The Metabolism *in vitro* and Hepatic Microsomal Interactions of some Enantiomeric Drug Substrates

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1. The metabolism *in vitro* and microsomal interactions of (+)-amphetamine, (–)-amphetamine, (+)-benzphetamine and (–)-benzphetamine were studied with hepatic microsomes from phenobarbitone-pretreated male rabbits. 2. (+)-Benzphetamine was *N*-demethylated 30–35% faster than (–)-benzphetamine, but the apparent Michaelis constants for the two enantiomers were similar. 3. (–)-Amphetamine was deaminated about 200% faster than (+)-amphetamine. 4. The benzphetamine enantiomers gave qualitatively and quantitatively identical type I microsomal difference spectra (peak, 390 nm; trough, 425 nm) indicating identical apparent binding affinities for microsomes and identical spectral changes at maxima ($\Delta E_{\max}$ values). 5. The amphetamine enantiomers gave qualitatively identical type II microsomal difference spectra (peak, 433 nm; trough, 395 nm). However, the type II spectral data indicated that (+)-amphetamine had a markedly higher apparent binding affinity than (–)-amphetamine for microsomes. The amphetamine enantiomers gave identical $\Delta E_{\max}$ values. 6. The benzphetamine enantiomers (0.5 mM) enhanced the rate of microsomal cytochrome *P*-450 reduction by NADPH by 400–500%, (+)-benzphetamine enhancing the rate 20–25% more than (–)-benzphetamine. 7. The amphetamine enantiomers decreased the rate of microsomal cytochrome *P*-450 reduction by NADPH. At a concentration of 2 mM, (+)-amphetamine decreased the rate more than (–)-amphetamine. 7. All four enantiomers enhanced microsomal NADPH oxidation.

Different drugs are metabolized at different rates in the same animal species, and the rate at which a drug substrate is oxidatively metabolized in hepatic microsomes can depend on the characteristics of the substrate and on its interactions with the microsomes.

It has been shown that many substrates and inhibitors of hepatic microsomal drug metabolism are reversibly bound to hepatic microsomes to give two types of difference spectra (Schenkman, Remmer & Estabrook, 1967*a*; Imai & Sato, 1967). The type I difference spectrum is characterized by a trough at about 420 nm and a peak at about 385 nm, whereas the type II difference spectrum is characterized by a peak at about 430 nm and a trough at about 395 nm. Both these spectral changes, it has been suggested (Schenkman *et al.* 1967*a*; Imai & Sato, 1967), involve an interaction with cytochrome *P*-450, the microsomal pigment that apparently mediates many oxidative and reductive drug biotransformations (Gillette, 1966;

Gillette, Kamm & Sasame, 1968; Hernandez, Mazel & Gillette, 1967). It was originally thought that the magnitudes of these spectra, especially type I spectra, could be correlated with rates of microsomal drug metabolism (Schenkman *et al.* 1967*a*; Schenkman, Frey, Remmer & Estabrook, 1967*b*). That this correlation is not universally applicable has been shown by Davies, Gigon & Gillette (1969), Holtzman, Gram, Gigon & Gillette (1968) and Gigon, Gram & Gillette (1969).

Gigon *et al.* (1969) found that the addition of type I or type II substrates to hepatic microsomes respectively enhanced or decreased the rate of reduction of cytochrome *P*-450 by NADPH. These workers found that, with rats, the sex difference in the magnitude of the stimulatory effect caused by the type I substrate ethylmorphine could be closely correlated with the sex difference in the maximal velocity of ethylmorphine *N*-demethylation. This sex difference in ethylmorphine demethylation could not be accounted for in terms of sex differences in hepatic microsomal cytochrome *P*-450 content, cytochrome *c* reductase activity, magnitude of type I spectral change, or cytochrome *P*-450 reductase activity in

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the absence of substrate. Accordingly Gigon *et al.* (1969) suggested that the rate-limiting step of hepatic microsomal drug oxidations, especially with type I substrates, may be the reduction of the cytochrome *P*-450-drug substrate complex.

