Biochemical Pharmacology, Vol. 23, pp. 1065–1071. Pergamon Press, 1974. Printed in Great Britain.

# THE HYDROXYLATION OF D-AMPHETAMINE BY LIVER MICROSOMES OF THE MALE RAT

HANS ROMMELSPACHER,* HENNING HONECKER, GERT SCHULZE and SABINE M. STRAUSS

Institut für Neuropsychopharmakologie der Freien Universität, 1000 Berlin 19, Ulmenallee 30, Germany

(*Received* 2 *July* 1973; *accepted* 13 *August* 1973)

**Abstract** This paper describes the conditions under which the hydroxylation of amphetamine to *p*-hydroxyamphetamine can be studied in isolated rat liver microsomes. The reaction depends on the concentration of NADP. The optimal pH is 6·9–7·0. As Michaelis parameters $1{\cdot}5 \times 10^{-4}$ M ($K_m$) and 2·16 nmoles (mg of microsomal protein)$^{-1}$ (10 min)$^{-1}$ ($V_{max}$) were calculated. The production of *p*-hydroxyamphetamine is not restricted to the microsomes but a smaller biotransformation of amphetamine was also found in mitochondria.

NUMEROUS investigations have been presented concerning the catabolism of amphetamine in rats *in vivo*.[1–9] It can be concluded, that the dominant pathway involves ring-hydroxylation at the 4-carbon atom, but deamination of the aliphatic side-chain also occurs.

While isolated microsomes of rat liver did not exhibit enzyme activity, Dingell and Bass[10] showed by perfusion of rat liver that this organ catabolizes amphetamine. Subsequently, Beckett and Al-Sarraj[11] found conversion of the side-chain using 10,000 *g* supernatant preparations of rat liver. In this paper we present an assay by which the hydroxylation of amphetamine to *p*-hydroxyamphetamine can be studied in isolated rat liver microsomes.

## MATERIALS AND METHODS

*Enzyme preparations.* Six grams of liver from male Wistar-rats (aged about 4 months) were homogenized with a four-fold volume of ice-cold sucrose (0·25 M) in a motor-driven Teflon-glass homogenizer. All succeeding tissue manipulations were performed at 0–4°. The homogenates were centrifuged twice, at 6000 *g* first and then at 10,000 *g* (10 min each time). The supernatant was centrifuged at 78,000 *g* for 60 min in a Spinco Model L ultracentrifuge. The pellet was washed with a Krebs–Ringer phosphate buffer (KRPB), pH 7·4. After recentrifugation (78,000 *g*, 60 min) the supernatant was discarded and the pellet was suspended in 15 ml of the phosphate buffer by gentle manual homogenization. All enzyme assays were performed immediately following the preparation of the microsomes. For each experiment the microsomes of the livers of three animals were pooled.

* Present address: The Johns Hopkins University School of Medicine, Department of Pharmacology and Experimental Therapeutics, Baltimore, Maryland 21205, U.S.A.

The mitochondria were prepared according to the method described by Neubert *et al.*[20] After the last centrifugation the pellet was suspended in a Krebs–Ringer phosphate buffer, pH 7·4.

*Enzyme assays.* One millilitre of the suspension described above was mixed with 1 ml of KRPB, pH 7·4, containing NADP (0·8 mM if not stated otherwise in the text) and a NADPH-generating mixture [glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (4 IU)], magnesium chloride (5 mM) and with 1 ml of a solution containing D-amphetamine (D-amphetamine was calculated as free base). As a substrate D-amphetaminesulfate (Merck, D-61 Darmstadt) and D-amphetamine-$^3$H (G) sulfate (5 Ci/m-mole; New England Nuclear Chemicals, D-6072, Dreieichenhain) were used. It was found to be necessary to remove from the $^3$H-amphetamine a small proportion of radioactive impurity with properties similar to those of the hydroxylated metabolite. This was done first by separating the pure $^3$H-amphetamine fraction by thin layer chromatography on commercial cellulose plates and then eluting a narrow areal of the amphetamine by 0·1 N HCl. After this manipulation the radiochemical purity was >99 per cent).

Prior to the addition of microsomes the assay was preincubated in a metabolic shaker at 37° under air for 15 min to ensure reduction of all of the NADP to NADPH. The incubation was started by adding 1 ml of the microsomal suspension. When the incubation time was over the enzymic activities were stopped by 3 ml of 1 N perchloric acid. The inclusion of control tubes during incubation showed that there was no measurable non-enzymatic degradation.

