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# Mechanism-Based Inactivation of Mitochondrial Monoamine Oxidase by *N*-(1-Methylcyclopropyl)benzylamine†

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ABSTRACT: Three different radioactively labeled *N*-(1-methylcyclopropyl)benzylamines [N-(1-Me)CBA] were synthesized and used to show which atoms of the inactivator remain bound to monoamine oxidase (MAO) after inactivation. Organic chemical reactions were employed to elucidate the structure of the enzyme adduct and clarify the mechanism of inactivation. Following inactivation and dialysis, the benzyl substituent is lost, but the methyl group and cyclopropyl carbons remain attached to the enzyme even after further dialysis against solutions containing 1 mM benzylamine or 8 M urea. Treatment of inactivated enzyme with sodium cyanoborohydride prior to dialysis results in the retention of the benzyl group, suggesting an imine linkage. One hydride from sodium boro[$^3$H]hydride is incorporated into the dialyzed inactivated enzyme consistent with a ketone functional group. When Pronase-digested N-(1-Me)CBA-inactivated MAO is treated with basic potassium triiodide, iodoform is isolated, indicating the presence of a methyl ketone. During inactivation, the optical spectrum of the covalently bound active site flavin changes from that of oxidized to reduced flavin. After urea denaturation, the flavin remains reduced, suggesting covalent linkage of the inactivator to the cofactor. On the basis of previous results [Silverman, R. B., Hoffman, S. J., & Catus, W. B., III (1980) *J. Am. Chem. Soc. 102*, 7126-7128], it is proposed that the mechanism of inactivation involves transfer of one electron from N-(1-Me)CBA to the flavin, resulting in an amine radical cation and a flavin radical. Then, either the cyclopropyl ring is attacked by the flavin radical or the cyclopropyl ring opens, and the radical generated is captured by the flavin radical. The product of this mechanism is the imine of benzylamine and 4-flavinyl-2-butanone, the proposed enzyme–inactivator adduct.

Mitochondrial monoamine oxidase (MAO,[1] EC 1.4.3.4), an enzyme containing covalently bound FAD, is one of the enzymes responsible for the catabolism of biogenic amines. Compounds that inhibit MAO are used clinically as antidepressant agents (Baldessarini, 1977; Berger & Barchas, 1977; Tyrer, 1976); many of the known inhibitors of MAO have been shown to be mechanism-based inhibitors (Maycock et al., 1976; Krantz & Lipkowitz, 1977; Kenney et al., 1979; Silverman & Hoffman, 1980). A mechanism-based inhibitor is an unreactive compound that is converted by an enzyme via its

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[1] Abbreviations: MAO, monoamine oxidase; N-CBA, *N*-cyclopropylbenzylamine; N-(1-Me)CBA, *N*-(1-methylcyclopropyl)benzylamine; FAD, flavin adenine dinucleotide; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)-aminomethane.

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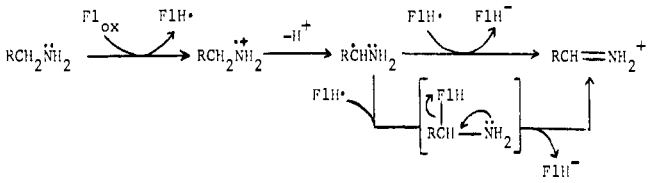
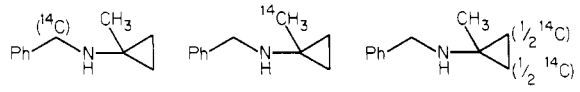
Scheme I: Mechanism Suggested for MAO-Catalyzed Amine Oxidation



catalytic mechanism to a species that produces inhibition of that enzyme without prior release from the active site (Rando, 1974; Abeles & Maycock, 1976; Walsh, 1982; Silverman & Hoffman, 1984). Previously, we showed that the *N*-cyclopropyl-*N*-arylalkylamine class of MAO inhibitors were mechanism based and that an adduct labile to denaturation and to benzylamine treatment was produced (Silverman & Hoffman, 1980). By use of radioactively labeled N-CBA, it was suggested that the mechanism of amine oxidation catalyzed by MAO was a two one-electron transfer process in which a nitrogen radical cation was initially generated by transfer of an amine nonbonded electron to the flavin cofactor (Scheme I) (Silverman et al., 1980). More recently, it was found that substitution of the C-1 proton of N-CBA by a methyl group also produced time-dependent inactivation of MAO; however, unlike N-CBA, N-(1-Me)CBA inactivation of MAO was not reversed by benzylamine treatment (Silverman & Hoffman, 1981a). The inability of benzylamine to cause reactivation after treatment of MAO by N-(1-Me)CBA suggested the formation of a stable adduct with the enzyme. Two possible pathways were suggested (Silverman & Hoffman, 1981a) and are shown in Scheme II. Recently, it was found that the product formed upon acid treatment of *trans*-2-phenylcyclopropylamine-inactivated MAO was cinnamaldehyde, and this was rationalized by a mechanism involving a one-electron transfer followed by cyclopropyl ring opening (Silverman, 1983).

In order to elucidate the mechanism of inactivation of MAO by N-(1-Me)CBA and to characterize the enzyme–inactivator adduct, three different radioactively labeled N-(1-Me)CBA's were synthesized (**3a–c**). Inactivation of MAO by each of



**3a** **3b** **3c**

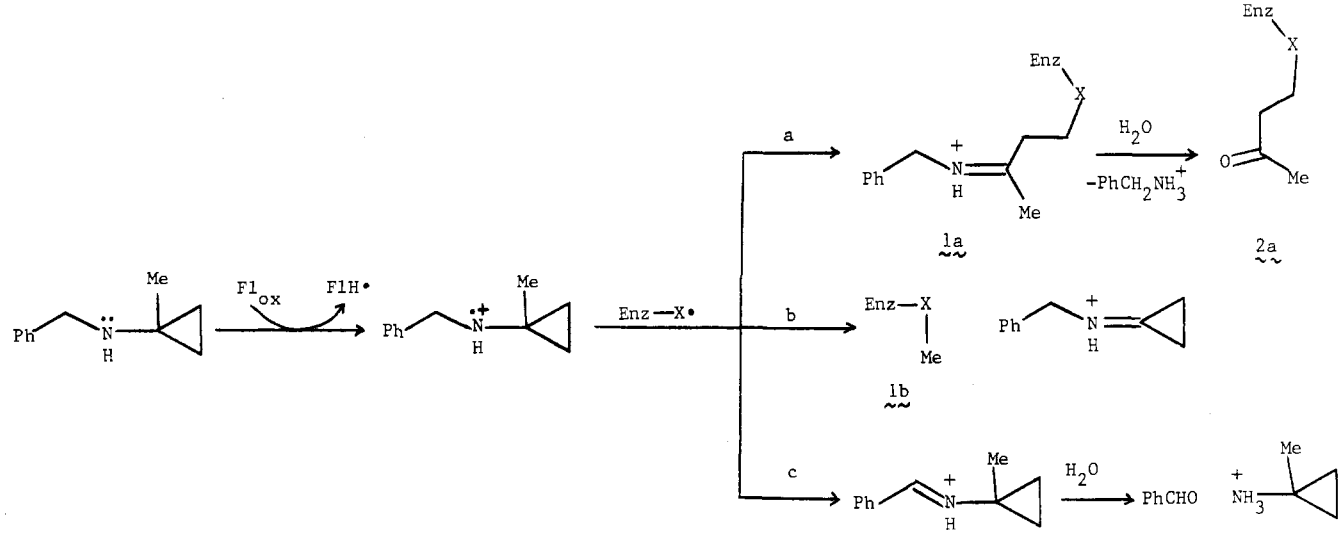
these established which atoms remained attached to the enzyme after inactivation. Further chemistry on the enzyme–inactivator adduct assisted in the elucidation of its structure and clarified the mechanism of inactivation. Most consistent with the data presented here is pathway a of Scheme II.

## Materials and Methods

*Analytical Methods.* Radioactivity was measured with a Beckman LS-3133T scintillation counter in 10 mL of 3a70B scintillation fluid from Research Products International. [$^{14}$C]Toluene ($4.7 \times 10^5$ dpm/mL) or [$^3$H]toluene ($1.68 \times 10^6$ dpm/mL, corrected for first-order decay), obtained from New England Nuclear, was used as internal standard. Optical spectra were recorded on a Beckman ACTA CIII spectrophotometer in quartz semimicrocuvettes. Melting points were obtained on a Fisher-Johns melting point apparatus and are uncorrected. NMR spectra were recorded on a Varian EM-390 spectrometer. All chemical shifts are expressed as parts per million ($\delta$) downfield from tetramethylsilane ($CDCl_3$ as solvent) or sodium 4,4-dimethyl-4-silapentane-1-sulfonate ($D_2O$ as solvent). Descending paper chromatography was performed on Whatman 3MM chromatography paper with 1-butanol–$H_2O$ (80:20, top layer) as the mobile phase. TLC was performed on silica gel 60 coated plastic plates (Merck) with 1-butanol–acetic acid–$H_2O$ (12:3:5) as the mobile phase unless indicated otherwise. Elemental analyses were performed by Micro-Tech Laboratories, Inc., Skokie, IL.