A property of a drug substrate that may be important in determining its rate of microsomal metabolism is its lipid solubility, i.e. a higher lipid solubility may favour a higher rate of metabolism (Gaudette & Brodie, 1959; McMahon, 1961; McMahon & Easton, 1961). However, enantiomeric drug substrates, that have identical lipid solubilities and many other identical physical and chemical properties, are often metabolized at different rates in hepatic microsomes (Axelrod, 1955; Axelrod, 1956; Furner, McCarthy, Stitzel & Anders, 1969).

In the present study enantiomers that are metabolized at different rates were compared with respect to their microsomal difference spectra and their effects on cytochrome *c* reduction, cytochrome *P*-450 reduction and NADPH oxidation. The enantiomers used were (+)-amphetamine, (–)-amphetamine, (+)-benzphetamine [(+)-*N*-benzyl-*N*- α -dimethylphenethylamine] and (–)-benzphetamine. Axelrod (1955) has demonstrated that the amount of (–)-amphetamine deaminated is markedly more than that occurring with (+)-amphetamine in rabbit hepatic 9000*g* supernatant. Hewick (1967) demonstrated that about 30% more *N*-demethylation occurred with (+)-benzphetamine than with (–)-benzphetamine in male rat hepatic microsomes. Preliminary studies had shown that benzphetamine and amphetamine enantiomers give type I and type II microsomal difference spectra respectively. Rabbits were used in this study since rabbits, unlike rats, give significant amphetamine deamination as assayed by the method of Axelrod (1954). The rabbits were phenobarbitone-pretreated to facilitate enzymic and spectral measurements.

MATERIALS AND METHODS

Treatment of animals. Male Dutch rabbits (1.5–2 kg) were used in all experiments and were allowed free access to food and water up to the time of being killed. Phenobarbitone pretreatment comprised five consecutive daily intraperitoneal injections of phenobarbitone sodium (30 mg/kg body wt.); the last injection was given approx. 24 h before death. The rabbits were killed by a blow to the head, and the livers were perfused *in situ* with ice-cold 0.9% NaCl to remove blood and then removed. All subsequent preparative operations were performed at 0–4°C.

Microsomal preparation. The livers were homogenized with 2 vol. of 0.15 M-KCl in 1.25 M-potassium phosphate buffer, pH 7.4, in a Potter-Elvehjem-type homogenizer equipped with a Teflon pestle. The homogenates were centrifuged for 20 min at 9000*g* in a B20 International ultracentrifuge. For sedimentation of microsomes the

9000*g* supernatant fraction (aspirated off carefully, avoiding the 9000*g* pellet and the waxy layer at the top of the centrifuge tube) was centrifuged at 180 000*g* for 30 min in a B60 International ultracentrifuge. The microsomes were resuspended in the KCl solution and resedimented. The washed microsomal pellet was suspended in 0.1 M-potassium phosphate buffer, pH 7.4, to give a protein concentration of 10 mg/ml. The microsomal protein concentration was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Miller (1959). Crystalline bovine serum albumin was used as the standard.

Assays for microsomal drug-metabolizing activity. Incubations were carried out in a Dubnoff shaker (100 oscillations/min) at 37°C under an atmosphere of O₂ (flow rate 1000 ml/min). A glass marble was added to each incubation beaker (capacity 15 ml) to facilitate mixing of the contents during incubation (Fouts, 1970). All incubations were performed in triplicate.

Incubation media contained 4 mM-glucose 6-phosphate, 5 mM-MgSO₄, 0.22 mM-NADP⁺, glucose 6-phosphate dehydrogenase (1.5 units; 1 unit reduced 1 μ mol of NADP⁺/min at pH 7.4 and 25°C), 10 mM-semicarbazide hydrochloride (for formaldehyde assays only), microsomes equivalent to 2 mg of protein, 0.12 M-potassium phosphate buffer, pH 7.4, and drug substrate, in a total volume of 2.5 ml.

The drug substrates were (+)-benzphetamine hydrochloride, (–)-benzphetamine hydrochloride, (+)-amphetamine sulphate and (–)-amphetamine sulphate. The specific optical rotations ($[\alpha]_D^{22}$) for these salts were 22.1, 20.4, 21.8 and 20.7° respectively (*c* 4 in water in each case). The benzphetamine enantiomers were a gift from Dr P. W. O'Connell, The Upjohn Co., Kalamazoo, Mich., U.S.A. The (–)-amphetamine sulphate was a gift from Dr E. A. Kimes, Smith, Kline and French Laboratories, Philadelphia, Pa., U.S.A. The (+)-amphetamine sulphate was obtained from DuMont Pharmacal Co., Philadelphia, Pa., U.S.A. The drug substrate concentrations ranged from 0.2 to 1.0 mM for the benzphetamine enantiomers and from 0.043 to 0.27 mM for the amphetamine enantiomers.

