THE MECHANISM OF THE OXIDATION OF d-AMPHETAMINE BY RABBIT LIVER OXYGENASE. OXYGEN-18 STUDIES.

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SUMMARY

The oxidation of d-amphetamine by rabbit liver microsomes has been studied using oxygen-18 as the source of oxygen. Incorporation of heavy oxygen into the two major metabolites phenylacetone oxime and phenylacetone, was 93-95% and 25-31% respectively. These data are consistant with a mechanism in which the initial step is the hydroxylation of the substrate at the carbon atom α to the amino group. The carbinol amine which is formed by this reaction then serves as the key intermediate from which ketone and oxime are formed. Thus, oxime can form from carbinol amine in two step, (1) dehydration of carbinol amine and (2) oxygenation of the resulting imine. Phenylacetone can form by two pathways (1) loss of a molecule of ammonia from carbinol amine (incorporation of oxygen from molecular oxygen) and (2) hydrolysis of oxime (incorporation of oxygen from water). In the case of d-amphetamine the hydrolytic route appears to be the more important as suggested by Hucker, et al. (4, 5).

The enzymatic formation of ketones from primary amines was first reported by Axelrod (1) who found that amphetamine was oxidized to phenylacetone by rabbit liver microsomes. Later, Brodie, et al. (2) proposed that this reaction occurs by an initial hydroxylation of the carbon atom adjacent to the amine nitrogen to form an unstable carbinolamine which then loses ammonia to form ketone ($R_2CHNH_2 \rightarrow R_2C(OH)NH_2 \rightarrow R_2CO$). As an alternative, Smith and Dring (3) have suggested that amphetamine might be directly dehydrogenated to an imine which would readily hydrolyze to ketone. Recently, Hucker, et al. (4, 5) using rabbit liver microsomes found phenylacetone oxime to be the major *in vitro* metabolite of amphetamine. These workers suggest that the phenylacetone arises from the subsequent

hydrolysis of this oxime. They also speculate that the oxime might arise from an intermediate imine.

In the preliminary study described below oxygen-18 labeling has been employed as a tool to investigate further the mechanism of d-amphetamine deamination. The results of the study support a mechanism in which the key step is an initial microsomal hydroxylation at the carbon atom adjacent to the nitrogen atom.

MATERIALS AND METHODS

The d-amphetamine sulfate used was obtained from Smith Kline and French Laboratories. Freshly prepared solutions in water (18.6 μmol/ml) were used for the enzyme runs. Oxygen-18 (92 atom %) was purchased from Miles Laboratories, Research Products Division. Experimental results were corrected to 100 atom % for oxygen-18.

To insure maximum yield of metabolites, phenobarbital 'induced' microsomes were used. Young male albino rabbits were treated with phenobarbital (15 mg/kg, intraperitoneally) twice daily for four days. On the fifth day the rabbits were sacrificed, and the livers were removed and placed in cold 0.25 M sucrose. Liver microsomes were then prepared at 0° by standard sedimentation procedures in 0.25 M sucrose. The washed microsomal pellets were resuspended in 1.15% KCl and used immediately for the enzyme studies.

The experiments with both oxygen-18 or oxygen-16 atmospheres were performed as follows. A 10 ml incubation mixture containing 20 μmol $MgCl_2$, 4 mmol buffer (pH 7.40), 5 μmol TPN^+, 50 μmol glucose-6-phosphate, 50 units glucose-6-phosphate dehydrogenase and microsomes equivalent to 2.5 g of liver was placed in a 25 ml round bottom flask. The flask was attached to a vacuum

line, frozen and degassed by thawing and refreezing. After adding 18.6 μmol of substrate, the flask was re-evacuated and 10 ml of $^{18}O_2$ (92 atom %) or 10 ml $^{16}O_2$ added. The reaction mixture was then incubated at 37° for 45 min. with vigorous shaking.

The reaction was terminated by the addition of 15 ml of ethyl acetate. The reaction mixture was then extracted with three 15 ml portions of ethyl acetate. The extracts were evaporated to dryness, dissolved in 0.1-0.2 ml of ethyl acetate, and a 2-3 μl aliquot was injected into a HP-5750 gas chromatograph. A 6 foot coiled glass column packed with 3 percent UC W-98 on diataport S was used. The temperatures were flash heater 110°, column 90°, and flame 130°. The flow rate of the helium carrier gas was 60 ml/min. The retention time of phenylacetone was 2.5 min. and that of its oxime, 13.5 min. Standard solutions of ketone and oxime were used to quantitate the amount of product formed in the reactions. The identity of phenylacetone and its oxime as well as their O^{16} and O^{18} content were determined using an LKB 9000 GLC Mass Spectrometer.

