Biochemical Pharmacology, Vol. 27, pp. 2525–2529.

0006-2952/78/1101-2525 $02.00/0

AROMATIC HYDROXYLATION OF AMPHETAMINE WITH RAT LIVER MICROSOMES, PERFUSED LIVER, AND ISOLATED HEPATOCYTES

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(*Received* 17 *December* 1977; *accepted* 7 *March* 1978)

Abstract—The hydroxylation of amphetamine to *p*-hydroxyamphetamine has been investigated. In 1 hr, perfused rat livers hydroxylated about 85 per cent of the administered 20 μmole dose of amphetamine. Ninety-four per cent of the *p*-hydroxyamphetamine was further metabolized by conjugation. Isolated hepatocytes are capable of hydroxylation of amphetamine and conjugation of hydroxyamphetamine at rates comparable to those of the perfused liver. The rate of hydroxylation with microsomes is about 50 per cent of that attained with isolated hepatocytes. With hepatocytes, the hydroxylation is inhibited by typical inhibitors of microsomal oxygenase(s) such as 2,6-dichlorophenyphenoxyethylamine (DPEA) and SKF-525A, and it is inhibited by iprindole. However, the rate could not be increased by treatments which induce cytochrome P450 and increase other mono-oxygenase reactions. The results obtained with both isolated hepatocytes and microsomes suggest that amphetamine is hydroxylated by a microsomal cytochrome P450-dependent enzyme system, but that amphetamine is not a typical substrate.

Axelrod [1] reported in 1954 that rats convert about 60 per cent of an administered dose of amphetamine (Amp) to *p*-hydroxyamphetamine (HO-Amp). Several investigators have confirmed the importance of this metabolic pathway in rats. The ability of rat liver microsomes to catalyze the hydroxylation of amphetamine was demonstrated in 1974, when Rommelspacher *et al.* [2] and Jonsson [3], using sensitive analytical techniques for the measurement of HO-Amp, determined that HO-Amp is formed from Amp upon incubation with rat liver microsomes and NADPH. This observation was confirmed by Cho *et al.* [4]. The NADPH-dependent rate with liver microsomes was reported to be slow relative to that obtained with common substrates of the microsomal mono-oxygenase system. The small amount of HO-Amp formed in microsomal incubations appeared not to account for the extensive hydroxylation which occurs *in vivo*.

Dingell and Bass [5] observed that the isolated perfused rat liver rapidly metabolizes Amp. However, the metabolic products were not measured. In a preliminary communication [6] we reported that Amp is extensively converted in the isolated perfused liver to HO-Amp, a finding which has recently been confirmed by Jonsson [7]. The details of our experiments are described in the present paper. In addition, we have compared the results obtained in the perfused liver to conversions obtained in liver microsomes and isolated hepatocytes. The latter preparation has been shown to be an excellent model of *in vivo* drug metabolism [8]. The properties of Amp hydroxylation in isolated hepatocytes have been delineated.

MATERIALS AND METHODS

Amphetamine, *p*-hydroxyamphetamine and SKF-525A were obtained from the Smith, Kline & French Laboratories, Philadelphia, PA. Butamoxane (2-butylaminomethyl-1,4-benzodioxane) and 3-amino-1-(4-hydroxyphenyl) butane were synthesized at the Lilly Research Laboratories, Indianapolis, IN. 6- and 7-Hydroxybutamoxane were synthesized by Professor A. Funke, Institut Pasteur, France [9]. Iprindole [5-(3-dimethylaminopropyl)-6,7,8,9,10,11-hexahydro-5H-cyclooct [b] indole·HCl] was a gift from Wyeth Pharmaceutical Co., Philadelphia, PA. All other chemicals were purchased from commercial suppliers.

Male Sprague–Dawley rats (180–220 g) were obtained from Cox Laboratories, Indianapolis, IN. They were maintained in stainless steel cages on Purina Laboratory Chow and water *ad lib.*

Microsomes were prepared on the day of use from livers which were homogenized with a Potter–Elvehjem homogenizer in 4 vol. of 1.15% KCl. The microsomal pellet was resuspended in 0.1 M phosphate buffer, pH 7.4. Incubations (1 ml) included 50 μmoles of phosphate buffer, pH 7.4, 4 mM $MgCl_2$, 0.5 mM $NADP^+$, 10 mM *dl*-sodium isocitrate, 80 μg isocitrate dehydrogenase (pig heart, 2 units/mg, Boehringer Mannheim, Indianapolis, IN) and microsomes from 100 mg liver.