*Reagents.* N-(1-Me)CBA·HCl was synthesized as previously described (Silverman & Hoffman, 1981a,b). 1-Methylcyclopropylamine hydrochloride, an intermediate in the synthesis of N-(1-Me)CBA, was recrystallized from ethyl acetate to give shiny white needles: mp 219–219.5 °C; NMR ($D_2O$) $\delta$ 0.79 (m, 2 H), 0.95 (m, 2 H), 1.45 (s, 3 H). *N*-Methylnitrosourea was prepared by the procedure of Arndt (1943). FAD was obtained from Sigma, urea was purchased from Mallinckrodt, and benzaldehyde, triethylamine, benzylamine cyanocyclopropane, and *n*-butyllithium were bought from Aldrich. The benzylamine was converted to its hydrochloride salt by bubbling HCl through an ethereal solution of the amine, and the product was recrystallized from ethanol. [7-$^{14}$C]Benzyl alcohol was a product of Pathfinder Laboratories, Inc. (St. Louis, MO), iodo[$^{14}$C]methane and sodium boro[$^3$H]hydride came from ICN Radiochemical Division (Irvine, CA), and *N*-[$^{14}$C]methylnitrosourea was bought from New England Nuclear (Boston, MA). [7-$^{14}$C]Pargyline was a gift of Prof. Roy McCauley (Wayne State University).

Scheme II: Pathways for Reaction of MAO with N-(1-Me)CBA



*N-(1-Me)C[7-$^{14}$C]BA·HCl* (**3a·HCl**). [7-$^{14}$C]Benzyl alcohol (0.25 mCi, 0.03 mmol) was diluted with nonradioactive benzyl alcohol (48 μL, 0.47 mmol) and oxidized by the method of Ratcliffe & Rodehorst (1970) to give [7-$^{14}$C]benzaldehyde (66% yield). The ethereal filtrate containing the crude [7-$^{14}$C]benzaldehyde was washed with 0.2 N NaOH (2.5 mL), followed by 1 N HCl (2.5 mL), dried ($CaSO_4$), filtered, and rotary evaporated under reduced pressure to remove the solvent. The [7-$^{14}$C]benzaldehyde (35 mg, 0.33 mmol) and 1-methylcyclopropylamine hydrochloride (36 mg, 0.33 mmol) were used to synthesize the HCl salt of **3a** by the method reported (Silverman & Hoffman, 1981b) for the synthesis of *N*-(1-deuteriocyclopropyl)benzylamine hydrochloride. The crude product was recrystallized from dichloromethane–ethyl acetate to give 23 mg (23% overall) of shiny white needles (sp act. 0.778 mCi/mmol), which proved to be radiopure as well as chemically pure by TLC and paper chromatography; all of the radioactivity comigrated with carrier N-(1-Me)-CBA·HCl.

*N-(1-[$^{14}$C]Me)CBA·HCl* (**3b·HCl**). To a stirred mixture of dry, distilled diisopropylamine (265 μL, 1.9 mmol) and dry, distilled tetrahydrofuran (2.0 mL) under argon at −78 °C was added dropwise, by syringe, 2.6 M *n*-butyllithium in hexane (0.73 mL, 1.9 mmol). The solution was stirred for 25 min, followed by the dropwise addition of distilled cyanocyclopropane (132 μL, 1.8 mmol). After a 5-min stirring at −78 °C, 800 μCi (2.84 mg, 0.02 mmol) of iodo[$^{14}$C]methane diluted with nonradioactive iodomethane (125 μL, 2.0 mmol) in dry, distilled tetrahydrofuran (400 μL) was added by syringe. The reaction mixture was stirred at −78 °C for 5 min, allowed to warm to room temperature over a 1-h period, and then poured into 25 mL of diethyl ether and washed with 2 M sodium bisulfite (3 mL). The aqueous wash was extracted with four 2-mL portions of ether, and the combined ether extracts were concentrated under reduced pressure to a yellow liquid that was refluxed under argon for 13 h in 3.0 mL of 6 N HCl, cooled to room temperature, and extracted with four 5-mL portions of ether. The combined ether extracts were washed with 0.1 N HCl (4 mL), dried ($CaSO_4$), filtered, and rotary evaporated to give 100 mg of a yellow oil that was dissolved in a mixture of chloroform (4 mL) and concentrated sulfuric acid (750 μL). The stirred mixture was immersed in an oil bath at 45 °C, and then, sodium azide (190 mg, 2.93 mmol) was added. After a 90-min stirring at 45 °C, 5 mL of ice–water was added, the mixture was stirred vigorously, and the two phases were separated. The organic layer was extracted twice with 5-mL portions of water; 20 mL of ether was added on the second aqueous extraction step. The combined aqueous layers were washed with three 10-mL portions of ether, then basified with 4 N NaOH (10 mL), and extracted with four 15-mL portions of ether. The combined ether extracts were washed twice with 3 mL of water, and the combined aqueous washes were back-extracted with 15 mL of ether. The combined ether extracts were extracted with three 2-mL portions of 1 N HCl and the combined aqueous layers were rotary evaporated in vacuo to dryness. The resulting solid was redissolved in 1 mL of water and reevaporated to give 66 mg of crude 1-[$^{14}$C]methylcyclopropylamine hydrochloride, which was converted into **3b**·HCl by the method used for preparing **3a**·HCl (vide supra). Recrystallization of the crude product from dichloromethane–ethyl acetate gave 30 mg (8.4% overall) of shiny white needles (sp act. 0.257 mCi/mmol). The recrystallized product was radiopure and chemically pure by TLC, and all of the radioactivity comigrated with carrier N-(1-Me)CBA·HCl.

*N-(1-Me)[2-$^{14}$C]CBA·HCl* (**3c·HCl**). *N*-Methylnitrosourea (103 mg, 1.0 mmol) and *N*-[$^{14}$C]methylnitrosourea (0.1 mCi, 15 mCi/mmol) were dissolved in dichloromethane (11 mL), and the solvent was evaporated. The resulting solid was added portionwise to a rapidly stirred mixture of 40% KOH (0.75 mL) and ether (3.0 mL) in an ice bath, and after a 10-min stirring, the yellow ether layer was separated. The aqueous layer was extracted with 1.0 mL and then 0.25 mL of ether, and the combined ether extracts containing diazo[$^{14}$C]methane were dried for 4 h over KOH in the refrigerator. Zinc chloride (68 mg, 0.5 mmol), which was heated with a Bunsen burner to the melting point under vacuum and then allowed to cool under argon, was dissolved in dry ether (1.0 mL). After being cooled in an ice bath under argon, methallyl alcohol (23 μL, 0.25 mmol) was added followed by the dried ethereal solution of diazo[$^{14}$C]methane. After being stirred for 3 h under argon at room temperature, the mixture was cooled in an ice bath, and 2.0 mL of saturated $NH_4Cl$ was added. The ether layer was separated, and the aqueous layer was extracted with five 5-mL portions of ether. The combined ether layers were washed with 2.0 mL of water, and the aqueous wash was extracted with 5 mL of ether. The ether extracts were combined, and the ether was evaporated on a steam bath. The residue was taken up in 1.0 mL of acetone and cooled in an ice bath, and Jones reagent (0.19 mL, 0.5 mmol) (Bowden et al., 1946) was added dropwise with stirring. After being stirred in an ice bath for 10 min and at room temperature for 20 min, the reaction mixture was cooled in an ice bath, and $H_2O$ (2.0 mL) was added. The mixture was extracted with ether (2 times 5 mL, 4 times 10 mL), and the combined ether extracts were washed with $H_2O$ (2.0 mL) and brine (2.0 mL) and dried ($MgSO_4$). After filtration, the ether was removed on a steam bath and then by rotary evaporation to give a colorless oil that was dissolved in chloroform (1.0 mL), filtered, and evaporated to 1-methyl[2-$^{14}$C]cyclopropanecarboxylic acid (15 mg) as a colorless oil. This carboxylic acid was converted to the corresponding amine hydrochloride (7 mg) by the method described above for 1-[$^{14}$C]methylcyclopropanecarboxylic acid and then to **3c**·HCl (6.2 mg after ethyl acetate recrystallization) as described above for **3b**·HCl. The product was radiopure and chemically pure by TLC (sp act. 0.076 mCi/mmol), and all of the radioactivity comigrated with carrier N-(1-Me)CBA.

