the present study also clearly indicates that DNA synthesis from glucose was more sensitive to IAA inhibition than were the other metabolic processes. At 10^{-5} M IAA, a concentration not affecting glycolysis, 36 per cent inhibition of DNA synthesis occurred. Since RNA synthesis was significantly *elevated* at this level of IAA, it is possible that one site of action for this inhibitor on DNA synthesis involves some process concerned with the formation of DNA precursors, such as the sulfhydryl-sensitive ribonucleotide reductase complex.[9] The present results suggest the need for caution when interpreting experiments which utilize IAA as a "specific" glycolytic inhibitor. The effects of IAA vary in a dose-dependent manner and include more than one metabolic pathway.

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Phenylacetone oxime—An intermediate in the oxidative deamination of amphetamine*

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AXELROD[1] first demonstrated the conversion, by rabbit liver microsomes, of amphetamine to phenylacetone. No reports, to our knowledge, have appeared concerning the nature of possible intermediates in the reaction referred to as oxidative deamination. Human liver microsomes also apparently convert amphetamine to phenylacetone.[2] Phenylacetone and benzoic acid were shown by Dring *et al.*[3] to be major metabolites of amphetamine in rabbit, dog and human urine and these authors concluded that oxidative deamination was the predominant metabolic pathway for amphetamine in these species. The corresponding imine, a possible phenylacetone precursor, was not found in urine after amphetamine administration.

The present report is concerned with the nature of the intermediate formed during oxidative deamination of amphetamine by a rabbit liver microsomal system.

* A preliminary account of this work has appeared in *Pharmacologist* **12,** 255 (1970).

Livers from male New Zealand white rabbits were homogenized with 2 vol. of ice-cold isotonic KCl and centrifuged at 9000 *g* for 20 min. Supernatant corresponding to 1 g liver was incubated with shaking for 1–3 hr at 37° in air in vessels containing the following components: *d*-amphetamine, 7·4 μmoles; 1 ml 0·2 M phosphate buffer, pH 7·4; NADP, 0·5 μmole; nicotinamide, 100 μmoles; glucose 6-phosphate, 25 μmoles and $MgCl_2$, 75 μmoles in water to a final volume of 5·95 ml.

Amphetamine disappearance was measured by assay of an aliquot of the incubation mixture according to the method of Axelrod.[1] About 65 per cent of the added amphetamine was metabolized in 2 hr. The remainder of the incubation mixture was shaken with 15 ml of heptane containing 3% isoamyl alcohol for 15 min. Ten ml of the heptane was evaporated to dryness *in vacuo* and the residue dissolved in 50 μl of ethyl acetate. One μl of the ethyl acetate solution was subjected to gas chromatography (GLC) under the conditions shown in Fig. 1.

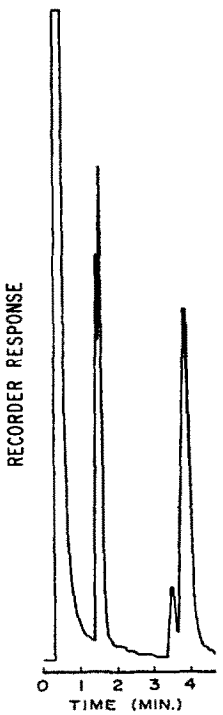

FIG. 1. Gas chromatogram of heptane extract of products of incubation of amphetamine with rabbit liver extract. A Hewlett–Packard (F&M) model 810 instrument fitted with a 6 ft × 0·25 inch glass column packed with 3% SE-30 on chromosorb G and flame ionization detector was employed. Helium was used as the carrier gas at a flow rate of 50 ml/min. Operating temperatures were as follows: flash heater, 150°; oven, 120°; detector, 180°.