*Extraction and separation of* D-*amphetamine and its metabolites.* The incubation mixture was centrifuged (18,000 *g* for 5 min) to remove the precipitated protein. Thereafter the supernatant was adjusted with 2 N KOH at pH 12. Then about 98 per cent of amphetamine was extracted by benzene (30 ml for an aliquot of 5 ml of the incubation sample). In this organic phase 0·4‰ of the *p*-hydroxyamphetamine (*p*-OHA) was detected. The remainder was in the aqueous phase.

After shaking and centrifugation, the aqueous phase was separated and adjusted at pH 6·4 by addition of 1 N perchloric acid. After running down the perchlorate by centrifugation 5 ml of the sample was lyophilized. The solide residue was dissolved in a methanol–water mixture (1 + 1, 0·6 ml). Amphetamine and its metabolites were separated by iteration of thin layer chromatography with cellulose as adsorbent using precoated sheets (Merck, Darmstadt). The multi-component solvent (butanol, toluene, glacial acetic acid, water, 2:2:1:1) was described by Wirkström and Salversen.[12] Before starting the separation some "carrier" of amphetamine and its metabolites was added to the prior applied sample at the starting point. Amphetamine and its metabolites were eluted by 0·1 N HCl.[13] The radioactivity of the eluate was measured with a liquid scintillation spectrometer (Packard). Each run included at least one sample the enzymic activities of which were stopped immediately after the start of the incubation. The metabolites of these samples were extracted and separated simultaneously with the other mixtures. The values of the respective zones served as a background activity and were subtracted from the gross cpm-values of each sample, incubated over the whole period.

Considering that hydroxylation of amphetamine results in a loss of one of the 13 tritium atoms of the generally labelled compound, we multiplied the net-counts/min of *p*-OHA by a factor of 1·08.

The amount of *p*-hydroxyamphetamine produced was calculated using the following formula:

$$x = \frac{\text{dis/min } p\text{-OHA}}{\text{SA}}; \quad \text{SA} = \frac{\text{dis/min A}}{\text{nrA}}$$

SA = specific activity of amphetamine
nrA = amount of non-radioactive amphetamine
dis/min A = dis/min amphetamine
dis/min *p*-OHA = dis/min *p*-OH amphetamine.

The recovery for *p*-OHA was 94 per cent.

It is known that *p*-OH norephedrine is formed from *p*-OHA. During the first 10 min of incubation no metabolism of *p*-OHA was detected. After 30 min incubation *p*-OH norephedrine and conjugated metabolites were formed. The conjugated metabolites were hydrolyzed by treating them with β-glucoronidase or HCl. The formed products were separated. *p*-OHA could be identified as the deconjugated metabolite. The amount of formed *p*-OHA during a certain incubation period was calculated by adding the yielded amount of *p*-OH norephedrine and that of the deconjugated *p*-OHA.

The protein content of the washed microsomal and mitochondrial suspensions, respectively, was determined in duplicates according to the method of Lowry *et al.*[14] with crystalline bovine serum albumin used as a standard.

## RESULTS AND DISCUSSION

After development of a suitable and reproducible method by which amounts of 0·1 pmole amphetamine and *p*-OHA were still detectable,[15] we investigated the biotransformation of amphetamine to *p*-OHA measuring the product formed. To find an optimal assay, the rate of hydroxylation was examined varying the concentration of one of the factors of the incubation mixture while the others were present in concentrations giving maximum activity. The effect of variations in NADP concentration is shown in Table 1.

TABLE 1. INFLUENCE OF INCREASING CONCENTRATIONS OF NADP ON THE PRODUCTION OF *p*-OHA. THE COMPONENTS OF THE ASSAY WERE DISSOLVED IN KREBS–RINGER PHOSPHATE BUFFER (KRPB). pH OF THE ASSAY: 7·0–6·9. THE VALUES ARE MEANS OF AT LEAST TWO EXPERIMENTS. INCUBATION TIME = 10 min

| NADP (mM) | Amphetamine (M) | nmoles *p*-OHA (mg of protein)$^{-1}$ (10 min)$^{-1}$ |
|---|---|---|
| 0·2 | $5 \times 10^{-7}$ | 0·0101 |
| 0·4 | $5 \times 10^{-7}$ | 0·0104 |
| 0·8 | $5 \times 10^{-7}$ | 0·0101 |
| 0·2 | $5 \times 10^{-4}$ | 1·47 |
| 0·4 | $5 \times 10^{-4}$ | 1·75 |
| 0·8 | $5 \times 10^{-4}$ | 1·87 |

The highest turnover rate was detectable at 0·8 mM NADP. It was, however, possible to improve the enzyme activity with higher concentrations of coenzyme, provided that the optimal pH range is preserved. This was only possible with a higher molar buffer. Therefore, a 0·1 M Sörensen–phosphate buffer was used for the following two experiments listed in Table 2.