The incubation media minus NADP⁺ were prepared at 0–4°C. The NADP⁺ was added to the incomplete media after they were warmed to 37°C by 3 min preincubation. Subsequent incubation was for 20 min or 6 min for benzphetamine metabolism (see the Results section) or for 25 min for amphetamine metabolism.

The *N*-demethylation of the benzphetamine enantiomers was measured by the formaldehyde produced in the reaction. The demethylation reaction was terminated by the addition of 5 ml of 0.6 M-HClO₄ to 2.5 ml of reaction medium. After removal of the protein by centrifugation, 3 ml of the supernatant was mixed with 2 ml of double-strength Nash (1953) reagent B and incubated at 50°C for 15 min. After the mixture had been cooled to room temperature, the intensity of the yellow colour was read at 415 nm. Formaldehyde produced by reactions not involving drug substrate was corrected for by performing incubations from which only the drug substrate was omitted.

The deamination of the amphetamine enantiomers was determined by the method of Axelrod (1954).

NADPH oxidase, NADPH-cytochrome *c* reductase and NADPH-cytochrome *P*-450 reductase assays. These

assays were performed in a Gilford 2000 spectrophotometer with silica cuvettes (10mm×10mm) containing 3ml of reaction mixture. In all cases the initial linear phase was used to calculate the rate of each reaction. NADPH oxidase and NADPH-cytochrome *c* reductase activities were measured at room temperature (20–24°C). NADPH-cytochrome *P*-450 reductase activity was measured at either room temperature or 37°C (see the Results section).

NADPH oxidase activity was determined by a modification of the method described by Gillette, Brodie & LaDu (1957). The reaction was carried out in 0.1M-potassium phosphate buffer, pH 7.4, and the microsomal protein concentration was 1.66mg/ml. After the addition of 0.18mM-NADPH the rate of decrease in E_{340} was measured. The rate of NADPH oxidation was calculated by using a molar extinction coefficient of 6230.

NADPH-cytochrome *c* reductase was determined by a modification of the methods of Phillips & Langdon (1962). The assay system contained 100µg of microsomal protein/ml, 0.1mM-NADPH, 2.1mM-KCN, 0.05mM-cytochrome *c* (type VI; Sigma Chemical Co., St Louis, Mo., U.S.A.) and 0.05M-potassium phosphate buffer, pH 7.4. The reduction of cyrochrome *c* was followed at 550nm. The molar extinction coefficient used in calculations was 18500.

The NADPH-cytochrome *P*-450 reductase activity was determined as described by Holtzman *et al.* (1968) except that we (1) suspended the microsomes in 0.1M-potassium phosphate buffer, pH 7.4, (2) bubbled all microsomal suspensions (at 0–4°C, 3mg of protein/ml, vol. about 40ml) with deoxygenated CO for an hour before transferring them to the Aminco anaerobic spectrophotometric cuvette, and (3) added 0.4mM-NADPH instead of an NADPH-generating system. This concentration of NADPH was optimum with rabbit liver microsomes (D. S. Hewick & J. R. Fouts, unpublished work). A molecular extinction coefficient of 91000 (Omura & Sato, 1964) was used to calculate the number of nmol of cytochrome *P*-450 reduced/min per mg of microsomal protein.

Measurement of substrate-induced microsomal difference spectra. Type I or type II difference spectra were measured essentially as described by Hewick & Fouts (1970). The microsomes were suspended in 0.1M-potassium phosphate buffer, pH 7.4, when benzphetamine enantiomers were added. Since higher concentrations of amphetamine enantiomers were used, the buffer concentration was increased to 0.2M for these experiments to keep the pH constant.

The magnitude of a type I or type II difference spectrum (ΔE) was measured as the difference in extinction between the trough and peak in the 350–450nm region of the microsomal-drug spectrum. Where used, the term K_s ('spectral dissociation constant', after Schenkman *et al.* 1967a) represents the concentration of drug-substrate giving a half-maximal type I or type II spectral change ($\Delta E_{\max}/2$).