As indicated, 4 peaks were observed after GLC, with retention times (T_R) of 1·5, 1·6, 3·5 and 3·8 min. No peaks were seen after incubation of substrate with boiled liver extracts. Phenylacetone oxime was synthesized from phenylacetone by the method of Hey,[4] except that the reflux period was extended to 18 hr. The product, m.p. 70–71° (lit. value,[4] 70°), was shown to be the oxime by elemental analysis. *Anal.* Calcd. for $C_9H_{11}NO$: C, 72·45; H, 7·43; N, 9·39. Found: C, 72·91; H, 7·58; N, 9·52. GLC of the authentic oxime under these conditions gave a single peak at 3·8 min. GLC of the metabolite extract to which authentic oxime was added gave the same pattern as before, except that the peak at 3·8 min was enlarged, suggesting the identity of the metabolite having T_R 3·8 min and phenylacetone oxime. The trimethylsilyl (TMS) derivative of phenylacetone oxime had a T_R of 5·0 min under these conditions. After trimethylsilylation, GLC of the metabolite extract showed a new peak of 5·0 min, but the peak at 3·8 min had disappeared. GLC of the trimethylsilylated metabolite extract to which the TMS ether of phenylacetone oxime had been added gave the same pattern as before except that the peak at 5·0 min was enlarged.

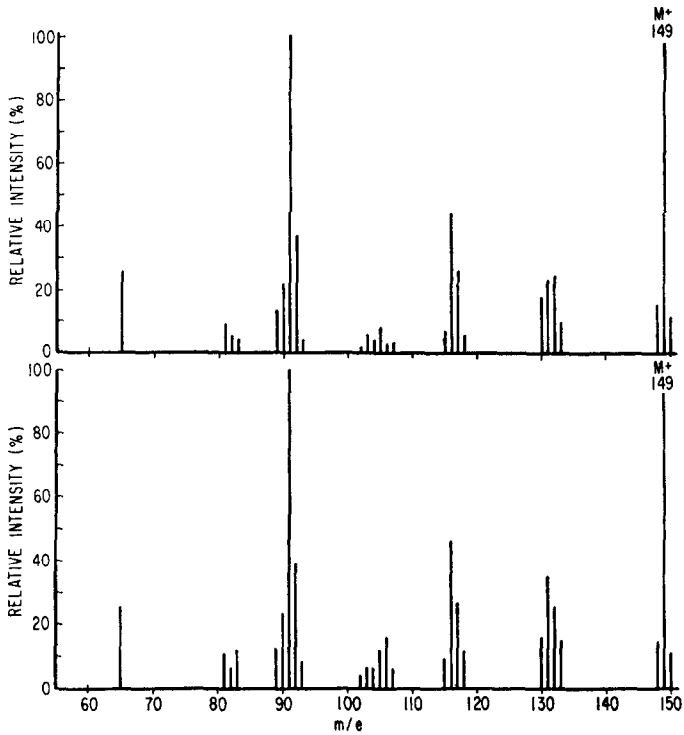

FIG. 2. Mass spectra of phenylacetone oxime (upper) and apparent oxime metabolite (lower) of amphetamine. Spectra were obtained on an A.E.I. MS-902 mass spectrometer using the direct probe inlet.

Preparative gas chromatography on a Perkin–Elmer Model 881 instrument was employed to collect larger quantities of the apparent oxime metabolite. The collected metabolite gave a single peak on GLC with T_R = 3·8 min. The mass spectra of the metabolite and authentic oxime shown in Fig. 2 were identical. In addition, treatment of the collected metabolite with 1 N HCl gave, after 5 min at room temperature, a new peak with T_R = 1·6 min, the same as that of authentic phenylacetone. After standing overnight GLC of the metabolite showed only 1 peak at 1·5 min, that at 3·8 min having completely disappeared. Co-injection of the acid-treated metabolite and authentic phenylacetone gave only a single peak at 1·5 min.

Axelrod[1] isolated and estimated phenylacetone as a metabolite of amphetamine by extraction of the ketone from an alkalinized sample into carbon tetrachloride. The extract was then treated with 2,4-dinitrophenyl-hydrazine in 2 N HCl to derivatize the ketone for subsequent colorimetry. Under these conditions, we have shown that the oxime metabolite was completely hydrolyzed to phenylacetone, as determined by GLC.

In other experiments requiring the use of radioactive amphetamine, the *l*-^{3}H-isomer was used. Gas chromatographic analysis of the products formed indicated that they were identical with those obtained using *d*-amphetamine except, as previously noted, that the *l*-isomer was metabolized more rapidly than the *d*-isomer.

