Mechanism of Inactivation of Monoamine Oxidase by 1-Phenylcyclopropylamine†

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ABSTRACT: 1-Phenylcyclopropylamine (1-PCPA) is shown to be a mechanism-based inactivator of mitochondrial monoamine oxidase (MAO). The strained cyclopropyl ring is important to inactivation since α,α-dimethylbenzylamine, the acyclic analogue of 1-PCPA, is neither an inactivator nor a substrate of MAO. Two different pathways occur during inactivation by 1-PCPA, both believed to be derived from a common intermediate. One pathway leads to irreversible inactivation of the enzyme and a 1:1 stoichiometry of radioactivity to the active site when 1-[*phenyl*-^{14}C]PCPA is used as the inactivator; the other pathway results in a covalent reversible adduct. Three organic reactions are carried out on the irreversibly labeled enzyme in order to determine the structure of the active site adduct. Sodium boro[^{3}H]hydride reduction results in the incorporation of 0.73 equiv of tritium, suggesting a carbonyl functionality. Baeyer–Villiger oxidation followed by saponification gives 0.8 equiv of phenol, indicating the presence of a phenyl ketone. Treatment of the labeled enzyme with hydroxide produces acrylophenone, as would be expected from the retro-Michael reaction of β-X-propiophenone. The identity of X is determined in two ways. The optical spectrum of the flavin cofactor is reduced during inactivation; no reoxidation occurs upon denaturation. Pronase treatment of the radioactively labeled enzyme produces fragments that contain both the radioactivity and the flavin. The X group, therefore, is the flavin. The results of two tests designed to differentiate N_5 from C_{4a} attachment to the flavin suggest an N_5 adduct. In addition to formation of this stable covalent adduct, another pathway occurs 7 times as often. This alternate reaction of 1-[*phenyl*-^{14}C]PCPA with MAO produces 7 equiv of [^{14}C]acrylophenone during the course of irreversible inactivation and is believed to arise from formation of the same type of adduct as described above except that X is something other than the N_5-flavin (Y). Upon denaturation of this labeled enzyme, the flavin is completely oxidized when most of the radioactivity is still bound to the enzyme. This indicates that Y is not a C_{4a}-flavin adduct and suggests attachment to an active site amino acid residue. More facile elimination of Y from this β-substituted propiophenone adduct would give acrylophenone on the time scale of the inactivation. Treatment of the reversible adduct with sodium borohydride prior to denaturation prevents release of radioactivity. This is further evidence for the involvement of a retro-Michael reaction in the formation of acrylophenone. The rate constant for the reversible reactivation component was determined to be 7.25 times larger than that for the irreversible inactivation component, and this is consistent with the observed 7:1 ratio for these reactions. Suggestions are made as to how and why attachment to the amino acid residue occurs. The mechanism that is consistent with these results is a one-electron transfer from 1-PCPA to the flavin followed by homolytic cyclopropyl ring opening to the common radical intermediate, which is trapped by two different active site radicals. Flavin radical combination yields the stable adduct; Y· capture produces the labile adduct.

Monoamine oxidase (EC 1.4.3.4) (MAO),[1] a FAD-containing enzyme, catalyzes the oxidative deamination of biogenic amine neurotransmitters. Inhibition of this enzyme has a pronounced clinical effect on mood; MAO inhibitors are used as antidepressant agents (Kaiser & Setler, 1981; Baldessarini, 1977; Berger & Barchas, 1977; Tyrer, 1976). The mechanism of inactivation of MAO by one of the earlier MAO inhibitors, *trans*-2-phenylcyclopropylamine, was recently elucidated (Silverman, 1983). This drug was shown (Silverman, 1983; Paech et al., 1980) to be a mechanism-based inhibitor of MAO that forms a labile covalent bond to an active site residue. A mechanism-based inhibitor (Rando, 1974; Abeles & Maycock, 1976; Walsh, 1984; Silverman & Hoffman, 1984) is a relatively unreactive compound that is converted by an enzyme into another compound that, without prior release from the active site, inactivates the enzyme, usually by covalent bond formation. Another cyclopropylamine, *N*-(1-methylcyclopropyl)benzylamine, was shown to be an irreversible mechanism-based inactivator of MAO; unlike *trans*-2-phenylcyclopropylamine, it formed a stable covalent bond to the covalently-bound FAD cofactor (Silverman & Yamasaki, 1984). In this paper we report the mechanism of inactivation of MAO by another cyclopropylamine, 1-phenylcyclopropylamine. This cyclopropylamine appears to combine the properties of *trans*-2-phenylcyclopropylamine and *N*-(1-methylcyclopropyl)benzylamine in that the results can be rationalized as covalent bond formation to both the flavin cofactor (stable adduct) and an active site residue (labile adduct).