Hepatocytes were isolated by the method of Ingebretsen and Wagle [10] and were incubated at 37° in 1 ml of Umbreit–Ringer buffer containing 25 mM D-glucose. All incubations were done in duplicate

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under a 95% O_2: 5% CO_2 atmosphere. The hepatocytes were incubated for 5 min before addition of the substrate. The incubations were terminated by sonicating the hepatocytes.

For the intact liver perfusion experiments the livers were perfused, as described previously [8], via the portal vein with 100 ml of Umbreit–Ringer buffer containing 1.5% bovine serum albumin (Sigma, Fraction V). The livers were allowed to equilibrate for 30 min before the addition of 20 μmoles *d*-amphetamine. One-millilitre aliquots of the perfusate were removed at 15, 30 and 60 min. The perfusion was terminated at 60 min and the liver was homogenized with a Polytron in 4 vol. of ice-cold 1.15% KCl. Triplicate samples (0.1 ml) of the perfusate, liver homogenate and bile were then analyzed for free and conjugated HO-Amp.

The various liver preparations were analyzed for protein by the biuret method [11]. The method of Omura and Sato [12] was used to determine cytochrome P450. For these assays, isolated hepatocytes were first homogenized with a Polytron in 0.1 M phosphate buffer, pH 7.4.

HO-Amp was quantified by the following gas chromatographic (g.c.) method. 3-Amino-1-(4-hydroxyphenyl)butane (0.2 μg) was added as an internal standard to 0.1-ml aliquots of the incubation. Protein was then precipitated by adding 2 ml acetone and it was removed by centrifugation. The acetone was evaporated *in vacuo* and 0.1 ml of 0.5 N HCl was added to the residue. The acidic solution was extracted twice with 4 ml of distilled *n*-butylchloride and the solvent was discarded. The aqueous phase was saturated with Na_2CO_3 and then was extracted twice with 4 ml of distilled *n*-butylchloride. The solvent was evaporated to dryness *in vacuo* and the residue was dissolved in 25 μl of distilled ethylacetate. Pentafluoropropionic anhydride (25 μl) was then added. The reaction mixture was allowed to stand at room temperature for 30 min; then it was evaporated to dryness under N_2. The residue was dissolved in 0.1 ml of distilled ethylacetate, and 2–5 μl was injected onto a 6-ft, 3% W98, column, attached to a Hewlett–Packard model 5710A gas chromatograph equipped with an electron capture detector. At a column temperature of 160°, HO-Amp had a retention time of 1.3 min and the internal standard emerged from the column in 2.4 min. HO-Amp was quantified from a standard curve constructed by adding known amounts of HO-Amp to blank samples and carrying these samples through the entire procedure. A linear response was obtained when samples containing between 20 and 400 ng HO-Amp were extracted.

Butamoxane hydroxylation was determined by measuring 6- and 7-hydroxybutamoxane by gas chromatography with electron capture detection, as previously described [8].

4-Hydroxybiphenyl was extracted from incubation mixtures as previously described [13]. The extracts were dissolved in 25 μl of distilled ethylacetate and 25 μl pentafluoropropionic anhydride. The mixture was heated at 37° for 60 min and then evaporated to dryness under a stream of N_2. The residue was dissolved in 0.1 ml of distilled ethylacetate, and 2–5 μl was injected onto the same gas chromatographic column used for HO-Amp analysis. At a column temperature of 145°, the pentafluoropropionic ester of 4-hydroxybiphenyl had a retention time of 3.5 min.

p-Nitroanisole *O*-demethylation was determined by assaying the incubation mixtures for *p*-nitrophenol by the method of Netter [14].

Sulfate and glucuronide conjugates were determined by hydrolyzing the samples with Glusulase (Endo Laboratories, Chicago, IL) prior to analysis.

