# MECHANISTIC ASPECTS OF THE OXIDATION OF AMINES BY MONOAMINE OXIDASE

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SUMMARY- Mechanistic studies on the oxidation by benzylamine by bovine hepatic monoamine oxidase at pH 7.4 have led to a formal mechanism. According to the proposed ping-pong mechanism, benzaldehyde and E-$FADH_2$ are produced first and this can be shown by carrying out the reaction anaerobically. However, the point of release of ammonia has not been studied directly. Therefore, $^{14}C$-methyl labelled N-methyl-benzylamine was synthesized and used as a substrate since it was difficult to show directly that ammonia is produced anaerobically because of contamination of solutions with $NH_3$. The experimental set-up and the results which show that ammonia is produced anaerobically before $H_2O_2$ are presented.

Monoamine oxidase is thought to be a very important enzyme which controls the level of certain neurohormones (1). Recent investigations have shown the enzyme to be a FAD-enzyme containing covalently bonded FAD (2). Although there are multiple forms of the enzyme, the smallest active subunit is reported to have a molecular weight of about 90-80,000 (3,4,5). The availability of highly purified enzyme has led to studies concerned with the mechanism of action of monoamine oxidase. Several formal mechanisms have been proposed for the bovine liver (6) and pig brain (7) enzymes. Chemical evidence has also been presented that benzaldehyde is produced anaerobically in amounts stoichiometric with the flavin content of the enzyme (8).

A working mechanism for the action of monoamine oxidase which takes into account most of the published data is shown below.

$$E\text{-}FAD + RCH_2NH_2 = E\text{-}FADH_2 + RCH{=}NH \quad \text{(Anaerobic reaction)}$$

$$RCH{=}NH + H_2O = RCHO + NH_3 \quad \text{(Anaerobic reaction)}$$

$$E\text{-}FADH_2 + O_2 = E\text{-}FAD + O_2 \quad \text{(Aerobic reaction)}$$

The formation of the aldimine intermediate is based on the report of Patek et al. (9). We have shown that RCHO and E-$FADH_2$ are produced

anaerobically when monoamine oxidizes benzylamine (10). Furthermore, the report of Smith et al. (11) have demonstrated from O-18 investigations that the oxygen in the aldehyde originates from $H_2O$. The only experiment which has not been checked is whether or not $NH_3$ is produced during the oxidation of benzylamine by monoamine oxidase under anaerobic conditions. All attempts to detect $NH_3$ directly failed because of the small amounts of ammonia produced during the reaction and because of the contamination of enzyme and buffer solutions by ammonia. Therfore, advantange was taken of the fact that monoamine oxidase oxidizes N-methyl amines in which case $CH_3NH_2$ is produced instead of ammonia. Thus, $^{14}C$-methyl labelled N-methylbenzylamine was synthesized and the experimental conditions for determining whether $CH_3NH_2$ is produced during the oxidation of amines by monoamine oxidase anaerobically are reported in the present communication.

## EXPERIMENTAL PROCEDURES AND RESULTS

Purification of bovine hepatic monoamine oxidase. The enzyme was purified by previously published procedures (10). Component 2 of specific activity 4,500 was used in all of the experiments. The enzyme was assayed by the spectrophotometric procedure of Tabor et al. (12). Protein was determined by the procedure described by Lowry et al. (13).

Synthesis of N-methylbenzylamine. The procedure of Rappaport et al. (14) was used. For the experiment, 2.33 mg (16.2 μmole) of $^{14}CH_3I$, specific activity 4.6 mc per mg was reacted with 2.33 mg (21.8 μmole) of benzylamine in 1 ml of acetone in a sealed tube for 24 hours. The sample was then heated for 10 minutes at 60°. The tube was opened and neutralized by adding 0.5 ml of 0.1 N HOAc. Carrier N-methylbenzylamine was added and the sample was chromatographed on 1 x 10 cm column of Dowex 50 ($H^+$ form) which had been equilibrated with pH 3.1, 0.2 N pyridinium acetate buffer. Gradient elution was

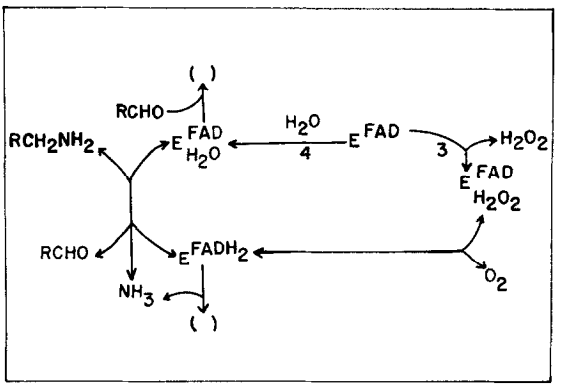


Figure 1. Design of the apparatus used to produce methylamine anaerobically. High purity nitrogen was passed through two alkaline pyrogallol traps, through a column of copper filings heated to 200°, through a solution of $H_2SO_4$ to remove $NH_3$, and finally through the reaction vessel and oxygen tester. The reaction vessel contained 0.4 ml $HClO_4$ and monoamine oxidase (.69 mg in 0.1 M phosphate buffer, pH 7.4, specific activity 4210) in two separate magnetic cups. Prior to starting the reaction, the vessel was evacuated with a water aspirator and then flushed with nitrogen. This process was repeated two times. Then nitrogen was passed through the system for 19 hours. At this time, the polyphenol oxidase (10 mg in 0.5 ml of 0.1 M phosphate buffer, pH 7.4) in the oxygen testing vessel was tipped into the solution of dopa (.5 mg) in 0.1 M phosphate buffer, pH 7.0. The lack of oxygen was shown by the fact that no hallochrome, which is pink in color, was produced. Therefore, the monoamine oxidase solution was dropped into the main compartment and reaction was allowed to proceed for 15 minutes at 25°. At this time, the $HClO_4$ solution was added and this terminated the reaction.