TABLE 2. INFLUENCE OF INCREASING CONCENTRATIONS OF NADP ON THE PRODUCTION OF *p*-OHA. SÖRENSEN-PHOSPHATE BUFFER WAS USED. pH OF THE ASSAY = 6·9. THE CONCENTRATION OF THE NADPH-GENERATING SYSTEM FOR 3·2 mM NADP WAS INCREASED = 12·5 mM GLUCOSE-6-PHOSPHATE, 10 IU GLUCOSE-6-PHOSPHATE-DEHYDROGENASE. THE VALUES ARE MEANS OF TWO EXPERIMENTS. INCUBATION TIME = 10 min

| NADP (mM) | Amphetamine (M) | nmoles *p*-OHA (mg of protein)$^{-1}$ (10 min)$^{-1}$ |
|---|---|---|
| 0·8 | $5 \times 10^{-4}$ | 1·91 |
| 3·2 | $5 \times 10^{-4}$ | 2·02 |

From the results shown in Table 2 it may be concluded that the maximal effect is almost reached at a concentration of 0·8 mM NADP.

Boot and Boyland[16] also found a dependence between enzyme hydroxylation by liver microsomes and the concentration of NADPH. Schenkman *et al.*[17] found no further increase with NADP concentrations higher than 0·3 mM.

An enhancement of the regenerating system does not improve the enzyme activity, examined up to a concentration of 12 IU glucose-6-phosphate dehydrogenase and 12 mM glucose-6-phosphate, respectively. As shown in Table 1 *p*-OHA was produced at a concentration of $5 \times 10^{-7}$ M amphetamine which can be expected approximately after injection of 10 mg/kg amphetamine in the liver *in vivo*.[8]

In Fig. 1 the rate of *p*-OHA produced within 30 min has been plotted as a function of amphetamine concentration. The activity of hydroxylase rose with increasing con-

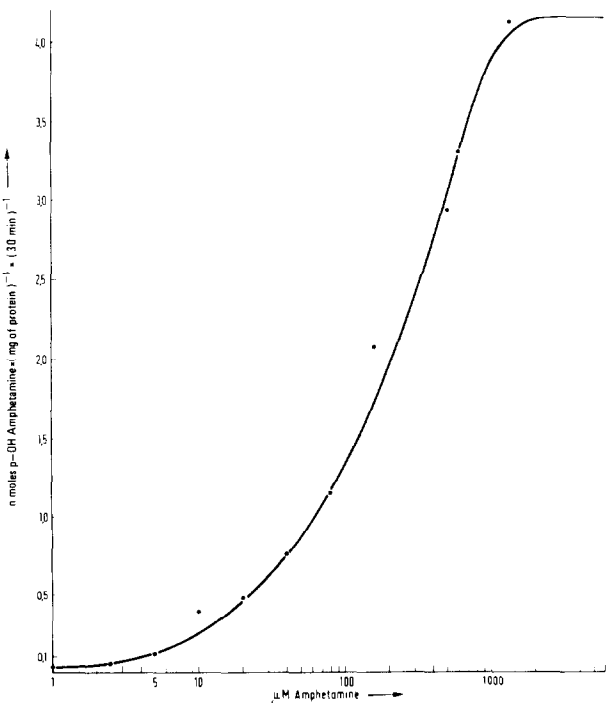


FIG. 1. Relationship between increasing concentrations of amphetamine and the production of *p*-OHA. KRPB, 0·8 mM NADP, pH of the assay 6·9. Incubation time = 30 min.

