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HYDROXYLATION OF AMPHETAMINE TO PARAHYDROXYAMPHETAMINE BY RAT LIVER MICROSOMES

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Abstract—Rat liver microsomes were shown to hydroxylate D- and L-amphetamine at the para position of the benzene ring. The identity of the product, *p*-hydroxyamphetamine, was established by the use of three different methods, paper chromatography, radio gas-chromatography and mass-spectral analysis. The hydroxylation reaction was found to be linear with time to 15 min and protein concentration to 2 mg/ml. The reaction followed Michaelis–Menten kinetics only at low substrate concentrations. When the concentration of D-amphetamine exceeded 0·5 M the plot indicated substrate inhibition. According to the Lineweaver–Burk plot the following apparent kinetic data were found: D-amphetamine, $K_m = 2{\cdot}4 \times 10^{-5}$ M, $V_{max} = 3{\cdot}7$ moles × mg protein^{-1} × 10 min^{-1}; L-amphetamine, $K_m = 1{\cdot}1 \times 10^{-6}$ M, $V_{max} = 2{\cdot}8$ nmoles × mg protein^{-1} × 10 min^{-1}.

THE METABOLISM of amphetamine by oxidation leading to *p*-hydroxyamphetamine (*p*OHA) and its consequences for the development of amphetamine tolerance have been studied extensively *in vivo*.[1–3] These studies were carried out on the rat as this species hydroxylates about 60 per cent of the amphetamine administered to *p*OHA.[4] This metabolic pathway can be blocked by many chemically different drugs,[5] of which the best known example is desipramine (DMI).[6]

The inhibitory effect of DMI has been demonstrated also in experiments on isolated perfused rat liver.[7] However, in rat liver microsomes kinetic data on the parahydroxylation are lacking since none or only a trace amount of *p*OHA has been detected. Thus, Daly *et al.*[8] found that only 0·002 per cent of incubated racemic amphetamine was converted into *p*OHA. Dingell and Bass[7] measured the disappearance of amphetamine but found no change of the initial concentration of substrate.

The present communication describes the parahydroxylation of D- and L-amphetamine by rat liver microsomes at low substrate concentrations and the identification of the product *p*OHA.

MATERIALS AND METHODS

Preparation of microsomes

Male Sprague–Dawley rats, 200–500 g body wt, were starved for 16 hr prior to decapitation. The liver was rapidly excised, freed from residual connective tissue and the microsomes were isolated by gel filtration on Sepharose 2B.[9,10] Protein was determined according to Lowry *et al.*[11]

Incubation experiments

Incubations were carried out at 37° in air. The incubation mixture consisted of 50 mM potassium phosphate buffer pH 7·5, 5 mM $MgCl_2$, 0·005 mM $MnCl_2$, 1 mM $NADP^+$, 5 mM D,L-isocitrate, 0·4 IU porcine heart isocitrate dehydrogenase, and amphetamine sulphate. The final volume was 2 ml. After a preincubation period of 5 min the reaction was started by the addition of the microsomes. Incubations were terminated by the addition of 1 ml 2 M perchloric acid. Time of incubation was 10 min unless otherwise stated.

Fluorometric analysis of p-*hydroxyamphetamine*

After the termination of the reaction the incubates were centrifuged and the supernatant was adjusted to pH 6 with 2 M potassium hydroxide. After another centrifugation the extract was applied to a column of weak cation exchange resin (Amberlite XE-64, H-form, 4 × 20 mm) to isolate the amine. The resin was prewashed with 10 ml 1 M sodium acetate pH 6·5 and 3 ml water. When the sample had passed through the column, it was washed with 10 ml 0·02 M potassium phosphate buffer–0·2 per cent EDTA, followed by 3 ml water. *p*-Hydroxyamphetamine was eluted with 3 ml 0·5 M hydrochloric acid. The eluate and standard solutions were treated with 1-nitroso-2-naphtole[12] to produce a fluorophor which was measured at 465/565 nM (uncorrected) in a spectrophotofluorometer. Recovery of *p*-hydroxyamphetamine added to the incubation mixtures was 81·6 per cent (81·6 ± 5·4, mean ± S.D.; n = 6).