Amphetamine hydroxylation *in vivo* was measured by injecting 200 g rats i.p. with 20 μmoles *d*-amphetamine. After 60 min, the rats were killed by decapitation, then skinned and homogenized in a Waring blender with 200 ml of distilled H_2O. Triplicate 0.1-ml aliquots of the homogenates were hydrolyzed with Glusulase and then analyzed for HO-Amp.

RESULTS AND DISCUSSION

Table 1 shows that the isolated perfused liver extensively metabolizes Amp to HO-Amp which is extensively metabolized by conjugation to sulfates and/or glucuronides. Very little HO-Amp was released into the perfusate during the first hour. However, in a separate experiment in which the perfusion time was increased to 120 min, the amount of total HO-Amp in the perfusate increased from 4.9 per cent at 60 min to 15 per cent at 120 min. The extensive hydroxylation in the isolated liver, 84 per cent of the dose in 60 min, strongly suggests that the liver is mainly responsible for Amp hydroxylation *in vivo*.

Figure 1 shows the time course of the hydroxylation of Amp in isolated rat hepatocytes. It is apparent that the rate of hydroxylation is linear for about 60 min. As with the perfused liver, most of the HO-Amp in the hepatocytes was present as conjugates. Hydrolysis of the samples with purified β-glucuronidase indicated that the HO-Amp was conjugated with glucuronic acid rather than with sulfate.

With rat liver microsomes, the hydroxylation was dependent upon NADPH (Table 2). Addition of NADH to NADPH-supplemented microsomes enhanced the activity slightly. NADH and $NADP^+$ alone were ineffective in supporting hydroxylation. A number of other additions were tried in order to assess their ability to inhibit or enhance the hydroxylation of amphetamine. Cyanide (1 mM) had no effect while 5 mM cyanide inhibited the reaction 43 per cent. Increasing the concentration of amphetamine from 0.2 to 1 mM showed no enhanced production of HO-Amp. Glutathione had no effect; neither did

Table 1. Hydroxylation of *d*-amphetamine in perfused rat liver

	HO-Amp (μmoles)
Perfusate	0.80 ± 0.044
Bile	0.092 ± 0.030
Liver	15.86 ± 0.657
Total	16.75 ± 0.713*

*Of this, 1.08 ± 0.50 was free HO-Amp; the rest was conjugated with glucuronic acid and/or sulfate. Results are the average ± S. E. of three perfusions and they are expressed as total HO-Amp found in the fraction after perfusion of 20 μmoles Amp for 60 min.

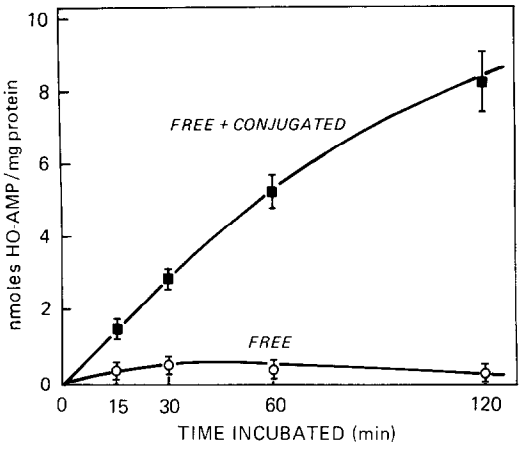

Fig. 1. Time course of the hydroxylation of *d*-amphetamine in isolated liver cells. The concentration of Amp was 2×10^{-4} M. Results are the average ± S. E. of duplicate determinations with three separately isolated cell preparations.

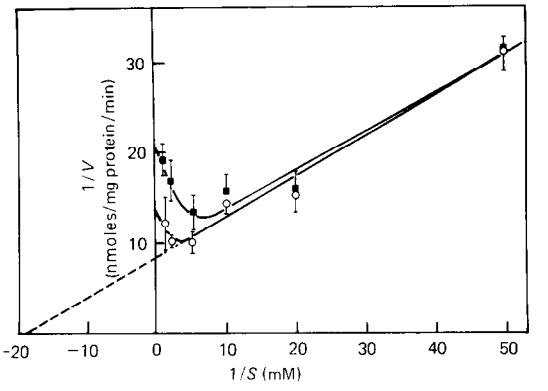

Fig. 2. Rate of hydroxylation of *d*- and *l*-amphetamine in isolated hepatocytes. *d*-Amphetamine (○) or *l*-amphetamine (●) was incubated for 15 min. Incubations were hydrolyzed with Glusulase prior to analysis. Results are the average ± S. E. of duplicate determinations with five separately isolated cell preparations. From this graph, the V_{max} was found to be 0.123 nmole/mg of protein/min and the apparent K_m was 5.49×10^{-5} M.