Statistical comparisons in this study were made by using Student's *t* test (Snedecor, 1956).

RESULTS

Hepatic microsomal drug metabolism. The enantiomers of benzphetamine have identical cofactor requirements, and in each case the optimum pH for

Fig. 1. *N*-Demethylation of (+)-benzphetamine or (–)-benzphetamine by hepatic microsomes from phenobarbitone-treated male rabbits in a 20min incubation time. ○, (+)-Benzphetamine; △, (–)-benzphetamine. Each point represents the mean of five experiments, the vertical lines representing $\pm$ S.E.M. Assay conditions are described in the Materials and Methods section.

Fig. 2. Hofstee (1959) plot (v versus $v/[S]$) for the *N*-demethylation of (+)-benzphetamine and (–)-benzphetamine by hepatic microsomes from phenobarbitone-treated male rabbits. ○, (+)-Benzphetamine; △, (–)-benzphetamine. Each point is derived from the mean of five experiments. Assay conditions are described in the Materials and Methods section. Data are taken from Fig. 1.

N-demethylation is pH 7.4. With saturating amounts of drug substrate present both isomers are *N*-demethylated linearly with time for 25min and

with microsomal protein concentration in the range used. The cofactor (e.g. NADP⁺, glucose 6-phosphate) concentrations used in these assays are also optimum.

In the substrate-concentration range 0.2–1.0 mM (+)-benzphetamine is *N*-demethylated 30–35% faster than (–)-benzphetamine (Fig. 1). If the mean values from Fig. 1 are plotted (v versus $v/[S]$, where v is the rate of demethylation and $[S]$ the corresponding substrate concentration) according to the method of Hofstee (1959) a pair of parallel curved lines is obtained, as shown in Fig. 2. The curvature of these lines (concave downwards) indicates that substrate inhibition and significant substrate depletion may have been occurring at high and low substrate concentrations respectively. It is not possible to improve the linearity of the plots of v versus $v/[S]$ by shortening the incubation time from 20 min to 6 min (to decrease substrate depletion) since incubation for the shorter time appears to increase substrate inhibition at the higher substrate concentrations (see Fig. 3 and compare with Fig. 1). However, as the lines in Fig. 2 are parallel, similar Michaelis constants (K_m values) are indicated for the two enantiomers. The points in Fig. 2 corresponding to the middle four concentrations of (+)-

Fig. 3. *N*-Demethylation of (+)-benzphetamine or (–)-benzphetamine by hepatic microsomes from phenobarbitone-treated male rabbits in a 6 min incubation time. O, (+)-Benzphetamine; Δ, (–)-benzphetamine. Each point represents the mean of three experiments, the vertical lines representing $\pm$ s.e.m. Assay conditions are described in the Materials and Methods section. Different rabbits were used in these experiments than in those giving the results in Figs. 1 and 2.

benzphetamine or (–)-benzphetamine are approximately on a straight line in each case. The slopes of the best straight lines through these points (least-squares method) give K_m values ($\pm$ s.e.m.) of 0.35 ± 0.05 mM and 0.30 ± 0.03 mM for the (+)-isomer and (–)-isomer respectively. These K_m values are not significantly different ($P > 0.05$). The intercepts of the best straight lines with the ordinate when the abscissa value is zero give maximal velocities (V_{max} values) of 31.1 ± 2.8 and 23.0 ± 1.8 nmol of formaldehyde/min per mg of microsomal protein for the (+)-isomer and (–)-isomer respectively. In agreement with Axelrod (1955) we found that (–)-amphetamine is deaminated at a markedly faster rate than (+)-amphetamine by rabbit liver microsomes. Fig. 4 shows that with hepatic microsomes from phenobarbitone-treated rabbits there is about a threefold difference in rates of deamination of the two amphetamines. Limitations of the amphetamine assay used make it impossible to obtain reliable kinetic data, especially for (+)-amphetamine.

Substrate-induced microsomal difference spectra. The addition of (+)-benzphetamine or (–)-benzphetamine to aerobic hepatic microsomes from phenobarbitone-treated male rabbits gives qualitatively identical type I difference spectra (peaks and troughs at 390 and 425 nm respectively). Similarly, qualitatively identical type II difference spectra (peaks and troughs at 433 and 395 nm respectively) are obtained by the addition of (+)-amphetamine or (–)-amphetamine to the microsomes.