*N-(3-Oxobutyl)piperidine Hydrochloride.* This compound was prepared by a modified method of Swaminathan & Newman (1958) for the synthesis of 1-(diethylamino)-3-butanone. To a stirred mixture of piperidine (1.98 mL, 20 mmol) and glacial acetic acid (45 μL, 0.79 mmol) under argon at 0 °C was added dropwise methyl vinyl ketone (1.62 mL, 20 mmol). The reaction mixture was stirred for 10 min at 0 °C and at room temperature for 3 h and then was poured into 50 mL of ether and washed successively with 0.1 N NaOH (10 mL), brine (10 mL), and water (10 mL). The ether layer was dried over $CaSO_4$ and filtered, and gaseous HCl was bubbled in. The precipitate was filtered, washed with anhydrous ether, and recrystallized from dichloromethane–ethyl acetate to give fine white platelets: mp 189–190 °C dec; NMR ($D_2O$) δ 1.78 (br m, 6 H), 2.25 (s, 3 H), 3.2 (br m, 8 H). Anal. Calcd for $C_9H_{18}ClNO$: C, 56.38; H, 9.48; N, 7.31; Cl, 18.49. Found: C, 55.99; H, 9.32; N, 7.25; Cl, 18.28.

*N-(3-Hydroxybutyl)piperidine Hydrochloride.* A solution of sodium borohydride (76 mg, 0.52 mmol) in 0.1 N NaOH (200 μL) was added dropwise with stirring to a mixture of *N*-(3-oxobutyl)piperidine hydrochloride (50 mg, 0.26 mmol), 0.2 N sodium borate, pH 9.5 (2.0 mL), and 0.1 N NaOH (2.0

mL). After being stirred at 25 °C for 12 h, 1 N NaOH (2.0 mL) was added, and the mixture was extracted with four 6-mL portions of ether. The combined ethereal extracts were washed with 1 N NaOH (3 mL) and then with water (3.0 mL). The combined aqueous washes were back-extracted with ether (5 mL), and the combined ether layers were dried over $CaSO_4$. After filtration, gaseous HCl was bubbled in, and the solvent was evaporated. The crude product was recrystallized from dichloromethane–ethyl acetate to give fine white needles: mp 149–150 °C; NMR ($D_2O$) $\delta$ 1.22 (d, 3 H), 1.8 (br m, 8 H), 2.6–4.1 (br m, 7 H).

*N-Methyl-N-(1-methylcyclopropyl)benzylamine Hydrochloride.* The method of Horrom & Martin (1963) was used to methylate N-(1-Me)CBA. To a solution of N-(1-Me)-CBA·HCl (105 mg, 0.53 mmol) in chloroform (3 mL) under argon was syringed triethylamine (148 $\mu$L, 1.06 mmol) and then ethyl chloroformate (51 $\mu$L, 0.53 mmol). After a 2-h stirring at room temperature, the solvent was evaporated. The white residue was taken up in ether (10 mL) and 0.1 N HCl (10 mL). The ether layer was separated, washed with 10 mL each of 0.1 N HCl, water, and brine, and dried ($MgSO_4$). The solvent was evaporated to a colorless liquid (107 mg), which was dissolved in 1 mL of dry ether and was syringed into a suspension of lithium aluminum hydride (26 mg, 0.68 mmol) in dry ether (5 mL) under argon. After being refluxed for 10 h, the excess lithium aluminum hydride was quenched with ethyl acetate, and then water and ether (10 mL each) were added. The ether layer was separated and washed with water (3 times 5 mL). The combined aqueous washes were back-extracted with ether, and then all of the ether extracts were combined and extracted with 1 N HCl (2 times 3 mL). The aqueous extracts were combined, and the solvent was evaporated to a colorless oil that was dissolved in water and re-evaporated. The resulting oil was crystallized and recrystallized from ethyl acetate–hexane to give the product as white crystals (29 mg): mp 131.5–133 °C; NMR ($CDCl_3$) $\delta$ 0.55 (s, 2 H), 1.2–2.0 (br m, 2 H), 1.43 (s, 3 H), 2.74 (s, 3 H), 4.21 (s, 2 H), 7.35 (m, 3 H), 7.67 (m, 2 H), 11.9 (br s, 1 H). Anal. Calcd for $C_{12}H_{18}ClN$: C, 68.07; H, 8.57; N, 6.62. Found: C, 67.99; H, 8.63; N, 6.59.

*Enzymes and Assays.* Mitochondrial MAO was purified from both bovine and porcine liver, obtained fresh from a local abattoir and kept on ice for an hour before processing by the procedure of Salach (1979). The specific activities of the purified enzymes varied from preparation to preparation and were generally in the range of 2.8–4.0 units/mg of protein. The enzyme was assayed by a modification of the method of Tabor et al. (1954) with 1 mM benzylamine in 20 mM Tris-HCl, pH 9.0 buffer at 30 °C. A unit of activity is defined as the amount of enzyme required to convert 1 $\mu$mol of benzylamine to benzaldehyde per minute at 30 °C. Pronase (Protease, type XIV), a nonspecific protease from *S. griseus*, was purchased from Sigma.

*Inactivation of MAO by N-(1-Me)CBA.* In a typical preparation of N-(1-Me)CBA-inactivated enzyme, 5 mM inactivator in 100 mM Tris-HCl, pH 9.0 (375 $\mu$L), was added to 160 $\mu$M MAO (porcine or bovine) in 50 mM potassium phosphate, pH 7.2 (250 $\mu$L), and the reaction mixture was incubated at 25 °C in the dark for 2 h. An inactivator control containing no enzyme and an enzyme control containing no inactivator also were carried out. Each mixture was microdialyzed against two changes (2.5 mL each) of 20 mM potassium phosphate, pH 7.0, at room temperature over a 3-h period, followed by exhaustive dialysis against three changes (200 mL each) of the same buffer. The combined outer portions from the microdialyses were stored frozen for further analysis as described (vide infra). The efficiency of microdialysis was typically greater than 90%. For kinetic studies, MAO was inactivated by the method described previously (Silverman & Hoffman, 1981a).

*Stoichiometry of Inactivator Binding and Effect of Urea and Benzylamine Treatment.* MAO (porcine or bovine) inactivated with radiolabeled N-(1-Me)CBA was prepared as described above. Aliquots were removed for MAO activity assays, scintillation counting, and for further dialysis against 300 mL of 8 M urea in 20 mM potassium phosphate, pH 7.0, or 1 mM benzylamine in 20 mM potassium phosphate, pH 7.0, for 14 h at room temperature in the dark. The samples, dialyzed against urea or benzylamine, were counted, and from the latter, aliquots were assayed for MAO activity. The original amount of active MAO was determined by titration with [$^{14}$C]pargyline (Chuang et al., 1974). Protein concentration was determined by the method of Lowry et al. (1951). Calculated stoichiometries were corrected for any remaining MAO activity and are expressed as moles of radiolabeled bound per mole of inactivated MAO.

*Identification and Quantitation of Nonbound Oxidation Products.* The outer portions of the microdialyses of MAO (porcine or bovine) inactivated by radiolabeled N-(1-Me)CBA (described above) were applied to Dowex 50X-8 (200–400 mesh; $H^+$ form) columns (0.5 × 8.5 cm) equilibrated with deionized water. The columns were eluted initially with deionized water (28 mL) and then with 1.5 N HCl at a flow rate of 1.2 mL/min. Fractions of 3 mL were collected, and 1-mL aliquots were removed from each fraction for counting. The same procedure was performed with the outer portions of microdialyses from the nonenzyme controls.

(*A*) *Non-Amine Oxidation Products.* When **3a** was used as the inactivator, the radioactive pool eluted from the Dowex 50 with deionized water was divided into two equal batches. The first batch was extracted with four 5-mL portions of ether, and the combined ether extracts were concentrated under reduced pressure (little or no radioactivity remained in the aqueous layer). The residue was analyzed by TLC with 1:1 ethyl acetate–*n*-hexane as the mobile phase. The developed chromatograms were cut into 5-mm fractions and counted. Carrier compounds were cochromatographed to allow identification of radioactive peaks. To the second batch was added 5 $\mu$L of 2,4-dinitrophenylhydrazine reagent (Shriner et al., 1964); the reaction mixture was allowed to stand in the dark at room temperature for 24 h and then was extracted with four 5-mL portions of ethyl acetate. The combined extracts were evaporated, and the residue was analyzed by TLC as described above.

(*B*) *Amine Oxidation Products.* Fractions containing radioactivity eluted with 1.5 N HCl were pooled according to their respective peaks on the radioactivity–elution profile. Each pool was rotary evaporated to dryness under reduced pressure, redissolved in less than 100 $\mu$L of water, and then analyzed by descending paper chromatography. After development, the chromatogram was cut into 1-cm fractions and counted. Some samples were analyzed by TLC, in which case the developed chromatograms were analyzed as described above. The ratio of benzyl side oxidation to cyclopropyl side oxidation (partition ratio) was determined from the amount of radioactive 1-methylcyclopropylamine produced and the amount of radiolabel bound to the enzyme.