Identification of p-*hydroxyamphetamine*

(a) *Paper chromatography.* 2·9 μCi (15·5 mCi/mM) of 7-^{14}C-D-amphetamine sulphate (sp. act. 15 mCi/mM, CEA, France, purity > 96 per cent by ion-exchange chromatography on Amberlite CG 120, type II) was incubated as described above. After the pH had been adjusted to 6 with 2 M potassium hydroxide and the precipitate sedimented by centrifugation in the cold, 50 μl of the extract was put on a Whatman No. 1 paper and the metabolites were separated according to Ellison *et al.*[13] The paper was cut and the radioactivity measured in a liquid scintillation spectrophotometer as described by Lewander.[14]

(b) *Radio gas chromatography.* 0·6 μCi of ^{14}C-D-amphetamine (1 mCi/mM) was incubated using the incubation system previously described. To obtain enough material seven incubates were pooled. The sample was extracted with five 20-ml portions of toluene at pH 11 to remove unchanged amphetamine and then applied to a strong cation exchange resin (Amberlite CG 120, type II), according to Lewander.[15] Eluted fractions were read against a standard solution in a spectophotofluorometer at 285/335 nm. Aliquots (0·1 ml) were taken for the measurement of radioactivity and the portion of the eluate containing *p*-hydroxyamphetamine was evaporated to dryness. The trifluoroacetyl-derivative was prepared by dissolving the residue in 2 ml ethyl acetate and heating at 60° for 30 min in the presence of 0·5 ml trifluoroacetic anhydride (TFA).[16] After evaporation of excess TFA the derivative was dissolved in 50 μl of ethyl acetate and analyzed on a Barber Colman radio gas chromatograph equipped with a 5 ft × 6 mm (i.d.) column packed with 5% OV-17 on Gas Chrom Q 100/200 mesh, column temperature 190°, inlet temperature 230° and detector temperature 250°.

(c) *Mass-spectrometry.* A Varian MAT CH7 mass-spectrometer coupled with a Varian Aerograph 1740 gas chromatograph was used. The gas chromatograph was equipped with a glass column packed with 1% OV-1 on Varaport 30 100–120 mesh, 2 ft × 2 mm. The oven temperature was programmed to rise at a rate of 6°/min, initial setting was 100°. The electrons had an energy of 70 eV.

RESULTS

Identification of the product. ^{14}C-D-Amphetamine was incubated with the microsomes and the *p*OHA formed identified by three different techniques. Paper chromatography of an aliquot of the neutralized incubate (Fig. 1) showed that about 3 per cent of the substrate was parahydroxylated to *p*OHA. The substrate concentration was 0·1 mM. A small amount (0·5 per cent) of an unidentified metabolite was also formed.

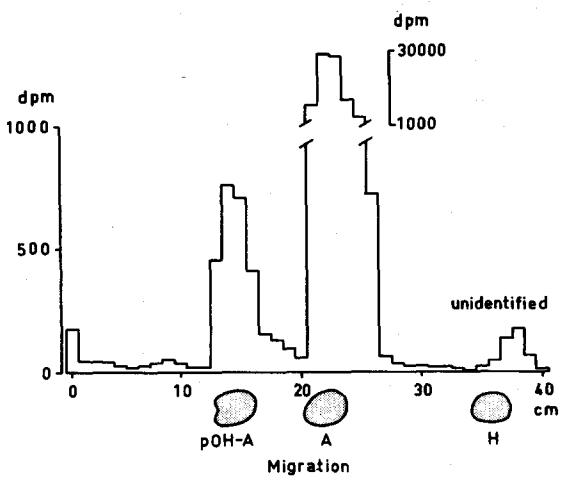

FIG. 1. Separation of amphetamine and metabolites by paper chromatography. 2·9 μCi (5·5 mCi/mM) of 7-^{14}C-D-amphetamine was incubated with 1·5 mg/ml of microsomes as described in Materials and Methods. 50 μl neutralized extract was spotted onto a Whatman No. 1 paper. Abbreviations: *p*OHA = *p*-hydroxyamphetamine, A = amphetamine, h = hippuric acid.

When the TFA-derivative of the labeled product was submitted to radio gas chromatography and detected by the proportional counter only one peak appeared. The retention time was identical with that of the TFA-derivative of *p*OHA (not shown in the figure.)

Figure 2 shows the relative abundance of fragments of the TFA-derivative of *p*OHA (A) and amphetamine product (B) as recorded on the combined gas chromatograph-mass spectrometer. The base peak was found to occur at *m/e* 140 which corresponds to the rupture of the bond beta to the nitrogen yielding the ion *m/e* 140, $[CH_3CHNHCOCF_3]^+$. Other ions appeared at *m/e* 203 $[CF_3OCOC_6H_5CH_2]^+$ and *m/e* 230 $[C_6H_5CH_2CH(CH_3)NHCOCF_3]^+$. No apparent differences in fragmentation pattern between the two mass-spectra were seen.