superoxide dismutase, dimethylpteridine, ascorbic acid nor norepinephrine. All of these properties seem to indicate that the hydroxylation of amphetamine is dependent upon a typical mono-oxygenase of the cytochrome P450 type. This idea is consistent with the recent observation of Jonsson [7] that CO inhibits microsomal AMP hydroxylation.

Based upon the cytochrome P450 content of the three liver preparations, the rate of Amp hydroxylation (nmoles/nmole of P450/min) was calculated to be 0.87 in the perfused liver, 0.80 in the isolated hepatocytes and 0.45 with microsomes. In contrast, it has been observed previously [8] that other substrates of the cytochrome P450 mono-oxygenase system are metabolized at a similar or slower rate in isolated hepatocytes than with microsomes. In the case of amphetamine hydroxylation the microsomes appear to have lost activity, since only the microsomal fraction was found to have detectable hydroxylase activity. Two experiments were performed to determine whether or not product inhibition was a significant factor in decreasing the rate of hydroxylation of amphetamine in microsomes. In one experiment the microsomal system was supplemented with 2 mM uridine diphosphoglucuronic acid (UDPGA). Under these conditions over 70 per cent of the HO-Amp produced was glucuronidated. Despite this concurrent conjugation, the total rate of hydroxylation was the same in the UDPGA-supplemented microsomes as it was in the microsomes that had only NADPH added. In the second experiment the methylene homologue of HO-Amp, which we have used as the internal standard in the HO-Amp assay, was added directly to the microsomes at the same time as amphetamine. Its concentration was 0.05 mM, which is a concentration 50- to 75-fold higher than the amount of HO-Amp found in an incubation with AMP alone. This hydroxylated analog, which is chemically similar to HO-Amp, showed no effect on the rate of hydroxylation of amphetamine in microsomes.

Some of the properties of the hydroxylation reaction have been determined using isolated hepatocytes. The substrate-dependent kinetics of the optical isomers of Amp are shown in Fig. 2. It is apparent that the hydroxylation of both isomers is inhibited by substrate at high concentrations. The hydroxylation of the *l*-isomer is inhibited at substrate concentrations greater than 100 μM, while a substrate concentration of more than 200 μM is necessary to inhibit the hydroxylation of the *d*-isomer. Nevertheless, the same theoretical maximum velocity was obtained with both isomers. Substrate inhibition of amphetamine hydroxylation has also been observed with microsomes [3, 4]. However, the pharmacological

Table 2. Pyridine nucleotide requirement for the hydroxylation of *d*-amphetamine*

Cofactor	Concn	Microsomes	Supernatant fraction (9000 *g*)
NAD^+	0.5	0·60	0.20
NADH	0.5	0	0.60
$NADP^+$	0.5	0	2.78
NADPH	0.5	3.97	4.30
NADPH	1.0	4.44	5.83
NADPH + NADH	0.5	5.89	4.77
None		0	0

*The concentration of *d*-Amp was 200 μM. Enzymatic activities are expressed as nmoles HO-AMP formed in 15 min with microsomes or a 9000 *g* supernatant fraction from 200 mg liver.

Table 3. Inhibition of aromatic hydroxylation in isolated rat hepatocytes*

Inhibitor	Concn (μM)	Per cent Inhibition	
		d-Amphetamine	Butamoxane
DPEA	5	39	9
	20	68	26
	100	—	52
SKF-525A	1	4	19
	5	14	28
	10	79	51
	20	88	56
	100	—	89
Iprindole	1	35	—
	5	81	0
	10	88	0
	100	—	19

*Hepatocytes were incubated with the inhibitor for 5 min prior to addition of 200 μM substrate. The incubation was then continued for 15 min. Samples were hydrolyzed with Glusulase before determination of *p*-hydroxyamphetamine or 6- and 7-hydroxybutamoxane. Results represent the average of three separate experiments.

significance of the substrate inhibition is questionable since it is unlikely that substrate concentrations higher than 100 μM are attained *in vivo*. Indeed, Fuller and Hines [15] have reported that *d*- and *l*-amphetamine disappear from brain with the same half-life.