*Sodium Cyanoborohydride Treatment of N-(1-Me)C[7-$^{14}$C]BA-Inactivated MAO.* To 75 $\mu$L of 5 mM **3a**·HCl in 100 mM Tris-HCl, pH 9.0, was added 50 $\mu$L of 160 $\mu$M MAO

(porcine or bovine) in 50 mM potassium phosphate, pH 7.2. The mixture was incubated at 23 °C in the dark for 2 h after which time the enzyme was found completely inactive. To a 50-μL aliquot of reaction mixture was added 1 mg of sodium cyanoborohydride in 10 μL of 0.2 N sodium borate, pH 9.5. The solution was incubated in the dark for 15 h at room temperature, diluted one-to-one with 8 M urea in 20 mM potassium phosphate, pH 7.0, and dialyzed exhaustively against three changes (150 mL each) of the same buffer medium. The remainder of the original reaction mixture, containing no added sodium cyanoborohydride, was dialyzed exhaustively against three changes (200 mL each) of 20 mM potassium phosphate, pH 7.0. Aliquots from the dialyzed samples were then removed for scintillation counting and normalized by Lowry assay (Lowry et al., 1951). A control was performed as above by starting with MAO that had been inactivated completely with nonradioactive pargyline (Hellerman & Erwin, 1968; Chuang et al., 1974).

*Treatment of N-(1-Me)CBA-Inactivated MAO with Sodium Boro[$^3H$]hydride.* Nonradioactive N-(1-Me)CBA-inactivated bovine MAO and an enzyme control, containing no inactivator, were prepared as described above [see Inactivation of MAO by N-(1-Me)CBA]. The inactivated enzyme was assayed for remaining MAO activity, and 100-μL aliquots of control and inactivated enzyme solutions were diluted with 100 μL of 8 M urea in 0.2 N sodium borate, pH 9.5. To each was added 1 μL of 0.1 M [$^3$H]$NaBH_4$ (diluted to sp act. 3.5 mCi/mmol with nonradioactive $NaBH_4$) in 0.1 N NaOH. The reaction mixtures were allowed to stand in the dark at room temperature for 10 h before neutralization with 1 N HCl (10 μL). The solutions were then dialyzed exhaustively against four changes (150 mL each) of 8 M urea in 20 mM potassium phosphate, pH 7.0. Aliquots were counted and normalized by the Lowry assay (Lowry et al., 1951).

*Reaction of Sodium Boro[$^3H$]hydride with N-(3-Oxobutyl)piperidine.* One milliliter of the same [$^3$H]$NaBH_4$ solution used above was added to 9 mg (47 μmol) of crystalline *N*-(3-oxobutyl)piperidine hydrochloride. After being stirred at room temperature for 13 h, 1 drop of 1 N NaOH was added. The reaction mixture was extracted with five 2-mL portions of ether, the combined ethereal extracts were washed with 1 N NaOH (1 mL), followed by water (1 mL), and the combined aqueous washes were back-extracted with ether (2 mL). The combined ether extracts were dried over $CaSO_4$ and filtered, and gaseous HCl was bubbled in. The ether was removed by evaporation, and the residue was recrystallized from dichloromethane–ethyl acetate to give fine white needles: mp 149–150 °C (sp act. 2.14 mCi/mmol). The recrystallized product was radiopure and chemically pure by TLC, and all of the radioactivity comigrated with carrier *N*-(3-hydroxybutyl)piperidine hydrochloride.

*Treatment of FAD with Sodium Boro[$^3H$]hydride.* To 1.0 mL of 1.0 mM FAD in 0.2 N sodium borate, pH 9.5, was added 0.1 mL of 0.1 M [$^3$H]$NaBH_4$ (sp act. 350 mCi/mmol) in 0.1 N NaOH. The reaction mixture was incubated at room temperature in the dark for 10 h and quenched with 1.0 mL of acetone. After a 15-min standing in the dark, the solution was rotary evaporated to dryness in vacuo. The residue was dissolved in 1.0 mL of 1 N HCl 3 times and rotary evaporated to dryness 3 times. The residue was then dissolved in 1.0 mL of $H_2O$ and rotary evaporated to dryness after a 10-μL aliquot was counted. Repeated wash cycles with water resulted in no further change in radioactivity.

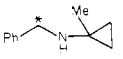
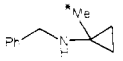
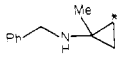
*Iodoform Reaction on N-(1-[$^{14}C$]Me)CBA-Inactivated MAO.* Bovine MAO inactivated with **3b**·HCl was prepared as described above [see Inactivation of MAO by N-(1-Me)-CBA]. Aliquots were removed for scintillation counting, and a 330-μL aliquot was diluted one-to-one with 8% trichloroacetic acid. The denatured protein was pelleted by centrifugation for 1 min in a Beckman Microfuge B, redispersed in 500 μL of 5% trichloroacetic acid, and pelleted in the same manner. The pellet was resuspended in 200 μL of methanol, diluted with 400 μL of chloroform, and pelleted by centrifugation. The pellet was washed successively with methanol (600 μL) and 20 mM potassium phosphate, pH 7.9 (600 μL). All supernatants were placed into scintillation vials and counted. The washed protein was dispersed in 500 μL of 20 mM potassium phosphate, pH 7.9, and digested at 37 °C for 12 h with 1 mg of Pronase. The digest mixture was diluted with 500 μL of 1 N NaOH, and the solution was divided into two equal batches. One batch (the control) was extracted with three 1-mL portions of dichloromethane after 90 min of standing at room temperature. To the second batch was added dropwise with stirring potassium triiodide solution (made by dissolving 0.25 g of potassium iodide and 0.125 g of iodine in 1.0 mL of distilled water) until a reddish color persisted for at least 5 min. The reaction mixture was stirred at 30 °C for 1 h, triiodide solution being added when the color dissipated. The excess iodine was destroyed by addition of 3 drops of 1 N NaOH, and the solution was stirred for another 30 min. The reaction mixture was extracted with three 1-mL portions of dichloromethane. The two dichloromethane extracts (from this and the control) were concentrated to ~50 μL by evaporation of the solvent with gentle heating. The residues were analyzed by TLC with *n*-pentane as the mobile phase. The developed chromatograms were cut into 5-mm fractions and counted.

*Release of Radioactivity from N-(1-[$^{14}C$]Me)CBA-Inactivated MAO with Base.* Porcine MAO inactivated with **3b**·HCl [see Inactivation of MAO by N-(1-Me)CBA] was diluted one-to-one with 8 M urea and further dialyzed against 8 M urea (300 mL) for 15 h at room temperature. A portion (720 μL) of the inactivated enzyme (the control) was incubated with 2 mg of $NaBH_4$ in 80 μL of 0.2 N sodium borate, pH 9.5, at room temperature for 1 h and dialyzed exhaustively against 8 M urea (200 mL). Aliquots of the enzyme with and without $NaBH_4$ treatment were counted and normalized (Lowry et al., 1951). The $NaBH_4$-treated enzyme (control) and enzyme not treated with $NaBH_4$ (540 μL each) were made basic with 5 N NaOH (60 μL each) and incubated at 37 °C. At various time intervals, aliquots (200 μL) were neutralized with 5 N HCl (22 μL), dialyzed exhaustively against 8 M urea (200 mL) at room temperature, and then counted and normalized (Lowry et al., 1951).

## Results

*Inactivation of MAO by N-(1-Me)CBA and Effect of β-Mercaptoethanol.* No lag time for inactivation of MAO by N-(1-Me)CBA was observed. A second aliquot of MAO was then added to this solution; the rate of inactivation was identical with that for the first aliquot of MAO. Addition of β-mercaptoethanol to the inactivation solution had a profound inhibitory effect on the rate of inactivation of MAO by N-(1-Me)CBA. However, β-mercaptoethanol was shown to be a potent reversible inhibitor of MAO. No enzyme activity was observed in the presence of 5 mM β-mercaptoethanol and 1 mM benzylamine (55 and 27% activity was observed with 0.5 mM and 1.0 mM β-mercaptoethanol, respectively). Upon dilution or dialysis against 20 mM Tris-HCl, pH 9.0, buffer, full enzyme activity was restored. Incubation of N-(1-Me)-CBA-inactivated MAO with 10 mM β-mercaptoethanol in

Table I: Stoichiometry of Incorporation of Radioactivity into MAO after Treatment with $^{14}$C-Labeled N-(1-Me)CBA's[a]

| inactivator | after dialysis[b] | after benzylamine treatment | after urea treatment | partition ratio | % return of MAO activity after $PhCH_2NH_2$ dialysis |
|---|---|---|---|---|---|
|  | 0.082[c]<br>0.088[d] | 0.040[c]<br>0.043[d] | 0.050[c]<br>0.037[d] | | 8[c]<br>4[d] |
|  | 1.31[c]<br>1.46[d] | 1.08[c]<br>1.34[d] | 1.01[c]<br>1.45[d] | 0.15[c]<br>0.18[d] | 3[c]<br>1[d] |
|  | 1.36[c]<br>1.32[d] | 1.36[c]<br>1.31[d] | 1.11[c]<br>1.13[d] | 0.10[c]<br>0.22[d] | 5[c]<br>6[d] |

[a] The procedure is described under Materials and Methods. The stoichiometry is the ratio of moles of radioactivity incorporated to mole of MAO and is relative to the ratio of moles of [7-$^{14}$C]pargyline incorporated per mole of MAO, which was given the value 1.0. [b] Gel filtration gave the same results. [c] Porcine liver MAO was used. [d] Bovine liver MAO was used.