Linearity and reaction kinetics. The formation of *p*OHA by the microsomes incubated with 0·8 μmoles of D-amphetamine was found to be linear with the protein concentration within the range tested (0·5–2 mg protein/ml) (Fig. 3 A). The reaction was

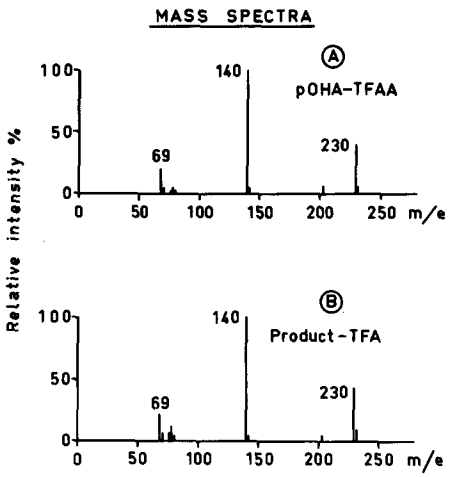

FIG. 2. Mass spectrum of the trifluoroacetyl (TFA) derivatives of *p*-hydroxyamphetamine (A) and product (B) found after incubation of microsomes with D-amphetamine. The experimental conditions are described in the text.

also found to be linear with time to 15 min using 1·5 mg of protein/ml (Fig. 3 B). A Lineweaver–Burk plot (Fig. 4) revealed that the reaction followed Michaelis–Menten kinetics only at low substrate concentrations (from 0·025 mM to about 0·5 mM). Above a concentration of about 0·5 mM the curve bent upwards indicating inhibition of the reaction due to excess substrate. The apparent K_m was found to be $8{\cdot}1 \times 10^{-5}$ M and V_{max} 3·6 nmoles × mg protein^{-1} × 10 min^{-1}.

Figure 5 shows a similar plot of the metabolism of D-amphetamine in comparison with L-amphetamine. The kinetic plots show a slower rate of parahydroxylation of L-amphetamine. In fact L-amphetamine had an apparent K_m of 1·1 × 10 min^{-1}, and V_{max} of 2·8 nmoles × mg protein^{-1} × 10 min^{-1}, and the optical antipode a K_m of $2{\cdot}4 \times 10^{-5}$ M and V_{max} of 3·7 nmoles × mg protein^{-1} × 10 min^{-1}. At a con-

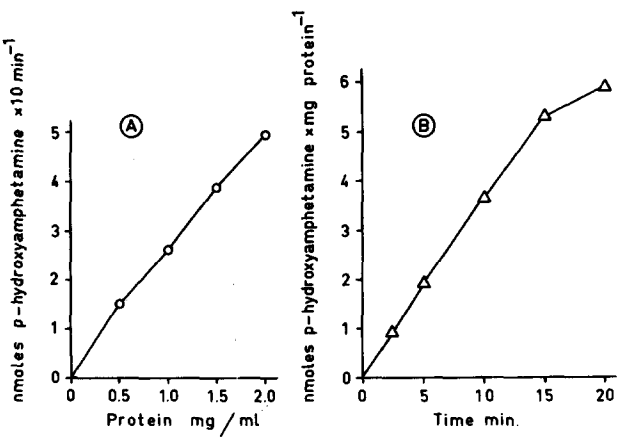

FIG. 3. Formation of *p*-hydroxyamphetamine with protein concentration and time. Incubations were performed as described in Materials and Methods. The concentration of D-amphetamine was 0·4 mM. Each point represents the mean of three experiments.

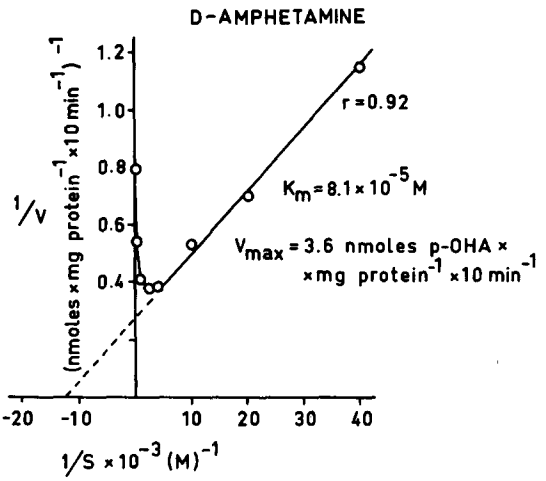

FIG. 4. Lineweaver–Burk plot of the formation of *p*-hydroxyamphetamine vs the concentration of D-amphetamine (range 0·025–10 mM). Incubations were performed as described in Materials and Methods. Each point represents the mean of three experiments. The K_m and V_{max} values were determined by the least square method.

centration of 0·38 mM the formation of *p*OHA was significantly different ($P < 0·01$), between D- and L-amphetamine.