No differences in the binding kinetics of (+)-benzphetamine and (–)-benzphetamine are detected from their type I difference spectra. With a different pool of microsomes for each, three similar experiments indicate that, within the substrate

Fig. 4. Deamination of (+)-amphetamine or (–)-amphetamine by hepatic microsomes from phenobarbitone-treated male rabbits. O, (+)-Amphetamine; Δ, (–)-amphetamine. Each plot represents the mean of four experiments, the vertical lines representing $\pm$ s.e.m. Assay conditions are described in the Materials and Methods section.

Fig. 5. Magnitudes of type I difference spectra caused by adding (+)-benzphetamine or (-)-benzphetamine to hepatic microsomes from phenobarbitone-treated male rabbits. $\circ$, (+)-Benzphetamine; Δ , (-)-benzphetamine. Each point represents the mean of three determinations, the vertical lines representing $\pm$ S.E.M. The microsomal protein concentration was 1.5mg/ml. The enantiomers were compared at identical concentrations; the points corresponding to (-)-benzphetamine have been displaced to the right for the sake of clarity. The curves connecting the experimental values are superimposable. The conditions were as described in the Materials and Methods section.

Fig. 6. (+)-Benzphetamine and (-)-benzphetamine (type I) spectral data plotted after the method of Hofstee (1959) (ΔE versus $\Delta E/[S]$). $\circ$, (+)-Benzphetamine; Δ , (-)-benzphetamine. Each point represents the mean of three determinations. The microsomal protein concentration was 1.5mg/ml. The conditions were as described in the Materials and Methods section.

concentration range 0.00166–0.133mM, identical concentrations of either enantiomer give type I difference spectra that are not significantly different in magnitude ($P > 0.05$). The data from one of these experiments are plotted in Fig. 5. The mean values from Fig. 5 are replotted in Fig. 6 (ΔE versus $\Delta E/[S]$, where $[S]$ is the corresponding substrate

concentration) after the method of Hofstee (1959). Fig. 6 shows that a linear relationship is not obtained, indicating that simple Michaelis–Menten kinetics are not followed. Similar non-linear plots were obtained by Hewick & Fouts (1970) using (+)-benzphetamine with hepatic microsomes from phenobarbitone-pretreated rats.

In contrast with the benzphetamine enantiomers, the binding kinetics of the amphetamine enantiomers, as determined from the type II spectral results, are markedly different. From three similar experiments the ratios of ΔE for (+)-amphetamine to that of (-)-amphetamine ($\pm$ S.E.M.) at 0.5, 2 and 10mM are 3.97 ± 0.25 , 2.41 ± 0.27 and 1.44 ± 0.14 respectively. Double-reciprocal plots ($1/\Delta E$ versus $1/[S]$) show that the line corresponding to (-)-amphetamine has a steeper slope than that corresponding to (+)-amphetamine (Fig. 7). However, at saturating substrate concentrations the same maximal spectral change ($\Delta E_{\max}$) is obtained with either enantiomer. The double-reciprocal plots for both enantiomers are slightly curved, again indicating that non-Michaelis–Menten kinetics are followed. The curvilinear relationship is more marked on plotting ΔE versus $\Delta E/[S]$, but we have used the double-reciprocal method of plotting to show more clearly that both amphetamine isomers give the same $\Delta E_{\max}$.

Fig. 7. Double-reciprocal plot of (+)-amphetamine and (-)-amphetamine (type II) spectral data. $\circ$, (+)-Amphetamine; Δ , (-)-amphetamine. The microsomal protein concentration was 3mg/ml. The conditions were as described in the Materials and Methods section.

Table 1. *Effects of benzphetamine and amphetamine enantiomers on NADPH oxidase and NADPH-cytochrome c reductase activities of hepatic microsomes from phenobarbitone-treated male rabbits*

Preparations and assays are described in the Materials and Methods section. The numbers of rabbits used are given in parentheses. With each rabbit three determinations were made. The assays were performed at room temperature (20–24°C).