Tris-HCl, pH 9.0, buffer at 25 °C did not restore enzyme activity even after 5.5 h.

*Stoichiometry of Inactivation of MAO by $^{14}$C-Labeled N-(1-Me)CBA's.* Each of the three labeled inactivators (**3a–c**) was used to inactivate both porcine and bovine liver MAO. The stoichiometry of radiolabel incorporation after exhaustive dialysis was determined and then redetermined following further dialysis in the presence of either 1 mM benzylamine or 8 M urea (Table I). After inactivation and dialysis, the benzyl methylene carbon did not remain bound to the enzyme (little radioactivity was found attached to MAO when **3a** was used). However, both **3b** and **3c** resulted in the incorporation of approximately 1 equiv of radioactivity. Neither benzylamine treatment nor urea denaturation following inactivation had a significant effect on the stoichiometry of inactivation. As seen in Table I, once inactivation with labeled N-(1-Me)CBA had occurred, very little MAO activity was regenerated upon treatment with benzylamine in agreement with Silverman & Hoffman (1981a).

*Identification and Quantitation of Radioactive 1-Methylcyclopropylamine from Inactivation of MAO by* **3b** *and* **3c**: *Partition Ratio.* Figure 1A shows the Dowex 50 elution profile of the outer portion from the microdialysis after MAO inactivation by **3b**. The peak at elution volume 30–39 mL corresponds to the fractions in which 1-methylcyclopropylamine hydrochloride eluted. TLC (Figure 1B) confirmed the identity of the radioactive material as 1-methylcyclopropylamine hydrochloride. The same treatment of the inactivator control, containing no MAO, did not produce any radioactive 1-methylcyclopropylamine hydrochloride. Similar results were found with **3c**. A ratio of oxidation of the benzyl methylene carbon to enzyme labeling (partition ratio) was calculated on the basis of the amount of radioactive 1-methylcyclopropylamine hydrochloride isolated relative to the amount of radioactivity bound to the enzyme after the urea treatment. The results for both porcine and bovine MAO are shown in Table I.

*Release of Radioactive Benzylamine and Benzaldehyde after Inactivation of MAO by* **3a**. Figure 2A shows the Dowex 50 1.5 N HCl elution profile of the outer portion from the microdialysis after MAO inactivation by **3a**. The fractions that were expected to contain any benzylamine hydrochloride were pooled, concentrated, and chromatographed by descending paper chromatography. As shown in Figure 2B, radioactive benzylamine hydrochloride was a product of the inactivation. The radioactivity at elution volume 5–15 mL of the Dowex chromatogram (Figure 2A) was shown to be [7-$^{14}$C]benzaldehyde by TLC analysis of the isolated radioactive compound and of its 2,4-dinitrophenylhydrazone derivative. The sum total of radioactive benzylamine hydrochloride and benzaldehyde produced gave a molar ratio of approximately 1.6 when compared to the amount of MAO inactivated.

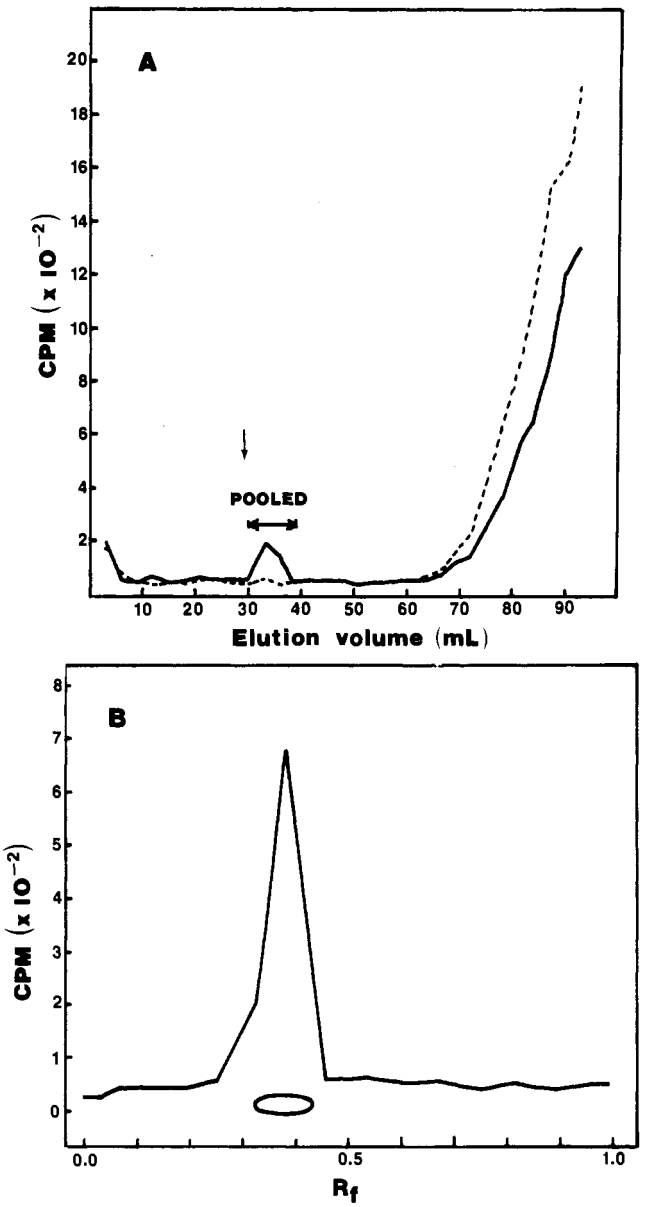



FIGURE 1: (A) Dowex 50 elution profile for the outer portion of the microdialysis following **3b** inactivation of MAO (solid line). The dotted line is the elution profile for the nonenzymatic control. The inverted arrow is the point at which water was replaced with 1.5 N HCl as the eluant. (B) TLC of pooled fractions from (A). The oval represents carrier 1-methylcyclopropylamine hydrochloride. See Materials and Methods.

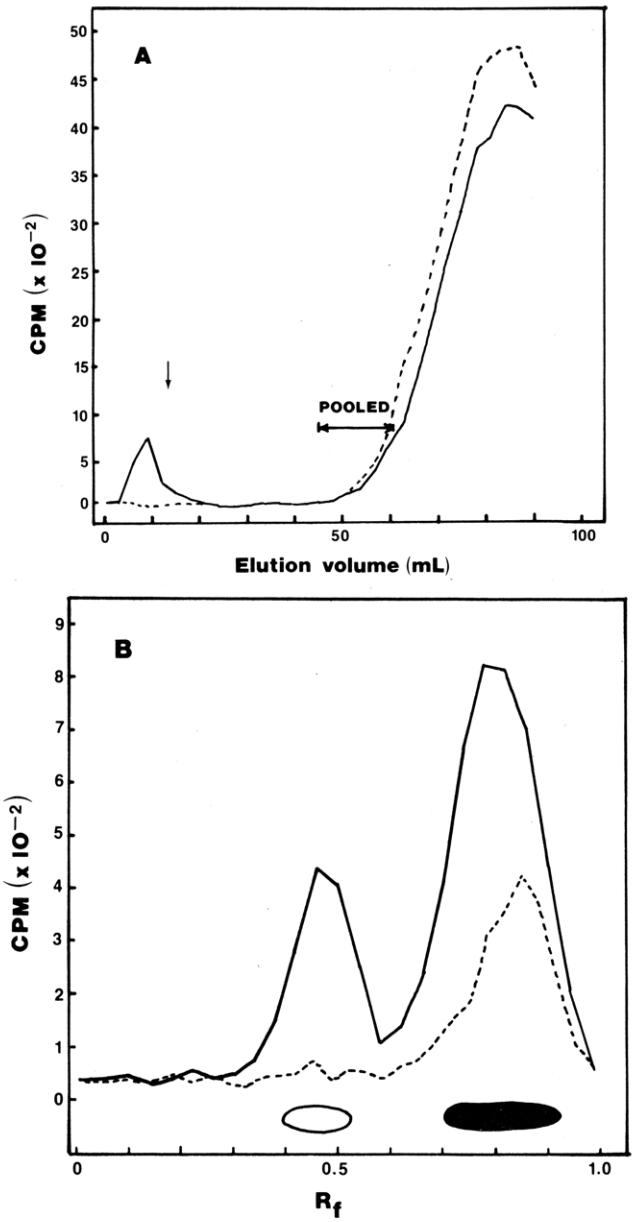



FIGURE 2: (A) Dowex 50 elution profile for the outer portion of the microdialysis following **3a** inactivation of MAO (solid line). The dotted line is the elution profile for the nonenzymatic control. The inverted arrow is the point at which water was replaced with 1.5 N HCl as the eluant. (B) Paper chromatography of pooled fractions from (A). The open oval represents carrier benzylamine hydrochloride; the solid oval represents carrier N-(1-Me)CBA·HCl. See Materials and Methods.