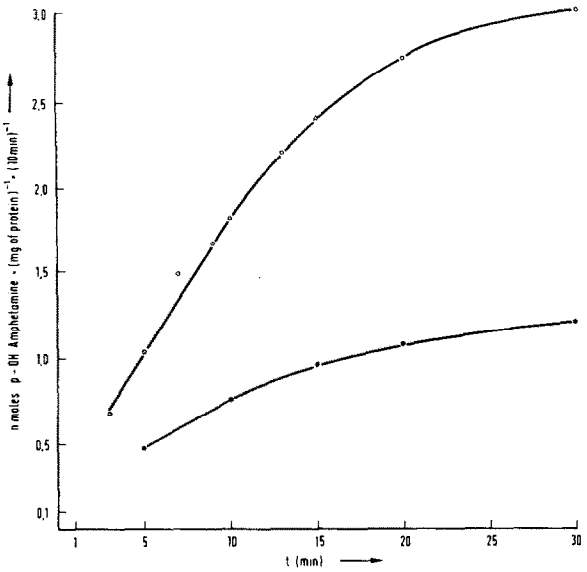


FIG. 2. Relationship between the incubation time and the production of *p*-OHA at two concentrations of amphetamine [(○) $5 \times 10^{-4}$, (●) $5 \times 10^{-5}$]. Routine assay.

centration of the substrate. The dependence between enzyme activity and amphetamine concentration may be expressed in a saturation curve. Experimental details are listed in the legend of the graphs.

In Fig. 2 the enzyme activity versus the incubation time was plotted. It shows a linear relation at least during the first 10 min for both concentrations of substrate examined. Therefore, in the following experiments the incubation period was limited to 10 min.

The effect of pH on the amphetamine hydroxylase activity is shown in Fig. 3. The optimal pH was 6·9–7·0. The microsomes and the factors of the assay were suspended in a Sörensen–phosphate buffer (0·1 M). The NADP concentration was 0·8 mM.

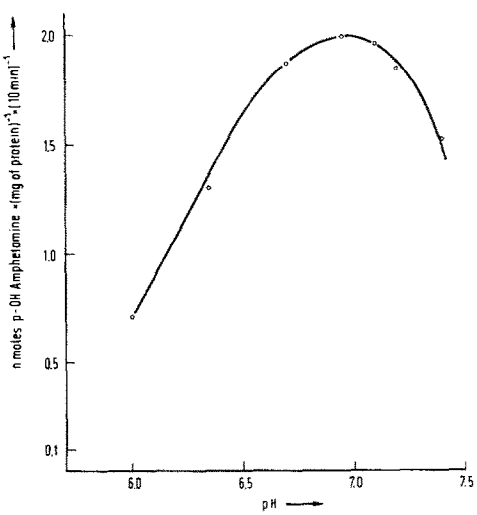


FIG. 3. Effect of pH on production of *p*-OHA. Sörensen–phosphate buffer, 0·1 m. 0·8 mM NADP. Incubation time = 10 min.

In Table 3 the concentration of the hydroxylating enzyme was varied.

TABLE 3. INFLUENCE OF INCREASING CONCENTRATIONS OF MICROSOMAL PROTEIN ON THE PRODUCTION OF *p*-OHA. ROUTINE ASSAY, 0·8 mM NADP, $5 \times 10^{-5}$ M AMPHETAMINE, KRPB, MEANS OF THREE EXPERIMENTS. INCUBATION TIME = 10 min

| Microsomes, equivalent of | mg of protein/ml of the assay | nmoles *p*-OHA (mg of protein)$^{-1}$ (10 min)$^{-1}$ |
|---|---|---|
| 267 mg liver (wet wt) | 2·4 | 0·61 |
| 400 mg liver (wet wt) | 3·6 | 0·67 |
| 800 mg liver (wet wt) | 7·1 | 0·46 |

The production of *p*-OHA was the same between 2·4 and 3·6 mg of protein/ml incubation mixture. At 7·1 mg of protein/ml a clear decrease of enzyme activity occurred. This may be due to a more rapid decline in enzyme activity[17] or to a non-specific binding of substrate.[18]

In Fig. 4 a typical course of one experiment is plotted according to the method of Lineweaver and Burk.[19] The apparent Michaelis parameters for the metabolism of amphetamine by microsomal preparations from rat liver were calculated from the results of five experiments. $1{\cdot}5 \times 10^{-4}$ M ($K_m$) was found as the amphetamine concentration at half maximal velocity. As the maximal production of *p*-OHA ($V_{max}$) 2·16 × nmoles/mg of microsomal protein and 10 min was calculated.