Enantiomer added	Concn. (mM)	Cytochrome c reductase activity [nmol/min per mg of microsomal protein ($\pm$ S.E.M.)]	NADPH oxidase activity	
			[nmol/min per mg of microsomal protein ($\pm$ S.E.M.)]	(% of control)
None (control)		117.0 $\pm$ 2.4 (3)	4.95 $\pm$ 0.17 (3)	100
(+)-Benzphetamine	0.5	108.0 $\pm$ 2.6 (3)	24.00 $\pm$ 2.62* (3)	485
(-)-Benzphetamine	0.5	108.2 $\pm$ 1.8* (3)	22.40 $\pm$ 2.09* (3)	452
(+)-Amphetamine	2.0	115.0 $\pm$ 1.4 (3)	6.58 $\pm$ 0.33* (3)	133
(-)-Amphetamine	2.0	114.7 $\pm$ 1.7 (3)	7.08 $\pm$ 0.41* (3)	143

* Significantly different from control ($P < 0.05$).

Table 2. *Effects of benzphetamine and amphetamine enantiomers on NADPH-cytochrome P-450 reductase activity in hepatic microsomes from phenobarbitone-treated male rabbits*

Preparations and assays are described in the Materials and Methods section. The numbers of rabbits used are given in parentheses. With each rabbit three to six determinations were made.

Enantiomer added	Concn. (mM)	Temperature at which assay performed (°C)	Cytochrome P-450 reductase activity	
			[nmol/min per mg of microsomal protein ($\pm$ S.E.M.)]	(% of control)
None (control)		20–24	1.01 $\pm$ 0.04 (5)	100
(+)-Benzphetamine	0.5	20–24	6.08 $\pm$ 0.24*† (5)	604
(-)-Benzphetamine	0.5	20–24	4.95 $\pm$ 0.19*† (5)	490
None (control)		37	4.06 $\pm$ 0.23 (4)	100
(+)-Amphetamine	2.0	37	3.02 $\pm$ 0.12*† (4)	74.5
(-)-Amphetamine	2.0	37	3.57 $\pm$ 0.18*† (4)	88.0
(+)-Amphetamine	10.0	37	2.38 $\pm$ 0.13* (4)	58.7
(-)-Amphetamine	10.0	37	2.70 $\pm$ 0.05* (4)	66.6

* Significantly different from corresponding control ($P < 0.05$).

† Rate with (+)-isomer significantly different from rate with (–)-isomer at the same concentration ($P < 0.05$).

Microsomal NADPH-cytochrome c reductase and NADPH oxidase. The concentrations of drug substrates given in Table 1 are those that give maximal enhancement of NADPH oxidase activity. At these concentrations the enantiomers have little effect on NADPH cytochrome c reductase activity except perhaps to decrease it slightly (Table 1).

In contrast, all substrates enhance NADPH oxidase activity (Table 1). The effect is most marked with the type I enantiomers, a 300–400% enhancement occurring. The marked enhancement of hepatic microsomal NADPH oxidase activity by (+)-benzphetamine has also been noted by Lu, Strobel & Coon (1969). Rates of NADPH oxidation in the presence of either of a pair of enantiomers are not statistically significantly different. However, it is noted that within each pair the computed effect

on the mean rate of NADPH oxidation appears to be higher with the more rapidly-metabolized enantiomer.

If carbon monoxide is bubbled through a microsomal suspension in the absence of drug substrate (control) the rate of NADPH oxidation is decreased by about 30%. This carbon monoxide treatment completely abolishes the substrate-induced enhancement of NADPH oxidation; the rate is decreased to that of the carbon monoxide-treated control (results not shown).

Microsomal NADPH-cytochrome P-450 reductase. At 37°C the rate of cytochrome P-450 reduction by NADPH in the presence of the benzphetamine enantiomers is too rapid to measure reliably with the apparatus used. Therefore all NADPH-cytochrome P-450 reductase assays with the benz-

phetamine enantiomers were performed at room temperature (20–24°C).

The addition of 0.5mM-(+)-benzphetamine or (-)-benzphetamine increases the rate of cytochrome *P*-450 reduction by NADPH by 400–500% (Table 2). The enhancement caused by (+)-benzphetamine is about 25–30% higher than that caused by (-)-benzphetamine.