2,6Dichlorophenylphenoxyethylamine(DPEA)and SKF-525A are typcial inhibitors of the cytochrome P450 substrate [8, 16]. The amount of inlibition is system. Table 3 shows that DPEA and SKF-525A inhibit amphetamine hydroxylation as well as the hydroxylation of butamoxane, a typical cytochrome P450 substrate [8, 16]. The amount of inhibition is dependent upon the concentration of the inhibitor. Iprindole has been reported by Lemberger *et al.* [17] and by Miller *et al.* [18] to be a rather specific inhibitor of amphetamine hydroxylation *in vivo*. In isolated hepatocytes, iprindole at a concentration as low as 1 μM inhibits amphetamine hydroxylation (Table 3). In contrast, butamoxane hydroxylation is not inhibited except at a high iprindole concentration.

To observe the effect of increasing the cellular concentration of cytochrome P450, isolated hepatocytes were prepared from rats which had been treated with known inducers of cytochrome P450 and mono-oxygenase reactions. Phenobarbital (PB), 3-methylcholanthrene (3-MC) and β-naphthoflavone (BNF) increased the cytochrome P450 concentration about 2-fold (Table 4). The rate of butamoxane hydroxylation was increased in hepatocytes from PB-treated rats, but was unaffected by 3-MC or BNF treatment. In contrast, the rate of Amp hydroxylation was not affected by PB induction of cytochrome P450 (Table 4). This observation is consistent with the finding both with liver microsomes [4] and *in vivo* [19, 20] that PB does not induce amphetamine hydroxylation. Hepatocytes from BNF- or 3-MC-treated rats hydroxylated Amp at only 30 per cent of the rate obtained with hepatocytes from control rats.

Two additional experiments were done to determine if the reduced activity is due to residual inducing agent. In the first experiment, 3-MC and BNF were added *in vitro* to the incubation mixtures. Neither agent, at a concentration of 1–20 μM, affected the hydroxylation rate in either isolated hepatocytes or microsomes. In the second experiment, BNF was given to rats in a single injection 48 hr before death. This dosage schedule should lead to lower concentration of inducer and/or its metabolites in the liver [21]. However, as shown in Table 5, the rate of Amp hydroxylation in isolated hepatocytes was still reduced. In contrast, the rate of butamoxane hydroxylation was unchanged, and the rate of *O*-demethylation of *p*-nitroanisole and the 4-hydroxylation rate of biphenyl were enhanced by BNF pretreatment. It is noteworthy that, with microsomes, the rate of Amp hydroxylation is not affected by BNF pretreatment, but the effect of this agent upon the oxidation of the other substrates is the same as with isolated hepatocytes.

In order to determine if BNF pretreatment inhibits the *in vivo* hydroxylation of Amp, a group of five rats was treated with BNF (16 mg in 0.5 ml dimethylsulfoxide) 48 hr prior to injection of Amp. Sixty min after Amp administration, whole body levels of HO-Amp were determined in these rats as well as in a group of five rats treated only with dimethylsulfoxide. It was found that BNF-treated rats formed 5.8 $\pm$ 0.49 μmoles HO-Amp compared to 8.6 $\pm$ 0.86 μmoles formed with vehicle-treated control rats. Thus, the effect of BNF-pretreatment is the same

Table 4. Effect of inducers of cytochrome P450 on aromatic hydroxylation in isolated hepatocytes*

Treatment	P450	HO-Amp	HO-B
Control	131 $\pm$ 9	1.6 $\pm$ 0.16	5.4 $\pm$ 0.47
Phenobarbital	339 $\pm$ 27	1.4 $\pm$ 0.23	10.8 $\pm$ 0.72
3-Methylcholanthrene	301 $\pm$ 14	0.47 $\pm$ 0.25	3.6 $\pm$ 0.97
β-Naphthoflavone	286 $\pm$ 23	0.49 $\pm$ 0.04	5.3 $\pm$ 0.70

*Rats were injected i.p. with inducers for 4 days prior to the preparation of the isolated hepatocytes. Phenobarbital was given in saline at a dose of 50 mg/kg. β-Naphthoflavone (40 mg/kg) and 3-methylcholanthrene (10 mg/kg) were given in PEG 200. Control rats received PEG 200. The substrate concentration was 200 μM. The incubations were hydrolyzed with Glusulase before determination of HO-AMP or 6- and 7-hydroxybutamoxane (HO-B). Results represent the average $\pm$ S. E. of three preparations and are expressed as nmoles product/mg of protein/15 min. The P450 concentration is expressed as pmoles/mg of protein.