Bovine and porcine MAO gave virtually identical results.

*Sodium Cyanoborohydride Treatment of MAO Inactivated by* **3a**. In separate experiments, MAO inactivated with **3a** (but not dialyzed to remove excess inhibitor) was treated with sodium cyanoborohydride. A control, in which MAO had been inactivated by nonradioactive pargyline (Hellerman & Erwin, 1968; Chuang et al., 1974) before the addition of **3a**, was included. The stoichiometry of the amount of radiolabel bound to the amount of inactivated enzyme was determined, and the results for both porcine and bovine MAO are shown in Table II.

*Sodium Boro[$^3$H]hydride Treatment of N-(3-Oxobutyl)-piperidine, N-(1-Me)CBA-Inactivated MAO, and FAD.* The specific activity (2.14 mCi/mmol) of the *N*-(3-hydroxy[3-$^3$H]butyl)piperidine hydrochloride produced from the reduction of *N*-(3-oxobutyl)piperidine was 0.61 times that of the

Table II: Stoichiometry of Incorporation of Radioactivity into MAO Inactivated by **3a** with and without Sodium Cyanoborohydride Treatment[a]

| with $NaCNBH_3$ treatment | | without $NaCNBH_3$ treatment | |
|---|---|---|---|
| MAO + **3a** | pargyline–MAO + **3a** | MAO + **3a** | pargyline–MAO + **3a** |
| 2.13[b] | 0.56[b] | 0.050[b] | |
| 1.50[c] | 0.30[c] | 0.037[c] | 0.027[c] |

[a] The procedure is described under Materials and Methods. [b] Porcine liver MAO was used. [c] Bovine liver MAO was used.

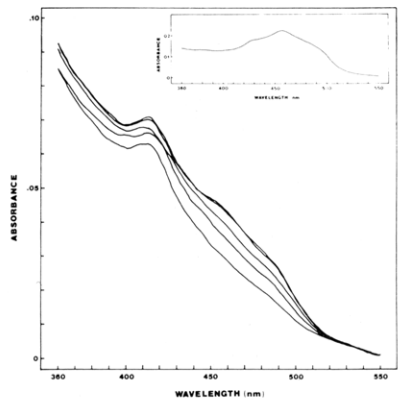



FIGURE 3: Change in absorption spectrum of MAO upon inactivation by N-(1-Me)CBA. Bovine liver MAO (2 $\mu$M) was incubated with 100 $\mu$M N-(1-Me)CBA in 100 mM Tris-HCl, pH 9.0, buffer. The five spectra tracings in order of decreasing $\epsilon$ value correspond to noninactivated enzyme (top trace) and enzyme activity at 100, 60, 25, and <1%, respectively. Addition of sodium dithionite (1 mg) or 8 M urea resulted in no significant change from the bottom trace. The inserted figure shows the difference spectrum of native enzyme vs. the N-(1-Me)CBA-inactivated enzyme (bottom trace in main spectrum).

[$^3$H]$NaBH_4$ used. This specific activity was used in calculations of the incorporation of tritium into noninactivated control MAO and N-(1-Me)CBA-inactivated MAO treated with [$^3$H]$NaBH_4$.

Treatment of denatured N-(1-Me)CBA-inactivated MAO with [$^3$H]$NaBH_4$ led to incorporation of 2.47 mol of tritium label/mol of active site compared with 1.16 mol of tritium label/mol of active site for the noninactivated control. A separate control reaction was carried out to compensate for the fact that the noninactivated MAO control contained oxidized FAD whereas the inactivated MAO contained reduced FAD (vide infra). Treatment of FAD with [$^3$H]$NaBH_4$ led to incorporation of 0.23 mol of tritium label/mol of FAD.

*Iodoform Reaction of N-(1-[$^{14}$C]Me)CBA-Inactivated MAO.* Dialyzed MAO inactivated by **3b** was denatured with trichloroacetic acid and washed to remove any nonspecific binding of inactivator. The amount of radioactivity removed proved to be small (13%). After treatment with basic potassium triiodide, 57% of the radioactivity was extracted into the organic phase, and 94% of this radioactivity comigrated with iodoform by TLC. The control MAO inactivated by **3b** but not treated with potassium triiodide gave no radioactive iodoform.

*Changes in the MAO Optical Spectrum during Inactivation by N-(1-Me)CBA.* Incubation of MAO with N-(1-Me)CBA produced a time-dependent change in the absorption spectrum measured at 360–550 nm (Figure 3). Addition of dithionite to the completely inactivated enzyme did not appreciably change the absorption spectrum. Similarly, no further change in the spectrum was seen after exposure of the inactivated

MAO to 6 M urea for 120 min (sufficient time to denature MAO).

*Release of Radioactivity from* **3b***-Inactivated MAO by Base.* The percent of radioactivity bound for the inactivated enzyme that was not treated with $NaBH_4$ was 46, 33, and 23% after a 1-, 6-, and 22-h incubation with 0.5 N NaOH; for the $NaBH_4$-treated inactivated enzyme, it was 70, 48, and 44% after the same incubation times. Sodium borohydride treatment removed only 3.5% of the total radioactivity prior to base treatment.

*Inactivation of MAO by N-Methyl-N-(1-methylcyclopropyl)benzylamine.* Incubation of MAO with different concentrations of *N*-methyl-*N*-(1-methylcyclopropyl)benzylamine gave pseudo-first-order rates of inactivation. From a plot of $1/[I]$ vs. $t_{1/2}$ (Kitz & Wilson, 1962), the $K_i$ for this compound was determined to be 1.75 mM, and the $k_{cat}$ was 0.0016 $s^{-1}$. Treatment of inactivated enzyme with 1 mM benzylamine resulted in return of only 3% of the enzyme activity.

## Discussion

Complementary results to our preliminary studies (Silverman & Hoffman, 1981a) were found to be in accordance with the criteria for mechanism-based inhibition (Silverman & Hoffman, 1984). It was found that following inactivation of MAO by N-(1-Me)CBA, a second aliquot of MAO was inactivated at the same rate as the first and no lag time of inactivation was observed for either aliquot. This suggests that N-(1-Me)CBA is not converted to a reactive species that escapes from the active site prior to inactivation. When inactivation was carried out in the presence of β-mercaptoethanol to test this hypothesis, a pronounced inhibitory effect on the inactivation by N-(1-Me)CBA was observed. The possibility that β-mercaptoethanol reactivated the N-(1-Me)CBA-inactivated MAO and thus produced the observed inhibitory effect was tested and ruled out. However, it was shown that β-mercaptoethanol was a potent reversible inhibitor of MAO at variance with a report of Rando & Eigner (1977). This is consistent with the work of others (Gascoigne & Radda, 1967; Yokoe & Bruice, 1975; Loechler & Hollocher, 1980a,b), who have shown that mercaptans reversibly react with flavins to form unstable adducts, which would reversibly inhibit the enzyme and also protect it from inactivation by N-(1-Me)-CBA.

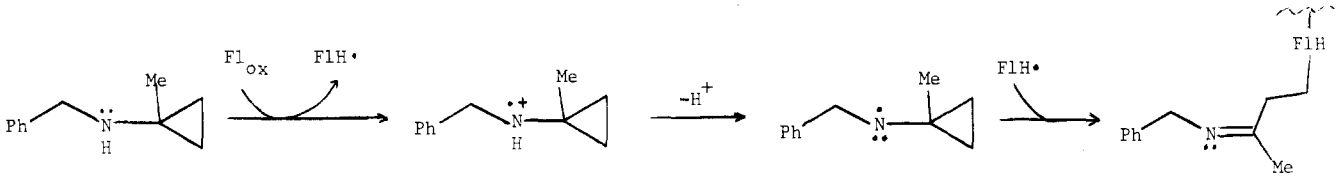
Compounds **3a–c** were designed to determine the stoichiometry of N-(1-Me)CBA binding to MAO. As shown in Table I (compound **3a**), the benzyl methylene carbon did not remain bound to the enzyme after exhaustive dialysis. However, both **3b** and **3c** resulted in the incorporation of approximately 1 equiv of radioactivity, indicating that *both* the methyl group and the cyclopropyl ring carbons comprise part of the stable adduct. Neither benzylamine nor urea denaturation following inactivation had a significant effect on the stoichiometry of inactivation by **3b** or **3c**. The adduct, therefore, is covalently and irreversibly bound to the enzyme. These results eliminate pathway b (Scheme II) as the mechanism of inactivation since, if this were the pathway, only **3b** would label the enzyme irreversibly. It is interesting to note that essentially the same results were obtained whether porcine or bovine MAO was used, suggesting that the enzymes from both species may have common mechanisms of action and possibly similar active site structures.