DISCUSSION

The present results show that amphetamine is metabolized by rat liver microsomes yielding *p*OHA in a NADPH dependent reaction. The evidence is a positive identification of the product by gas chromatography and mass-spectral analysis. The relative abundance of fragments corresponds with other published data.[16]

The product also had the same characteristics as *p*OHA when analyzed by paper chromatography, radio gas chromatography and ion-exchange chromatography. Further, the metabolite had the same fluoroscence spectrum as the parent compound both as such and when coupled with nitroso-naphtole.

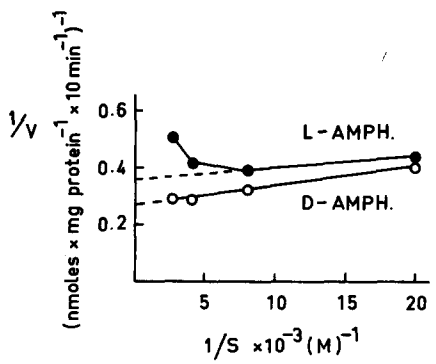

FIG. 5. Lineweaver–Burk plot of the formation of *p*-hydroxyamphetamine vs the concentration of D- and L-amphetamine (range 0·05–0·38 mM). Incubations were performed as described in Materials and Methods. D-Amphetamine: $K_m = 2·4 \times 10^{-5}$ M; $V_{max} = 3·7$ nmoles × mg protein^{-1} × 10 min^{-1}. L-Amphetamine: $K_m = 1·1 \times 10^{-6}$ M; $V_{max} = 2·8$ nmoles × mg protein^{-1} × 10 min^{-1}. At a concentration of 0·38 mM the formation of *p*-hydroxyamphetamine from D- and L-amphetamine was significantly different ($P < 0·01$) Each point represents the mean of three experiments.

Since only a small amount of the substrate was hydroxylated into *p*OHA it was necessary at all times to measure the formation of the metabolite and not only the disappearance of amphetamine. Dingell and Bass[7] measuring the disappearance of amphetamine failed to detect any metabolism in the microsomal fraction of the liver. Furthermore, their incubation medium contained nicotinamide which is a known inhibitor of drug metabolism *in vitro*.[17] Trace amounts (0·002 per cent) of *p*OHA were found by Daly *et al.*,[8] in their studies on the NIH-shift. They incubated the microsomes with 5 μmoles of D,L-amphetamine and recovered 0·002 per cent as *p*OHA. Their high substrate concentration may partly explain the small amount of *p*OHA which they found (see Fig. 4). Their use of racemic amphetamine may depress further the hydroxylation of the D-isomer since L-amphetamine more rapidly reaches the level of substrate inhibition (Fig. 5). The latter finding implies that the L-isomer may inhibit the hydroxylation of D-amphetamine *in vivo*. This type of interaction is currently under investigation.

The activity of the amphetamine hydroxylase (Fig. 4) followed Michaelis–Menten kinetics at low substrate concentrations when plotted according to Lineweaver and Burk.[18] When higher concentrations of amphetamine were used the plot curved upwards, showing substrate inhibition characteristics.[19]

It is now well established that a prerequisite for hydroxylation reactions is binding of the substrate to oxidized cytochrome P-450 of the liver microsomal mixed function oxidase to form a complex.[20,21] Mainly two types of spectral changes, type I and type II, have been found to occur when substrates are added to a microsomal suspension. It has been suggested that the type I spectral change is related to metabolism of the substrate, while the type II spectral change is related to interaction of the substance with the haeme iron of cytochrome P-450. Substances of the latter class are basic amines and it has been suggested that the nitrogen forms a bond with the sixth ligand of the iron atom.[22] Thus, like several other amines, amphetamine has been shown to cause a type II spectral change both with rabbit and rat* liver microsomes.[23,24] When D-amphetamine was added to rabbit microsomes at low concentrations, a type I spectral change occurred. Rat liver microsomes on the other hand showed a type II spectrum at all concentrations, which were used (2·6 μM–1·0 nM). However, amphetamine† like aniline[25] could be shown to produce a hidden type I component as studied by the hexobarbital saturation method. Since aniline displaces carbon monoxide from cytochrome P-450[22] such a competition might possibly occur also between other type II compounds and oxygen. By competing with oxygen in such a way amphetamine at high concentrations may inhibit its own metabolism. This has been suggested to occur for other amines.[26]

Although, the mechanism discussed above may explain the substrate inhibition at relatively high amphetamine concentrations, the influence of other factors should be considered. A typical type II substrate like aniline, has not been shown to inhibit its own parahydroxylation at concentrations comparable with those of amphetamine used in the present experiment.[27,28] On the contrary, these authors found evidence for enzyme activation by increasing the aniline concentrations. Hence, amphetamine and aniline seem to interact in different manners with the mixed function oxidase of rat liver microsomes.

*† I. Hoffström, personal communications.

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