The amphetamine enantiomers inhibit cytochrome *P*-450 reduction by NADPH (Table 2). At a concentration of 2M (+)-amphetamine decreases the rate of cytochrome *P*-450 reduction about twice as much as (-)-amphetamine (26% compared with 12%). On increasing the concentration of the amphetamine to 10mM the rate of cytochrome *P*-450 reduction by NADPH is further decreased, but the enantiomers inhibit it to similar extents (41% and 33%).

At the concentrations used none of the enantiomers alters the extinction coefficient of the reduced cytochrome *P*-450–carbon monoxide complex.

DISCUSSION

Gigon *et al.* (1969) have suggested that the mechanism of oxidative hepatic microsomal metabolism for type I drug substrates involves the following sequence. The drug substrate reacts with oxidized cytochrome *P*-450 and the drug–cytochrome *P*-450 complex is reduced by NADPH either directly or indirectly by NADPH–cytochrome *c* reductase. This reduced drug–cytochrome *P*-450 complex then combines with atmospheric oxygen and decomposes with the oxidation of both the drug and the cytochrome *P*-450 and with the formation of water. These workers suggest that the mechanism of microsomal metabolism of type II substrates is more complex, probably involving an inhibitory substrate–cytochrome *P*-450 interaction.

We find that the enantiomers tested, all of which are metabolized by hepatic microsomes, increase the rate of microsomal NADPH oxidation. This increased microsomal NADPH oxidation is consistent with the general mechanism proposed by Gigon *et al.* (1969). The lower enhancement of NADPH oxidation by the type II enantiomers (amphetamine) probably reflects their lower rates of metabolism (deamination) as compared with the type I substrates (benzphetamine; demethylation). The drug-induced enhancement of microsomal NADPH oxidation is not caused by alterations in the activity of NADPH–cytochrome *c* reductase, since the enantiomers have a negligible effect on the activity of this enzyme.

However, in common with the type I substrates tested by Gigon *et al.* (1969), (+)-benzphetamine and (-)-benzphetamine enhance the rate of cyto-

chrome *P*-450 reduction by NADPH. However, the greater enhancement of cytochrome *P*-450 reduction by (+)-benzphetamine is not a result of its faster metabolic rate (demethylation), since the cytochrome *P*-450 reduction assay is performed anaerobically with microsomes saturated with carbon monoxide, conditions under which it is highly unlikely that oxidative demethylation could occur. It may be rather that, as suggested by Gigon *et al.* (1969), a higher rate of metabolism (demethylation) is a result of a faster rate of cytochrome *P*-450 reduction.

It seems possible that the type I substrate-induced enhancement of cytochrome *P*-450 reduction by NADPH is a result of an enzyme conformation change and that the absolute configuration of (+)-benzphetamine induced a more favourable change in microsomal binding sites or enzyme active centres than that caused by (-)-benzphetamine.

As with the type II substrates tested by Gigon *et al.* (1969), (+)-amphetamine and (-)-amphetamine decrease the rate of cytochrome *P*-450 reduction by NADPH.

It might be thought that the type I substrate-induced enhancement of NADPH oxidation (i.e. NADPH oxidase, measured at 20–24°C in the absence of carbon monoxide) is a result of the type I substrate-induced enhancement of cytochrome *P*-450 reduction (measured at 20–24°C in the presence of carbon monoxide). However, this is difficult to reconcile with the observation that the substrate-induced enhancement of NADPH oxidation is completely blocked by carbon monoxide. In addition, with the type II enantiomers, although NADPH oxidation is measured at 20–24°C and cytochrome *P*-450 reduction is measured at 37°C, it is still puzzling that either 2mM-(+)-amphetamine or 2mM-(-)-amphetamine increases microsomal NADPH oxidation (NADPH oxidase), but decreases the rate of cytochrome *P*-450 reduction by NADPH.

With the substrates used there is clearly no correlation between the rates of microsomal drug metabolism and the magnitudes of type I and type II microsomal difference spectra. (+)-Benzphetamine is *N*-demethylated about 35% faster than (-)-benzphetamine, but the two enantiomers are identical in their type I spectral properties (both K_s and $\Delta E_{\max}$). (+)-Amphetamine is deaminated at about one-third the rate of (-)-amphetamine, but induces greater type II spectral changes (except at a very high substrate concentration). At a concentration of 0.5mM the magnitude of the type II difference spectrum caused by (+)-amphetamine is about four times that induced by (-)-amphetamine. This difference would be even more marked in the substrate range used for metabolism studies (approx. 0.04–0.3mM).