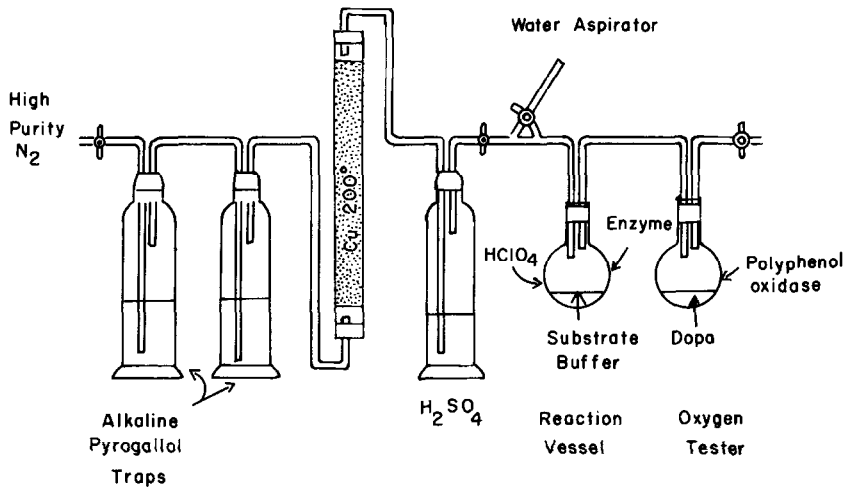


Figure 2. Proposed formal mechanism for the oxidation of benzylamine by hepatic monoamine oxidase at pH 7.4.

obtained by mixing 100 ml of pH 3.1 buffer with 100 ml of pH 5.0, 2.0 N pyridinium acetate buffer. The benzylamine was eluted first and at pH 4.0 in the gradient, N-methylbenzylamine was eluted.

Further purification of the N-methylbenzylamine. In order to get the last traces of benzylamine removed from the sample of N-methylbenzylamine, 3.0 mg of N-methylbenzylamine preparation was treated with 400 units of bovine plasma amine oxidase (15) in 1.0 ml of 0.1 M phosphate buffer, pH 7.2. The absorbance at 250 nm of the sample was measured till no further increase in absorbance occurred which took about 28 hours. The pH of the sample was lowered to pH 5.2 and it was applied to a 0.6 x 10 cm column of Dowex 50 which had been equilibrated with 2 N pyridinium-acetate buffer, pH 5.8. Fractions of 4 ml were collected. Benzaldehyde was found in tubes 4-7 by testing aliquots of the samples with phenylhydrazine. Tubes 8-13 contained the N-methylbenzylamine. The latter was acidified to pH 4.5 with glacial acetic acid and taken to dryness.

Anaerobic test for the production of $CH_3NH_2$. The objective of the experiment was to determine whether or not $NH_3$ or its

TABLE I

Methylamine Production Anaerobically from the Oxidation of N-methylbenzylamine by Monoamine Oxidase

| Experiment number | cpm | μmoles $^{14}CH_3NH_2$ produced | moles of $^{14}CH_3NH_2$ per mole of FAD in MAO |
|---|---|---|---|
| 1 | 4499 | $7.3 \times 10^{-3}$ | 1.1 |
| 2 | 3681 | $6.1 \times 10^{-3}$ | 0.9 |

The specific activity of the N-methylbenzylamine was 601,900 cpm/μmole and $50 \times 10^{-3}$ μmole was used for the experiment. Other experimental conditions are given in the legend to Figure 1.

equivalent in the case of N-methylbenzylamine, $CH_3NH_2$ is produced anaerobically when bovine liver mitochondrial monoamine oxidase acts on substrates. Special conditions necessary for an anaerobic reaction led to the experimental set-up shown in Fig. 1. After the reaction, the contents of the reaction mixture were transferred quantitatively to a centrifuge tube and the precipitate was removed by centrifugation. The supernatant was purified as previously described on a column of Dowex 50 and eluted with 2 N pyridinium acetate buffer, pH 5.2. Methylamine was isolated by concentrating fractions 9-24 while N-methylbenzylamine was isolated from fractions 28-40 ml. Both fractions were acidified by adding 1 ml of glacial HOAc and concentrated with a stream of nitrogen. The final volume of the methylamine fraction was 2 ml of the N-methylbenzylamine fraction was 5 ml. A 0.5 ml aliquot of methylamine fraction was mixed with 10 ml of PPO-2.5-Diphenyloxazole in dioxane and counted in a Packard Liquid Scintillation Counter. The results are summarized in Table I.

## DISCUSSION

There is general agreement that the oxidation of amines by

monoamine oxidase occurs by a ping-pong mechanism (6-8). In particular, the bovine hepatic monoamine oxidase-benzylamine reaction has been studied in some detail (6,8). These investigations have led to the proposed formal mechanism shown in Fig. 2. The present demonstration that ammonia or its equivalent, N-methylamine, is produced anaerobically in stoichiometric amounts to the FAD present in the hepatic enzyme, when N-methylbenzylamine is utilized as substrate, concludes our chemical proof for the mechanism shown in Fig. 2.

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