As was described for the case of N-CBA inactivation of MAO (Silverman & Hoffman, 1980), enzyme inactivation does not occur every turnover. If a nitrogen radical cation were generated, it would be expected that reactions could take place at *both* substituents attached to the electron deficient center. Cleavage of the benzyl methylene C–H bond (pathway c, Scheme II) would produce the imine of benzaldehyde and 1-methylcyclopropylamine. It was shown previously (Silverman & Hoffman, 1980) that the imine of benzaldehyde and cyclopropylamine is not an inactivator of MAO but rather hydrolyzes to benzaldehyde and cyclopropylamine. As shown in Table I, the partition ratio for the production of 1-methylcyclopropylamine (Figure 1) to the inactivation of enzyme is finite but quite low (0.10–0.22) and is essentially invariant regardless of the location of the label in the inactivator. This partitioning is consistent with an amine radical cation intermediate and suggests that ring opening is 5–10 times faster than benzyl methylene proton abstraction. Although the rate of proton removal adjacent to an amine radical cation is not known, it must be a very facile process since the rate of opening of a cyclopropyl ring adjacent to an amine radical was so fast it could not be measured by kinetic EPR spectroscopy at 135 K (Maeda & Ingold, 1980). As was found with the radioactive labeling results described above, the partition ratio results for both porcine and bovine liver MAO were remarkably similar.

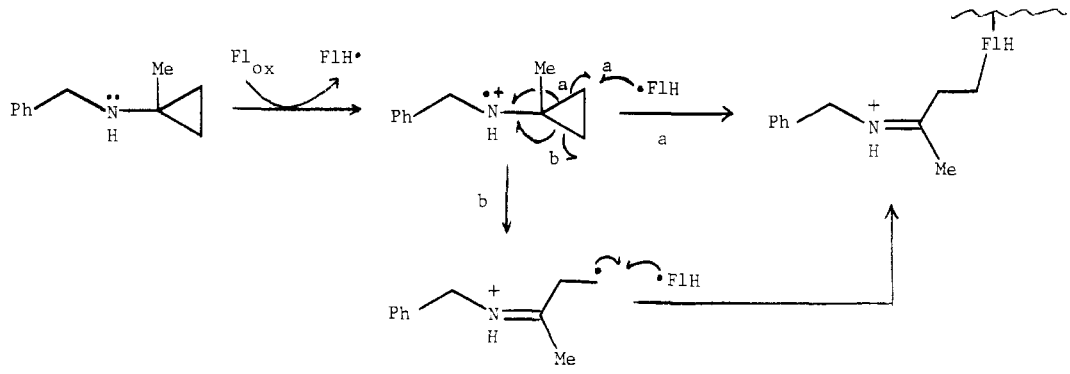
The structures of **1a** and **2a** (Scheme II) were further characterized by the utilization of standard organic chemical reactions. The lack of attachment of the benzyl methylene carbon following exhaustive dialysis (Table I) is consistent with the lability to hydrolysis of Schiff bases of aliphatic ketones (Feeney et al., 1975) and is evidence for a conversion of **1a** to **2a**. Isolation of the small molecules formed after inactivation by **3a** revealed the formation of benzylamine (Figure 2), the expected product of oxidation and hydrolysis by pathway a. Structure **2a** requires the retention of all carbon atoms except those of the benzyl group as was observed (Table I). Further confirmation for structure **1a** as an intermediate was obtained by treatment with sodium cyanoborohydride of the enzyme inactivated by **3a**. Sodium cyanoborohydride is a hydride-reducing agent that reacts rapidly with protonated imines but very slowly with aldehydes and ketones at or above neutral pH (Borch et al., 1971). When the inactivated enzyme was dialyzed following sodium cyanoborohydride treatment, the benzyl group was found to be attached to the enzyme in accordance with reduction of an imine to an amine, which is stable to hydrolysis. As shown in Table II, even after pargyline treatment, 0.3–0.6 equiv of radioactivity was reduced onto the enzyme; this apparently is nonspecific labeling outside of the active site since pargyline is known to titrate the MAO flavin (Chuang et al., 1974). It also should be noted that the 1.2–1.6 equiv of active site labeling after reduction (i.e., when nonspecific labeling is subtracted) is a maximum number. It was shown (Table I) that 0.1–0.25 equiv of benzaldehyde is generated during inactivation and this could be reduced onto protein amino groups (Means & Feeney, 1968; Geoghegan et al., 1981), which would account for additional benzyl labeling.

Another experiment was carried out to confirm that adduct **2a** was a ketone. Treatment of **2a** with sodium boro[$^3$H]-hydride would be expected to result in reduction of the ketone to the alcohol and in incorporation of 1 equiv of tritium, assuming no tritium isotope effect. As a control for possible tritium isotope effects, a model for **2a** where X is reduced flavin (vide infra), *N*-(3-oxobutyl)piperidine, was synthesized and treated with [$^3$H]$NaBH_4$ under the same conditions as the enzyme reduction. *N*-(3-Hydroxybutyl)piperidine was isolated and shown to contain 0.61 equiv of tritium, indicating only a small isotope effect was apparent. MAO that had been

Scheme III: Inactivation Mechanism Involving an Amine Radical Intermediate




Scheme IV: Proposed Mechanism of Inactivation of MAO by N-(1-Me)CBA




inactivated by N-(1-Me)CBA and dialyzed (presumably producing **2a**) was incubated with [$^3$H]$NaBH_4$. After exhaustive dialysis, 2.47 equiv of tritium was incorporated compared to a control reaction with native enzyme that incorporated 1.16 equiv of tritium. It was shown in a separate control reaction of FAD with [$^3$H]$NaBH_4$ that only 0.23 mol of tritium is incorporated into oxidized flavin; consequently, most of the 1.16 equiv of tritium found after treatment of native MAO with [$^3$H]$NaBH_4$ must be bound to something other than the cofactor. The difference in tritium incorporation into *N*-(1-Me)CBA-inactivated MAO vs. that into native MAO suggests that approximately one hydride is incorporated into the adduct as would be expected for a ketone.

Since **2a** is a methyl ketone, the iodoform reaction, i.e., conversion of a methyl ketone to a carboxylic acid and iodoform, was performed on the labeled enzyme. With **3b** as the inactivator, the adduct (**2a**) would be expected to have the label at the methyl group of the methyl ketone, and this would result in transfer of the radioactive label to any iodoform produced in this reaction. The labeled enzyme was degraded with Pronase prior to treatment with basic triiodide, and 54% of the previously bound radioactivity was isolated as iodoform, thus confirming a methyl ketone.

The X shown in Scheme II could represent the flavin in the active site or some active site nucleophile; for example, it has been suggested that there is a sulfhydryl group at the active site of MAO (Singer & Barron, 1945). Since thiols are excellent hydrogen atom donors (Knight, 1974), X· could represent either the flavin semiquinone or, after hydrogen atom abstraction of a cysteinyl thiol by the flavin radical, it could be a thiyl radical. So that differentiation between these two possibilities could be made, the changes in the flavin optical spectrum during inactivation and after urea treatment of the inactivated enzyme were recorded (Figure 3). A time-dependent conversion of the oxidized to the reduced form of the covalently bound flavin was observed during inactivation. This suggests either that the inactivator is becoming attached to the flavin and thereby locking the flavin in the reduced form or that it is becoming attached to an active site nucleophile and thereby is blocking the entrance of $O_2$ and preventing oxidation of the reduced flavin. The changes in the optical spectrum of MAO during inactivation by N-(1-Me)CBA appear qualitatively similar to those reported (Silverman & Hoffman, 1980) during the inactivation of MAO by N-CBA. However, unlike the case of N-CBA (Silverman & Hoffman, 1980), this quandary can be resolved because the enzyme adduct is stable to urea treatment. Denaturation of the inactivated enzyme in 6 M urea resulted in no change in the flavin spectrum from the reduced form, indicating that the X group in **1a** is the covalently bound flavin cofactor. This was not the case with N-CBA. The exact location on the flavin where the new bond forms is unknown, but the N-5 and C-4a positions are most likely since other MAO inactivators have been reported to bond to those positions of the flavin (Maycock et al., 1976; Kenney et al., 1979).