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 or Ready-Solve from Beckman. [^{14}C]Toluene (4.7×10^5

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[1] Abbreviations: MAO, monoamine oxidase; FAD, flavin adenine dinucleotide; 1-PCPA, 1-phenylcyclopropylamine.

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dpm/mL) and [^{3}H]toluene (1.68×10^6 dpm/mL, corrected for first-order decay) were obtained from New England Nuclear for use as internal standards. Optical spectra were recorded with semi-microcuvettes either on a Beckman ACTA CIII spectrophotometer or on a Perkin-Elmer 330 spectrophotometer equipped with a Perkin-Elmer data station. Infrared spectra were obtained on a Perkin-Elmer 283 spectrophotometer. NMR spectra were recorded on a Varian EM-390 90-MHz spectrometer. All chemical shifts are expressed as parts per million (δ) downfield from tetramethylsilane when $CDCl_3$ was used as solvent and from sodium 3-(trimethylsilyl)-1-propanesulfonate when D_2O was used as solvent. Melting points were obtained on a Fisher-Johns or Mel-Temp melting point apparatus and are uncorrected. Thin-layer chromatography was performed on silica gel 60 coated plastic plates (Merck) in the following solvent systems: (A) 1:1 *n*-hexane–ethyl acetate; (B) 3:1 *n*-hexane–ether; (C) 12:3:5 1-butanol–acetic acid–water; (D) 2:1:1 *n*-hexane–ether–ethanol; (E) 1:1 ethyl acetate–ether; (F) 1:1 ethyl acetate–ethanol. MAO activity assays were carried out on a Gilford Model 222 update system with a Beckman DU monochrometer or a Perkin-Elmer Lambda 1 UV/vis spectrophotometer, and a Klett-Summerson Model 800-3 photoelectric colorimeter was used for protein concentration determinations. An Orion Research Model 601A pH meter with either a general combination electrode or a microcombination probe (Microelectrodes, Inc., Model MI-410) was used to measure pH values.

Reagents. Benzylamine hydrochloride was prepared by bubbling HCl gas through an anhydrous ethereal solution of benzylamine and then recrystallizing the salt from ethanol. [*ring*-U-^{14}C]Benzyl cyanide was purchased from Pathfinder Laboratories, Inc. (St. Louis, MO) and sodium boro[^{3}H]-hydride from ICN Radiochemical Division (Irvine, CA). [7-^{14}C]Pargyline was a gift of Prof. Roy McCauley (Wayne State University). 1-Phenylcyclopropanecarbonitrile and leuco crystal violet were bought from Aldrich, and benzoylacetonitrile was purchased from Pfaltz and Bauer. Acrylophenone and *N*-(3-oxo-3-phenylpropyl)piperidine hydrochloride were prepared by the method of Angeloni & Tramontini (1964). α,α-Dimethylbenzylamine was synthesized by the reported procedure (Cope et al., 1949).

1-Phenylcyclopropylamine Hydrochloride. 1-Phenylcyclopropanecarbonitrile (1.7 mL, 11.9 mmol) was added to a solution of sodium hydroxide (2 g) in water (60 mL). The reaction mixture was stirred at reflux for 20 h; then, the solution was cooled to room temperature, washed with ether (2×25 mL), acidified with 6 N HCl, and extracted with ether (3×30 mL). The combined ether extracts were washed with brine (2×40 mL), dried ($MgSO_4$), and filtered, and the ether was removed by rotary evaporation, yielding 1.98 g (98%) of 1-phenylcyclopropanecarboxylic acid as white crystals: mp 84–85 °C; NMR ($CDCl_3$ + [$^2H_6$]Me_2SO) δ 1.2 (m, 2 H), 1.6 (m, 2 H), 7.3 (br m, 5 H), and 12.5 (br s, 1 H). This compound can be purchased from Aldrich.