An experiment was carried out to determine whether the breakdown of amphetamine to *p*-OHA was restricted to the microsomes or whether mitochondria were also able to hydroxylate this amine. Mitochondria of liver of rats were prepared by the method described by Neubert *et al.*[20] The substrate concentration was $5 \times 10^{-4}$ M. Krebs–Ringer phosphate buffer (pH 7·4) was used as a medium. In six experiments a turnover of 0·36 ± 0·06 nmoles (mg of protein)$^{-1}$ (10-min)$^{-1}$ was found. Therefore, in mitochondria the production of *p*-OHA is detectable *in vitro* which is much smaller (5·4-fold) than the turnover in microsomes related to mg of protein.

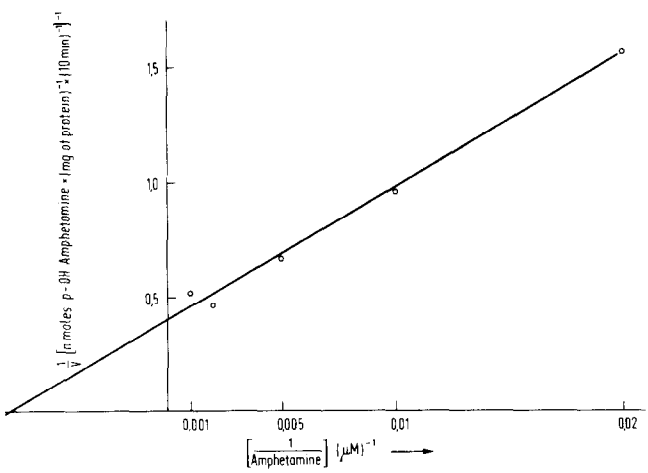


FIG. 4. Reciprocal plots for turnover velocity and various amphetamine concentrations. Routine assay. Incubation time = 10 min.

We think that the method described above is suitable and reproducible and can be used to study the activity of the amphetamine hydroxylase under different conditions.

## REFERENCES


1. J. AXELROD, *J. Pharmac. exp. Ther.* **110,** 315 (1954).
2. J. J. ALLEVA, *J. Med. Chem.* **6,** 6 (1963).
3. M. GOLDSTEIN and B. ANAGNOSTE, *Biochem. biophys. Acta* **107,** 166 (1965).
4. T. ELLISON, L. GUTZAIT and E. J. VAN LOON, *J. Pharmac. exp. Ther.* **152,** 383 (1966).
5. L. VALZELLI, E. DOLFINI, M. TANSELLA and S. GARATTINI, *J. Pharm. Pharmac.* **20,** 595 (1968).
6. T. LEWANDER, in *Abuse of Central Stimulants.* Almquist & Wiskell, Stockholm (1969).
7. L. G. DRING, R. L. SMITH and R. T. WILLIAMS, *Biochem. J.* **116,** 425 (1970).
8. M. ZIEM, H. COPER, I. BROERMANN and S. STRAUSS, *Naunyn-Schmiedeberg's Arch. Pharmak.* **267,** 208 (1970).
9. H. ROMMELSPACHER and H. HONECKER, *Naunyn-Schmiedeberg's Arch. Pharmak.* **277,** R 63 (1973).
10. J. V. DINGELL and A. D. BASS, *Biochem. Pharmac.* **18,** 1535 (1969).
11. A. H. BECKETT and S. AL-SARRAJ, *J. Pharm. Pharmac.* **24,** 174 (1972).
12. A. WIRKSTRÖM and B. SALVERSEN, *J. Pharm. Pharmac.* **4,** 631 (1952).
13. T. LEWANDER, *Psychopharmacologia (Berl.)* **13,** 394 (1968).
14. O. LOWRY, H. ROSEBROUGH, N. J. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
15. H. ROMMELSPACHER, H. HONECKER and S. STRAUSS, manuscript in preparation.
16. J. BOOTH and E. BOYLAND, *Biochem. J.* **66,** 73 (1957).
17. J. B. SCHENKMAN, J. B. BALL and R. W. ESTABROOK, *Biochem. Pharmac.* **16,** 1071 (1967).
18. J. R. GILETTE, *Proc. 2nd Int. Pharm. Meeting,* Vol. 4, p. 9 (1965).
19. H. LINEWEAVER and D. BURK, *J. Am. chem. Soc.* **56,** 658 (1934).
20. D. NEUBERT, E. OBERDISSE and R. BASS, in *Biochemical Aspects of the Biogenesis of Mitochondria,* pp. 123. Adriatica Edictrice, Bari (1968).