Ketones containing $\beta$-leaving groups (e.g., **2a** where X–Enz is the leaving group) are known to undergo retro-Michael reactions (elimination of HX to give $\alpha,\beta$-unsaturated carbonyl compounds) in the presence of base (Gutsche, 1967). However, alcohols containing $\beta$-leaving groups are generally stable to this base reaction. Therefore, MAO was inactivated with **3b**, then was treated with base, and was compared to another batch of **3b**-inactivated MAO that was first reduced with sodium borohydride before treatment with base. In both cases, radioactivity was released slowly from the protein; release from the batch that was not $NaBH_4$ treated was only about twice as fast as that from the $NaBH_4$-treated batch. An explanation for this could be that there is a competing reaction common to both modified flavins. The flavin of MAO is known to be attached to the protein through an 8$\alpha$ linkage of the flavin to a cysteine residue (Walker et al., 1971). Hydroxide is known to cleave lanthionine (Cecil & McPhee, 1959) and cystine residues in proteins (Nashef et al., 1977) by $\beta$-elimination. Consequently, both the ketone and alcohol adducts may be released from the protein still attached to the flavin (or a modified hydrolyzed flavin, Wadke & Guttman, 1966) as a result of hydroxide ion mediated $\beta$-elimination of the flavin (or hydrolyzed flavin) from the cysteine residue. Since the adduct in the ketone form did not release radioactivity upon base treatment considerably faster than the adduct in the alcohol form, it suggests that the leaving group, i.e., the flavin (or hydrolyzed flavin) in this case, is a poor one. This argues more in favor of N-5 than C-4a attachment to the inactivator because the flavin N-5 anion would be a poorer leaving group than the flavin C-4a anion ($pK_a$ differences). This question, however, is yet to be answered.

Rando & Eigner (1977) showed that allylamine was an inactivator of MAO and proposed that the structure of the adduct was the aldehyde analogue of **2a**. This adduct, however, was unstable to benzylamine treatment, which led to reactivation of the inactivated enzyme. It also was mentioned in that paper that 2-amino-3-butene slowly inactivated MAO, but no comment was made regarding the stability of the adduct produced. Inactivation of MAO by 2-amino-3-butene would be expected to produce **2a** if the reasonable mechanism proposed by Rando & Eigner (1977) is operative. Therefore, the adduct formed from 2-amino-3-butene inactivation should have the same stability as the adduct formed from N-(1-Me)CBA inactivation. We have found this to be the case,[2] which further corroborates **2a** as the structure of the adduct.

On the basis of the work of Lewis & Correa (1981), the mechanism shown in Scheme II may not be complete. They have shown that photochemical oxidation of secondary amines, which are known to proceed by an initial one-electron excitation to give the amine radical cation, lead to two competing pathways. In addition to the favored C–H proton transfer to give a carbon radical, a competing reaction is N–H proton transfer, which produces the amine radical. If this is a predominant pathway, then reaction of Enz–X· would occur on the amine radical rather than on the amine radical cation (Scheme III). For differentiation of these two possibilities, *N*-methyl-*N*-(1-methylcyclopropyl)benzylamine was prepared. This molecule would not have a proton on the amine when the radical cation was generated. Had this compound inactivated MAO at a much slower rate or not at all, it would have suggested that removal of the amine proton to give the amine radical rather than amine radical cation is an important part of the mechanism of inactivation. Although the $K_i$ for *N*-methyl-*N*-(methylcyclopropyl)benzylamine was 9.7 times larger than that of the non-methylated compound, the $k_{cat}$ was virtually identical with that for N-(1-Me)CBA (Silverman & Hoffman, 1981a). The product of inactivation also was stable to benzylamine treatment as is the case with N-(1-Me)CBA. This suggests that removal of the amine proton is not an important pathway in the mechanism.

One further mechanism was considered, namely, attack of oxidized flavin by the cyclopropyl ring of N-(1-Me)CBA to give **1a** directly. As a test of this mechanistic possibility, **3a** (free base) was heated with a 5-fold excess of 3-methyllumiflavin in dimethyl sulfoxide at 95 °C for 12 h. Paper chromatography analysis of the reaction mixture showed that 93% of the starting material was recovered unchanged, indicating that this reaction probably is not relevant to the inactivation process.

All of the data presented here are consistent with an initial one-electron transfer from N-(1-Me)CBA to the flavin to give a flavin radical and a highly reactive amine radical cation (Scheme II). This intermediate can form a covalent bond to the flavin either by direct attack of the flavin radical on the cyclopropyl ring or by initial rapid cyclopropyl ring opening (Maeda & Ingold, 1980) to give a primary radical, which then combines with the flavin radical (Scheme IV).

**Registry No.** **3a**·HCl, 88887-82-5; **3b**·HCl, 88887-83-6; **3c**·HCl, 88887-84-7; *N*-(3-oxobutyl)piperidine hydrochloride, 6631-71-6; *N*-(3-hydroxybutyl)piperidine hydrochloride, 88887-85-8; *N*-methyl-*N*-(1-methylcyclopropyl)benzylamine hydrochloride, 88887-86-9; [7-$^{14}$C]benzaldehyde, 14734-27-1; 1-methylcyclopropylamine hydrochloride, 88887-87-0; cyanocyclopropane, 5500-21-0; 1-[$^{14}$C]-methylcyclopropylamine hydrochloride, 88887-88-1; *N*-methyl-nitrosourea, 684-93-5; 1-methyl[2-$^{14}$C]cyclopropanecarboxylic acid, 88887-89-2; 1-methyl[2-$^{14}$C]cyclopropylamine hydrochloride, 88887-90-5; piperidine, 110-89-4; methyl vinyl ketone, 78-94-4; monamine oxidase, 9001-66-5.

[2] MAO was inactivated with 2-amino-3-butene as described under Materials and Methods for the inactivation of MAO by N-(1-Me)CBA. The 2-amino-3-butene was prepared by converting (carbobenzyloxy)-alanine to (carbobenzyloxy)alaninal with borane followed by pyridinium dichromate and then treating the aldehyde with triphenylphosphonium methylide. Hydrolysis produced 2-amino-3-butene. Unlike allylamine-inactivated MAO, no reactivation occurred upon treatment of 2-amino-3-butene-inactivated enzyme with 1 mM benzylamine for 15 h.

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# Inhibition of Xanthine Oxidase by Various Aldehydes†

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ABSTRACT: The inactivation of bovine milk xanthine oxidase by various aldehydes has been investigated. For each aldehyde, the inactivation reaction gives rise to a unique molybdenum(V) electron paramagnetic resonance signal from xanthine oxidase (the Inhibited signal). Of the aldehydes tested, only a few (mainly aromatic) failed to undergo this reaction. The $g$ values of the Inhibited signals vary systematically from one aldehyde to another. As the substituents of the $\alpha$-carbon atom become more electron withdrawing, so the $g_{av}$ increases. The inactivation rate depends on both enzyme and aldehyde concentration. Oxygen or another oxidizing substrate is also required for inhibition by 3-pyridinecarboxaldehyde and butyraldehyde but not formaldehyde. Reactivation of xanthine oxidase inhibited by an aldehyde occurs spontaneously after removal of excess aldehyde. For butyraldehyde or 3-pyridinecarboxaldehyde, greater than 95% recovery of activity was observed. The rate of reactivation is dependent both on the nature of the molecule bearing the aldehyde group and on a p$K$ (6.6) of the complex with the enzyme. Evidence is presented that the modifying aldehyde in the Inhibited signal-giving species has (contrary to earlier assumptions) not been oxidized. These results are discussed in relation to the structure of the molybdenum center, and a mechanism for the inhibiting reaction is suggested.

Booth (1938) carried out the first, and until very recently [see Morpeth (1983)] the most rigorous, examination of the specificity of xanthine oxidase toward aldehyde substrates. In the course of these studies, he found that formaldehyde inactivates xanthine oxidase. This phenomenon was apparently not investigated further until Pick et al. (1971) provided evidence that the inactivation of xanthine oxidase by methanol (Polonovski et al., 1947; Rajagopalan & Handler, 1964; Coughlan et al., 1969; Bray et al., 1968) which results in a unique electron paramagnetic resonance (EPR)[1] signal, due to molybdenum(V) and given the name Inhibited, was in fact due to the enzyme slowly oxidizing methanol to formaldehyde. Since then, several other alcohols and aldehydes have been reported to react similarly with xanthine oxidase, always yielding the Inhibited signal (Tanner & Bray, 1978a,b; Malthouse et al., 1981a). Another carbonyl compound, formamide, will also give the Inhibited signal (F. F. Morpeth, G. N. George, and R. C. Bray, unpublished results). At least with formaldehyde and methanol, this signal is obtainable not only from xanthine oxidase but also from the related enzymes xanthine dehydrogenase (Barber et al., 1976) and aldehyde oxidase (Bray et al., 1982).

The Inhibited signal differs from other molybdenum(V) species from active xanthine oxidase in that it is air stable (Bray et al., 1968). However, little is known about the nature of the inhibitory reaction, though Bray & Gutteridge (1982) have proposed a structure for the Inhibited species on the basis of $^{17}O$-substitution studies.

The present work was undertaken to determine the nature of the inhibitory side reaction, occurring during the turnover of aldehyde substrates by xanthine oxidase, which gives rise to the Inhibited signal. The main approach we have used has been to vary the nature of the aldehyde molecule and to study the effect of this, and of other variables such as pH, on the formation and breakdown of the species formed in the inhibitory reaction.

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[1] Abbreviations: EPR, electron paramagnetic resonance; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; DPPH, diphenylpicrylhydrazyl; EDTA, ethylenediaminetetraacetic acid.

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