The procedure for the conversion of 1-phenylcyclopropanecarboxylic acid to 1-PCPA is a modification of the procedure of Kaiser et al. (1962). Concentrated sulfuric acid was added to a stirred solution of 1-phenylcyclopropanecarboxylic acid (0.75 g, 4.6 mmol) dissolved in chloroform (25 mL). The reaction mixture was heated to 50 °C, and sodium azide (0.92 g, 14.2 mmol) was then added in portions. Ice water (50 mL) was added after 3 h, and the layers were separated after being stirred an additional 15 min. The organic layer was extracted with 0.5 N HCl (2×10 mL), and the combined aqueous layer and extracts were washed with ether (2×25 mL) and made basic with 6 N NaOH. The basic solution was extracted with ether (3×40 mL), and the ether extracts were washed with brine (2×50 mL) and dried ($MgSO_4$). The hydrochloride salt was obtained by bubbling HCl gas into the filtered ethereal solution and was recrystallized twice from chloroform–ethyl acetate to give the product as white crystals (245 mg, 31%): mp 201.5–202 °C (sealed tube); NMR of amine ($CDCl_3$) δ 0.9 (m, 4 H), 1.8 (br s, 2 H), and 7.2 (s, 5 H); NMR of salt (D_2O) δ 1.0 (m, 4 H), and 7.2 (m, 5 H). Anal. Calcd. for $C_9H_{12}ClN$: C, H, N.

1-[phenyl-^{14}C]Phenylcyclopropylamine Hydrochloride. To a stirred mixture of freshly distilled diisopropylamine (147 μL, 1.1 mmol) and anhydrous tetrahydrofuran (1 mL) under argon at −78 °C was added dropwise, by syringe, 2.6 M *n*-butyllithium in hexane (425 μL, 1.1 mmol). The solution was stirred for 25 min, and then a solution of benzyl cyanide (43 μL, 0.36 mmol) and [*ring*-U-^{14}C]benzyl cyanide (15.6 μL, 0.13 mmol, 0.49 mCi, 3.77 mCi/mmol) in anhydrous tetrahydrofuran (1.5 mL) was added dropwise by syringe. After 15 min the temperature of the solution was allowed to rise to −20 °C, the dibromoethane (46 μL, 0.53 mmol) dissolved in anhydrous tetrahydrofuran (0.5 mL) was added via syringe. The solution was stirred for 2 h at 0 °C, and then the temperature was allowed to rise to room temperature over a period of 2 h. At this time, ether (5 mL) was added, and the solution was washed with 2 N HCl (2×5 mL) and brine (2×5 mL). After removal of the solvent by rotary evaportion to give 1-[*phenyl*-^{14}C]phenylcyclopropanecarbonitrile, the remainder of the procedure followed that for the preparation of nonlabeled 1-PCPA as described above. The crude product was recrystallized from chloroform–ethyl acetate to give 17 mg (21% overall) of radiolabeled 1-PCPA as white crystals (sp act. 0.975 mCi/mmol), which proved to be radiopure as well as chemically pure by TLC (solvent system C); all of the radioactivity comigrated with carrier 1-PCPA.

1-Oxo-1-phenyl-3-hydroxy-2-propene. This compound was prepared by the procedure of Ainsworth (1957) for the preparation of 2-(hydroxymethylene)cyclohexanone (method 1) on a $^1/_{20}$ scale, substituting acetophenone for cyclohexanone. The compound was obtained as an intensely yellow oil, which was converted to its cupric salt, recrystallized, and regenerated according to the procedure of Claisen & Fischer (1888): mp of the cupric salt, 112–115 °C dec; NMR of enol ($CDCl_3$) δ 6.2 (d, 1 H), 7.4 (m, 3 H), 7.9 (m, 2 H), 8.3 (d, 1 H), and 11–14 (br m, 1 H); NMR of dicarbonyl δ 4.0 (d, 2 H), 7.4 (m, 3 H), 7.9 (m, 2 H), and 9.9 (t, 1 H). The ratio of enol to dicarbonyl was 15:1.

N-(3-Hydroxy-3-phenylpropyl)piperidine. *N*-(3-Oxo-3-phenylpropyl)piperidine hydrochloride (120 mg, 0.47 mmol) was added to a solution of sodium borohydride (40 mg, 0.27 mmol) in 0.1 N sodium hydroxide (10 mL). After being stirred for 15 h at room temperature, the white crystals (95 mg, 92%) that had formed were collected, washed with water, and suction dried: TLC showed one spot eluting in system E (R_f 0.10) or F (R_f 0.38); mp 64–65 °C; NMR ($CDCl_3$) δ 1.6 (m, 6 H), 1.8 (t, 2 H), 2.5 (m, 7 H), 4.9 (t, 1 H), and 7.3 (s, 5 H).