In the first of these experiments, 2·3 μc (1 mg) of *l*-amphetamine-^{3}H was incubated for 2 hr in duplicate vessels with rabbit liver extract and cofactors as described above. The mixture was extracted with heptane as before and authentic phenylacetone oxime (300 mg) was added to the heptane solution. After equilibration, the solvent was evaporated and the residue recrystallized from boiling petroleum ether (40–60°). The sp. act. (3000 counts/min/5 mg) and m.p. (70–71°) did not change significantly over 3 succeeding crystallizations indicating that the metabolite was, in fact, phenylacetone oxime. The results also indicated that the oxime metabolite represented about 60 per cent of the radioactivity present in the incubation mixture (corrected for 60 per cent extraction into heptane).

Thin-layer chromatography was used to identify the oxime metabolite in a subsequent experiment. *l*-Amphetamine-^{3}H was incubated as before and a methylene chloride extract of the incubation mixture subjected to TLC on silica gel using chloroform as a solvent. After autoradiography for 1·5 days at − 40° using Kodak Royal Blue safety film (plate was initially sprayed with Omnispray intensifier obtained from New England Nuclear Corp.), the developed film showed two intense dark spots. The first of these (R_f 0·13) corresponded to that of authentic phenylacetone oxime. The second (R_f 0·21) corresponded to that of phenyl-2-propanol (see below). Fainter spots were seen at R_f 0·05 (amphetamine), 0·46 (phenylacetone) and 0·76 (unknown). Radioactivity in the spots was determined by direct counting in a liquid scintillation counter. The results showed that about 55 per cent of the label on the plate was present in the spot corresponding to phenylacetone oxime. Since approximately 90 per cent of added oxime was recovered by methylene chloride, the oxime metabolite represented about 60 per cent of the label in the incubation mixture, in agreement with the reverse isotope dilution results.

In addition to this evidence that phenylacetone oxime is a major metabolite of amphetamine *in vitro*, subsequent experiments were performed to identify the remaining metabolites demonstrated to be present by GLC (see Fig. 1). The metabolite with T_R = 1·5 min at 120° on 3% SE-30 had a T_R = 6·0 min on GLC at 90° on 1% QF-1 (on Chromosorb Q). These retention times corresponded with those of phenylacetone and co-injection of the metabolite and the authentic ketone gave only a single peak in each case.

The metabolite with T_R = 1·6 min at 120° on 3% SE-30 had a T_R = 3·2 min at 90° on 1% QF-1, values which corresponded exactly with those observed for phenyl-2-propanol. Co-injection of the metabolite and phenyl-2-propanol gave a single peak. Likewise, the trimethylsilyl derivative of the metabolite had, on GLC at 90° on 1% QF-1, a T_R = 2·7 min, the same as that of trimethylsilylated phenyl-2-propanol.

When phenylacetone oxime was incubated with a rabbit liver extract (as described above for amphetamine) followed by methylene chloride extraction and GLC, peaks were seen which corresponded to those of phenylacetone and phenyl-2-propanol. No metabolism occurred with boiled liver extracts. Quantitative gas chromatography on 1% SE-30 showed that the peak height and amount of phenylacetone oxime injected were linearly related over a range of 100–1000 μg. Measurement of oxime remaining after incubation of 6·8 μ-moles of oxime as described above for 2 hr showed that approximately 70 per cent of the added oxime was metabolized. In experiments in which *d*-amphetamine (7·4 μmoles) was incubated for 2 hr, an average of 1·6 μmoles of oxime was present at the end of the incubation period during which 4·8 μmoles of amphetamine was metabolized. Since approximately 70 per cent of the oxime present was in turn metabolized, it was calculated that 4·9 μmoles of oxime had actually been formed. These results indicated that amphetamine was stoichiometrically converted to the oxime by the rabbit liver extract.

Other experiments were devoted to the identification of metabolites *in vitro* of phenylacetone and phenyl-2-propanol. Incubation of the former (7·5 μmoles in 0·25 ml ethanol) for 2 hr with rabbit liver extract followed by methylene chloride extraction and GLC indicated that about 70 per cent of the added ketone had been metabolized to a compound with the same T_R values as phenyl-2-propanol. Co-injection of the ketone metabolite and phenyl-2-propanol gave only a single peak.

