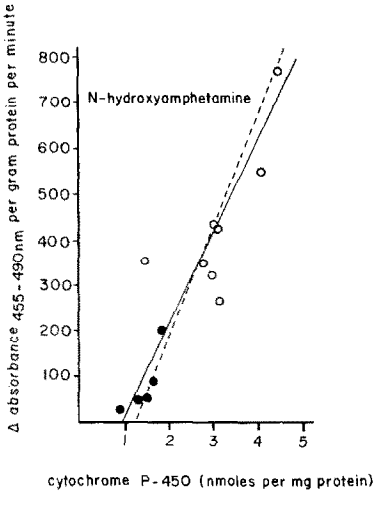

Fig. 2. Rate of 455 nm complex formation from *N*-hydroxyamphetamine, using microsomes from untreated and phenobarbital-pretreated rabbits. The determination of the rate of complex formation and method of presentation were the same as in Fig. 1.

Table 1. Specific activity of the rate of 455 nm complex formation from amphetamines and related compounds*

Drug	Rabbit		Rat	
	Untreated	Phenobarbital-pretreated	Untreated	Phenobarbital-pretreated
d-Amphetamine	0·71 ± 0·20 (6)	2·54 ± 0·20 (9)	0·73 ± 0·05 (12)	2·25 ± 0·13 (13)
l-Amphetamine	1·92 ± 0·60 (6)	4·01 ± 0·34 (9)	0·26 ± 0·04 (5)	0·98 ± 0·08 (11)
d-Methamphetamine	0·84 ± 0·20 (6)	3·1 ± 0·24 (9)	0·76 ± 0·08 (7)	2·15 ± 0·09 (13)
d-Norbenzphetamine	1·31 ± 0·25 (6)	5·1 ± 0·37 (9)	1·37 ± 0·08 (13)	11·96 ± 0·88 (13)
d-Benzphetamine	0·61 ± 0·15 (6)	4·43 ± 0·49 (9)	0·03 ± 0·02 (6)	5·69 ± 0·34 (13)
N-hydroxyamphetamine	59·7 ± 24·0 (5)	151·1 ± 18·3 (8)	69·3 ± 2·7 (7)	128·4 ± 9·0 (8)
SKF 525-A	0·45 ± 0·20 (5)	1·17 ± 0·18 (6)	3·6 ± 0·29 (11)	3·92 ± 0·21 (12)
Propoxyphene	0·13 ± 0·05 (3)	0·69 ± 0·18 (4)	1·1 ± 0·19 (11)	2·44 ± 0·20 (12)
p-Hydroxyamphetamine	0·0	0·0	0·0	0·0

* The rate of 455 nm complex formation was examined with microsomes from untreated and phenobarbital-pretreated animals, with 400 μM NADPH and the following concentrations of the substrates: 250 μM (1 mM in rat) l-amphetamine, *d*-amphetamine, methamphetamine and *p*-hydroxyamphetamine, 100 μM norbenzphetamine, 50 μM (66 μM in rat) benzphetamine, 33 μM SKF 525-A and propoxyphene, and 330 μM *N*-hydroxyamphetamine. The rates are quoted as specific activities in Δ absorbance/μmole of cytochrome P-450/min. Also shown is the standard deviation of the mean followed, in parentheses, by the number of microsomal preparations examined.

specific activity increases to a slightly greater extent in the rabbit. Both species show a low ability to form a 455 nm complex from benzphetamine with microsomes from untreated animals, but both show a high degree of induction of this ability upon phenobarbital pretreatment. Surprisingly, the rat which has the lower 455 nm complex-forming ability from benzphetamine in microsomes from untreated animals shows the higher specific activity after phenobarbital pretreatment. In fact, the ability to form a 455 nm complex from both the benzyl substituted amines (benzphetamine and norbenzphetamine) is stimulated to a much greater extent by phenobarbital pretreatment in the rat than in the rabbit. For *N*-hydroxyamphetamine, where the maximum rate of formation of the 455 nm complex formation is an order of magnitude higher than the maximal rates for the other compounds investigated, similar values were obtained with microsomes from untreated animals of both species and after phenobarbital pretreatment of both species. Although the rate of formation of a 455 nm complex from SKF 525-A was much smaller in rabbit than in rat microsomes, the specific activity in rabbit, in contrast to rat, was increased by phenobarbital pretreatment.