The concentrations of (+)-benzphetamine or (-)-benzphetamine giving half-maximal rates of *N*-demethylation (K_m values) are not significantly different. Similarly, no difference is observed between the concentrations of these enantiomers giving half-maximal type I spectral changes (K_s values, as defined by Schenkman *et al.* 1967a). However, the K_m values (about 0.3 mM) are markedly higher than the K_s values (about 0.01 mM from Fig. 5). A similar relationship between K_m and K_s values has been observed by Castro, Greene, Gigon, Sasame & Gillette (1968) with the type I substrate ethylmorphine and rat hepatic microsomes. Furner *et al.* (1969), studying the hepatic microsomal metabolism of hexobarbitone (type I) enantiomers, found that (+)-hexobarbitone was metabolized 70% faster than (-)-hexobarbitone, but, as in the present work, the enantiomers did not appear to have significantly different K_m values.

Although the double-reciprocal plots of the amphetamine type II spectral data give curves (Fig. 7), the fact that the overall slope of the line corresponding to (-)-amphetamine is steeper than that corresponding to (+)-amphetamine does indicate that the spectral binding (dissociation) constants are higher for (-)-amphetamine. Since it has been suggested (Schenkman, 1968) that type II spectral changes are a result of substrate interaction with the haem moiety of cytochrome *P*-450, one interpretation of our spectral data is that (+)-amphetamine has a greater affinity than (-)-amphetamine for the haem of cytochrome *P*-450. A greater (inhibitory) substrate-haem interaction could account for the observed lower rate of microsomal deamination of (+)-amphetamine.

The greater inhibitory nature of (+)-amphetamine-microsomal interaction can also be seen in effects on NADPH-cytochrome *P*-450 reduction. With respect to the rate of cytochrome *P*-450 reduction by NADPH, the increase in inhibition with increase in concentration of each amphetamine enantiomer is roughly paralleled by the corresponding increase in magnitude of the type II spectral change (ΔE) caused by adding the amphetamine to a microsomal suspension. At a concentration of 2 mM (+)-amphetamine inhibits the rate of reduction of cytochrome *P*-450 by NADPH about twice as much as does (-)-amphetamine. At this concentration the addition of (+)-amphetamine to microsomes gives a ΔE value that is about 2.4 times that given by (-)-amphetamine. On increasing the substrate or ligand (amphetamine) concentration to 10 mM, at which concentration the difference in ΔE given by the two enantiomers is less marked [(+)-isomer/(-)-isomer ΔE ratio is about 1.4], each enantiomer inhibits cytochrome *P*-450 reduction by NADPH to a similar extent.

It is noteworthy that the benzphetamine en-

antiomers exhibit no stereoselectivity in their spectral interaction with microsomes (type I spectral properties). Possibly a type I spectrum is a result primarily of substrate solvation in a lipid membrane, a process that probably would not be stereoselective. After dissolving in the lipid, the substrate could diffuse to an active or binding site. That type I binding sites are susceptible to lipases (Chaplin & Mannering, 1969) and the lipid-dissolving solvent 2,2,4-trimethylpentane (Leibman & Estabrook, 1969) supports the idea that type I binding is at least partly dependent on a lipid interaction. The greater stability of type II binding sites to agents that destroy the integrity of lipid membranes (Chaplin & Mannering, 1969; Leibman & Estabrook, 1969) and the generally greater water solubility of type II substrates (see listed type II compounds in Imai & Sato, 1967; and Schenkman *et al.* 1967a) indicates that ligand-lipid membrane interaction is less important with type II binding.

If it is assumed that type II spectral changes are a result of substrate or ligand-haem interactions, it is probable that, like oxygen, type II substrates (ligands) approach the haem of cytochrome *P*-450 via an aqueous route. The (+)-isomer of amphetamine seems to fit the type II binding site(s) better than the (-)-isomer. It is possible that the haem of cytochrome *P*-450 is in a crevice, the conformation of which favours the approach of (+)-amphetamine.

The possible significance of non-Michaelis-Menten kinetics associated with type I or type II spectral data has been discussed by Hewick & Fouts (1970).

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