Similarly, incubation of phenyl-2-propanol (7·5 μmoles in 0·25 ml ethanol) for 2 hr with rabbit liver extract followed by methylene chloride extraction and GLC indicated that about 20 per cent of the substrate had been metabolized to a compound with GLC properties identical with those of phenylacetone.

Excretion of phenylacetone oxime in urine of rabbits given amphetamine was determined in the following experiment. Two rabbits received 5·8 mg/kg of *l*-amphetamine-^{3}H, i.v. Urine collected for 24 hr was assayed for phenylacetone oxime by GLC. None was detected. However, when an aliquot of the urine was incubated with a β-glucuronidase/sulfatase preparation (Glusulase, Endo) before extraction with methylene chloride and GLC, a peak was seen with T_R corresponding to phenylacetone oxime. The amount was estimated to equal about 2 per cent of the dose in each urine sample (38 and 53 per cent of the dose was excreted by each rabbit, respectively, in this period). Trimethylsilylation of the urine extract followed by GLC gave a new peak which corresponded in T_R to that of the trimethylsilyl derivative of phenylacetone oxime.

Metabolism of phenylacetone oxime was studied in two rabbits given 10 mg/kg of the oxime, i.v. No oxime was detected in 24-hr urine by GLC. However, incubation of the urine with glusulase followed by extraction with methylene chloride and GLC gave a peak in each sample which corresponded to the oxime or, after trimethylsilylation, with the derivative. The amount present was estimated to be about 2 per cent of the dose, indicating that the oxime itself was extensively metabolized *in vivo*.

Phenylacetone oxime produced no alterations in gross behavioral activity of the mouse (1–324 mg/kg, i.p.). Corresponding doses (1–9 mg/kg, i.p.) of *d*-amphetamine produced readily observable stimulant effects in all animals.

Results of experiments described above represent conclusive evidence that phenylacetone oxime is an important intermediate in the oxidative deamination of amphetamine by a rabbit liver microsomal system. Possible mechanisms of its formation are illustrated in Fig. 3. One involves conversion to the

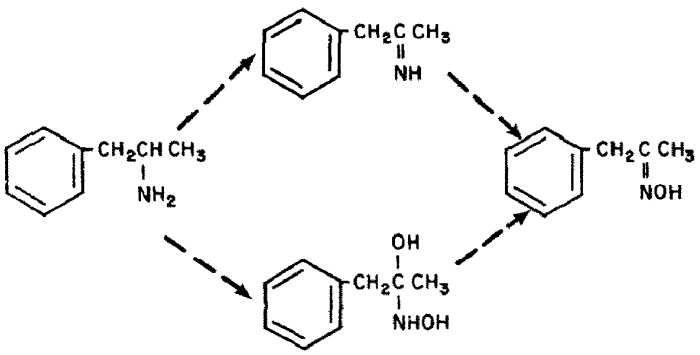

FIG. 3. Possible mechanisms of formation of phenylacetone oxime by rabbit liver *in vitro*.

imine which is, in turn, hydroxylated to yield the oxime. It is of interest that the imine was previously proposed[5] as an intermediate of amphetamine metabolism but none was detected in rabbit urine. A second possibility requires initial hydroxylation of the amino carbon atom as well as hydroxylation of the amino group, followed by loss of water to give the oxime. This intermediate is the same as that known to be formed during hydrolysis of oximes.[6] A third possibility (not shown) might be hydroxylation of the amino group to form a hydroxylamine followed by loss of hydrogen to give the oxime.

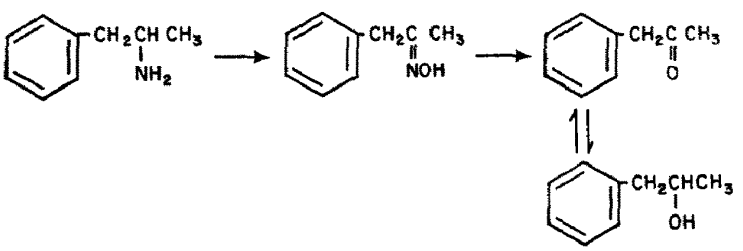

FIG. 4. Mechanism of amphetamine metabolism by rabbit liver *in vitro*.