DISCUSSION

It is known that the metabolism of amphetamine differs in rat and rabbit [16]. In the rat, *para*-hydroxylation is the main metabolic route *in vivo*, whereas in the rabbit deamination to phenylacetone is the predominant route. An interesting feature of metabolism in the rat is that, although deamination is a very minor route in control animals, phenobarbital pretreatment enhances deamination several-fold [17]. With propoxyphene the predominant reactions observed for amphetamine are reversed; demethylation of the nitrogen of propoxyphene is the main metabolic route in rat [18], while in rabbit a much greater proportion of the metabolites occur in ring hydroxylated form (R. E. McMahon, personal communication). These metabolic differences between these two compounds in each of the two species parallel the rate of 455 nm complex formation, it being reduced where ring hydroxylation is the predominant reaction.

The rabbit which shows a greater preference for the *l*-isomer of amphetamine in the deamination reaction [19] also shows this stereospecificity in its ability to form a 455 nm complex. Likewise, the rat which preferentially deaminates the *d*-isomer of amphetamine *in vivo* reflects this in the rate of formation of the 455 nm complex, the rate for the *d*-isomer being much faster than for the *l*-isomer. The rabbit which preferentially deaminates amphetamine shows a greater ability to form a 455 nm complex from its favored isomer, *l*-amphetamine, than the rat does from its favored isomer, *d*-amphetamine. Also, phenobarbital pretreatment, which increases deamination, increases the rate of 455 nm complex formation in both rabbit and rat. These observations reinforce the previously suggested idea that oxidative metabolism at or around the nitrogen is implicated in the reaction necessary for forming the 445 nm complexes [6, 8, 20]. Also, as mentioned above, the preferential metabolism of propoxyphene at the nitrogen in the rat and the phenyl groups in the rabbit, coupled with differences in their ability to form a 455 nm complex, is further evidence to support that theory.

The nitrogen substituted amphetamines further suggest nitrogen involvement in 455 nm complex formation. With the secondary amines, neither methyl nor benzyl substitution on the nitrogen of *d*-amphetamine hinders formation of 455 nm complexes in either animal species. In fact, with benzyl substitution the rate is considerably enhanced. With the tertiary amine, benzphetamine, which has a methyl in addition to the benzyl substituent, the lower rate of complex formation suggests that a tertiary substituted amine, especially in untreated animals, is a hindrance to 455 nm complex formation. It has been suggested (J. Werringloer, personal communication) that the 455 nm complex formed from benzphetamine is, in fact, formed entirely from norbenzphetamine (i.e. after benzphetamine has been demethylated to norbenzphetamine). If this is the case, the large increase in the rate of complex formation from benzphetamine after phenobarbital pretreatment could be explained in part

by the induction of the demethylase activity. Preliminary data, showing that under conditions used for 455 nm complex formation the production of norbenzphetamine only occurs to an appreciable extent in microsomes from phenobarbital pretreated rats, substantiate this idea.

The involvement of an oxidative reaction at or around the nitrogen being responsible for the 455 nm complex is also suggested by the results using *N*-hydroxyamphetamine as substrate, since this compound shows the highest rate of 455 nm complex formation of any substrate investigated [20]. It is interesting to note that the high rate of complex formation from *N*-hydroxyamphetamine is nearly identical for both species. This may indicate that the species variations occur in reactions preceding the formation of this or a similar compound prior to the actual reaction which is responsible for the formation of the 455 nm complex.

In summary, the formation of a 455 nm complex, when correlated with the metabolism of these compounds in the rabbit and rat, suggests that some metabolic intermediate of a reaction around the nitrogen is combining with cytochrome P-450 to form a 455 nm complex. The complete mechamism remains to be elucidated, but evidence presented here implicates the nitrogen either directly or indirectly as an oxidized intermediate, where resonance forms may have the ability to donate electrons and form a coordinate covalent (ligand type) bond with cytochrome P-450 [21]. This would correlate with the ligand type interaction proposed for the piperonyl butoxide 455 nm complex [22].

It is interesting that man metabolizes amphetamine much like the rabbit (mainly deamination) and metabolizes propoxyphene much like the rat [18], and thus there exists the possibility that he may also form these 455 nm complexes. Since all metabolically formed 455 nm complexes so far examined have proved to be inhibitory to cytochrome P-450-dependent mixed-function oxidation, there exists the possibility of drug interactions resulting from impaired metabolizing capabilities when multiple drug therapy includes compounds related to amphetamines.

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