Table 5. Effect of β-naphthoflavone pretreatment on drug metabolism in isolated hepatocytes and liver microsomes*

Reaction investigated†	Isolated hepatocytes		Microsomes	
	Control	BNF	Control	BNF
Amphetamine hydroxylation	316 ± 57	126 ± 11	177 ± 27	170 ± 6
Butamoxane hydroxylation	1030 ± 205	747 ± 68	2080 ± 254	2070 ± 209
p-Nitroanisole *O*-demethylation	348 ± 57	743 ± 50	520 ± 39	1070 ± 58
Biphenyl 4-hydroxylation	129 + 32	327 ± 61	144 ± 19	364 ± 22

*BNF (80 mg/kg) was given in dimethylsulfoxide to rats 48 hr prior to preparation of isolated hepatocytes or microsomes. Control rats received dimethylsulfoxide alone. In cells from control rats the concentration of cytochrome P450 was 110 pmoles/mg of cellular protein. The microsomes contained 395 pmoles cytochrome P450/mg of microsomal protein. The hepatocyte incubations were hydrolyzed with Glusulase before determination of the metabolic products. Results are the average ± S. E. of three preparations in each group and they are expressed as nmoles product formed/min/mg of microsomal protein. Microsomal protein in isolated hepatocytes was determined from the concentration of cytochrome P450 relative to the concentration in microsomes.

†The concentration of biphenyl was 1 mM and that of *p*-nitroanisole was 0.5 mM. Butamoxane and *d*-amphetamine were incubated at a concentration of 0.2 mM. The incubation volume was 1 ml except in incubations containing *p*-nitroanisole. These incubations were 3 ml.

in vivo as in isolated hepatocytes, but this effect is not seen with microsomes. One explanation for these results is that, after BNF or 3-MC treatment, endogenous compounds, present in the intact hepatocytes but not in microsomes, inhibit amphetamine hydroxylation. For example, if amphetamine is metabolized by cytochrome P450, then these endogenous compounds could compete with amphetamine for the electrons which are transferred to the cytochrome via NADPH–cytochrome P450 reductase.

The observations that liver microsomes can catalyze Amp hydroxylation [2–4] and that CO can inhibit the hydroxylation [7] indicate that the cytochrome P 450 system may be involved. However, the reaction is characterized by some unusual properties. The present studies indicate that the NADPH-dependent activity in microsomes is about 50 per cent of that attained with isolated hepatocytes. With hepatocytes, the hydroxylation is inhibited by the typical inhibitors of cytochrome P450, DPEA and SKF-525A, and is particularly sensitive to inhibition by iprindole. The formation of HO-Amp is not increased when the concentrations of cytochrome P450 are increased by induction. Thus, although these studies support the idea that Amp is hydroxylated by a liver microsomal cytochrome P450-dependent enzyme system, they also indicate that the hydroxylation is not a typical cytochrome P450 reaction. Cytochrome P450 is apparently a family of enzymes, and it seems reasonable to speculate that the form of cytochrome P450 involved in amphetamine hydroxylation may be different from that involved with most drug substrates. Induction by PB or 3-MC may not induce the cytochrome P450 involved in Amp hydroxylation. Additional evidence that the enzyme may be different is the observation that iprindole is a rather specific inhibitor of amphetamine hydroxylation. Purification of cytochrome P450(s) which is/are capable of Amp hydroxylation seems to be the only definitive route to answering these questions.

Acknowledgements—The authors are grateful to Ms. Ann Miller and Nancy Bridgeforth for skilful technical assistance in the preparation of isolated hepatocytes and to Mr. Gary Whitaker for help with the analyses.

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