The findings reported here are summarized in Fig. 4. Amphetamine is converted by rabbit liver *in vitro* to phenylacetone oxime, which in turn is enzymatically hydrolyzed to phenylacetone and then reduced to phenyl-2-propanol. The alcohol, previously shown[3] to be a urinary metabolite of amphetamine, is partially oxidized to phenylacetone.

No previous reports appear to have been published on the nature of intermediates in the oxidative deamination of foreign compounds. We have demonstrated that the oxime is the intermediate formed during deamination of amphetamine and, to date, of one other compound, an α-substituted benzylamine,* and suggest that the oxime may be the common (if somewhat unexpected) intermediate for all amino compounds which are metabolized by oxidative deamination.

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Different sensitivities of the phosphodiesterases (adenosine-3′,5′-cyclic phosphate 3′-phosphohydrolase) of dog cerebral cortex and erythrocytes to inhibition by synthetic agents and cold

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AFTER the initial report describing the effect of 4-(3,4-dimethoxybenzyl)-2-imidazolidinone as both a lipolytic agent and inhibitor of phosphodiesterase (PD),[1] an even more potent inhibitor of the PD from rat erythrocytes was found in the 3-butoxy-4-methoxybenzyl derivative (Ro 20-1724).[2] In the course of these and other studies, differences were noted in the sensitivity of PD from a variety of tissues to some of these compounds. A systematic examination of the effects of several inhibitors on the PD preparations of the cerebral cortex and erythrocytes of the dog and rat was undertaken.

The assay of PD involved a measurement of the conversion of adenosine (-8-^{3}H)-3′,5′-cyclic phosphate (C-AMP-^{3}H; 4 μM) to AMP in the presence of the enzyme.[1] Incubations were carried out in quadruplicate at 37° or in an ice bath as specified. The erythrocyte PD preparations represent ghost-free hemolysates prepared by mixing 2 ml of washed (three times) erythrocytes with 26 ml of 7 mM phosphate buffer and centrifuging at 15,000 rev/min for 40 min. The cerebral cortex PD preparation was obtained by homogenizing portions of tissue with 5 vol. of 1·15% KCl solution using a glass homogenizer for 0·5 min. The homogenate and washings (5 vol.) were then centrifuged at 105,000 *g* to obtain a relatively particulate-free supernatant. All incubations of the cerebral cortex PD were carried out in the presence of 2×10^{-4} M AMP to trap the AMP-^{3}H and minimize its conversion to adenosine by the 5′-nucleotidase activity of the preparation. This concentration of AMP did not significantly alter the PD activity.

TABLE 1. INHIBITION OF THE PHOSPHODIESTERASE FROM DOG CEREBRAL CORTEX AND DOG AND RAT RED BLOOD CELLS BY THEOPHYLLINE, PAPAVERINE, RO 20-1724, APOMORPHINE AND 3,4-DIHYDROXYPHENYLACETIC ACID (DOPAC)

	I_{50} (μM)				
Tissue	Theophylline	Papaverine	Ro 20-1724	DOPAC	Apomorphine
Rat RBC	820	12·0	0·17	> 10,000	170·0
Dog RBC	920	21·0	0·2	> 10,000	220·0
Dog cerebral cortex	600	12·0	275·0	630	15·0

In Table 1 it can be seen that the dog red blood cell (RBC) enzyme behaves very much like that from the rat in that inhibition by Ro 20-1724 > papaverine > apomorphine > theophylline > 3,4-dihydroxyphenylacetic acid (DOPAC). With the dog cerebral cortex, however, the inhibition by papaverine = apomorphine > Ro 20-1724 > DOPAC = theophylline.

The I_{50} values of theophylline for the two RBC preparations are not significantly different, but both are significantly higher ($P < 0·05$) than that for the cerebral cortex. The I_{50} of papaverine with the