RESULTS AND DISCUSSION

Control experiments with unlabeled oxygen were performed to determine yields and to establish the nature of the mass spectra of the metabolites under the experimental conditions involved. The results of the control experiments showed that an accurate assay of the oxygen-18 content would be possible by combined gas chromatography-mass spectroscopy since both phenylacetone and its oxime had prominent molecular ion peaks and no M+2 peaks. The absence of an M+2 peak in the ketone spectrum also showed that the phenylacetone GC-peak was uncontaminated by

the corresponding carbinol, a minor metabolite of amphetamine.

The results of the oxygen-18 studies are summarized in Table 1. The yield of phenylacetone was about one-half of that of oxime and was very similar to the yields in the control runs. Oxygen-18 incorporation amounted to 25-31% of theoretical. The fraction of the phenylacetone (25-31%) that contained oxygen-18 could not have been formed by either of the hydrolytic routes proposed by Smith and Dring (3) or by Hucker, et al. (4). The most reasonable explanation is that of Brodie, et al. (2), i.e. initial hydroxylation (with molecular oxygen as the source of oxygen) to carbinol amine followed by loss of a molecule of ammonia to form phenylacetone. This mechanism is formally the same as the mechanism of the microsomal N-dealkylation of tertiary amines which has recently been shown to utilize molecular oxygen as the source of oxygen (6).

The major portion (69-75%) of phenylacetone contained oxygen-16 rather than oxygen-18. The proposal of Hucker, et al. (4, 5) that phenylacetone could arise from hydrolysis of oxime readily explains this observation, since this pathway would result in the incorporation of oxygen from water rather than from labeled molecular oxygen.

TABLE I

INCORPORATION OF MOLECULAR OXYGEN INTO d-AMPHETAMINE METABOLITES BY RABBIT LIVER MICROSOMES

	METABOLITES			
	PHENYLACETONE		PHENYLACETONE OXIME	
Run No.	Yield	Oxygen-18 Incorporation	Yield	Oxygen-18 Incorporation
1	37.3μg	25.0%	79.7μg	93%
2	36.3μg	31.0%	50.8μg	95%

Incorporation of molecular oxygen into oxime was almost quantitative, indicating that it is formed by direct hydroxylation of an intermediate. The suggestion that this intermediate might be the imine is supported by a recent study (7) in which it was shown that liver microsomes readily hydroxylate 2,4,6-trimethyl acetophenone imine to the corresponding oxime. Isotopic oxygen studies showed that molecular oxygen served as the oxygen source. The formation of imine can be rationalized through the dehydration of the carbinol amine intermediate. Ample analogy for this step exists in organic chemical experience.

Figure 1 summarizes the proposed pathways by which rabbit liver microsomes convert d-amphetamine to phenylacetone and phenylacetone oxime.

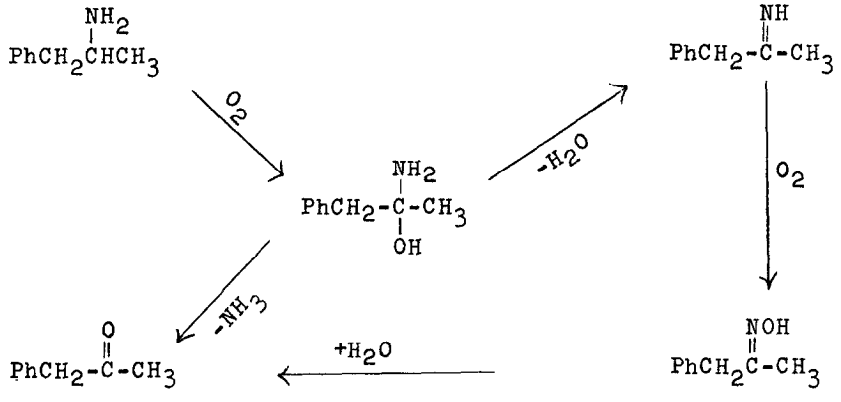

Figure 1. Microsomal oxidation of d-Amphetamine: Proposed reaction intermediates.

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