β-Aminopropiophenone Hydrochloride. A stirred solution of benzoylacetonitrile (0.5 g, 3.4 mmol), trimethylorthoformate (8.0 mL, 73 mmol), and *p*-toluenesulfonic acid (50 mg) in ethylene glycol (25 mL) was heated to 95 °C for 4 h. The methanol and methylformate produced were removed by distillation during the reaction; then, the remaining trimethylorthoformate was removed by distillation. The solution

was washed with aqueous sodium bicarbonate (5 mL) and extracted with ether (3 × 20 mL). The combined extracts were washed with saturated sodium bicarbonate–sodium chloride solution (2 × 20 mL) and brine (2 × 20 mL). The ethereal solution was dried ($MgSO_4$), filtered, and concentrated to a volume of approximately 10 mL. Lithium aluminum hydride (0.2 g, 5.3 mmol) was then added, and the mixture was stirred at reflux temperature for 15 h. After being cooled to room temperature, water (0.3 mL) was added, followed by 15% sodium hydroxide (0.3 mL) and water (0.8 mL). The solution was filtered and then extracted with 1 N HCl (3 × 10 mL). The combined extracts were basified with 6 N NaOH and extracted with ether (3 × 20 mL). The combined ether extracts were dried (K_2CO_3) and filtered, and the solvent was evaporated: NMR ($CDCl_3$) δ 1.23 (m, 4 H), 2.6 (m, 2 H), and 7.2 (m, 5 H); IR (KBr film) 3420 (N–H) and 1685 (C=O) cm^{-1}. The hydrochloride salt was prepared by treating the amine in ethanol (10 mL), with 1 N HCl (5 mL), and evaporating the solvents. The product was recrystallized from ethanol–ether to give 50 mg of the product as white crystals: mp 124–125 °C [lit. (Becker & Fanghänel, 1964) mp 128 °C].

Enzymes and Assays. Mitochondrial MAO was purified from bovine liver, obtained fresh from a local slaughterhouse and kept on ice for 1 h before processing by the procedure of Salach (1979). The specific activities of the purified MAO 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 μmol of benzylamine to benzaldehyde per minute at 30 °C. Pronase (protease, type XIV, a nonspecific protease from *S. griseus*) and horseradish peroxidase (160 purpurogallin units/mg) were obtained from Sigma. Protein determinations were made by the method of Lowry et al. (1951).

Formation of Irreversible Adduct of MAO with 1-PCPA. In a typical preparation of 1-PCPA-inactivated enzyme, 4 mM inactivator (either ^{14}C labeled or unlabeled) in 50 mM potassium phosphate, pH 7.2 (250 μL), was added to 60 μM MAO in the same buffer (500 μL), and the mixture was incubated at 25 °C in the dark for 8–10 days. An inactivator control containing no enzyme, as well as an enzyme control containing no inactivator, also was carried out. Each mixture was microdialyzed against 50 mM potassium phosphate, pH 7.2 (15 mL), for 2.5 h at room temperature in the dark followed by exhaustive dialysis against three changes (100 mL each) of the same buffer. This procedure typically produced inactivated enzyme with an activity less than 10% of the enzyme control. The outer portions from the microdialyses were stored frozen for further analysis as described (vide infra). The efficiency of microdialysis was typically greater than 90%.

Rate of Irreversible Inactivation of MAO by 1-PCPA. 1-PCPA (6 mM) in 50 mM potassium phosphate, pH 7.2 buffer (500 μL), was added to 8 μM MAO in the same buffer (1 mL), and the mixture was incubated at 25 °C. Aliquots (200 μL) were removed at 0, 1, 2.5, 4, 6, 8.5, and 24 h and were applied to Sephadex G-25 (0.7 × 10 cm) equilibrated with 20 mM potassium phosphate, pH 7.2 buffer and eluted with the same buffer at a flow rate of 0.5 mL/min. A 0.5-mL fraction containing the majority of the enzyme was collected for each aliquot, which was dialyzed against three changes of 250 mL of 50 mM potassium phosphate, pH 7.2 buffer. Aliquots of the dialyzed samples were assayed for enzyme activity and normalized for protein concentration.

Sodium Boro[^3H]hydride Treatment of 1-PCPA-Inactivated MAO. 1-PCPA-inactivated MAO and an enzyme control containing no inactivator were prepared as described above. Aliquots of each solution (100 μL) were diluted 1:1 with 8 M urea in 0.2 N sodium borate, pH 9.5 buffer. To each was added 0.1 M sodium boro[^3H]hydride (1.5 μL, diluted in sp act. 0.35 mCi/mmol with nonradioactive sodium borohydride) in 0.1 N NaOH. The mixtures were stored at room temperature for 14 h in the dark and then neutralized with 1 N HCl (10 μL). The solutions then were exhaustively dialyzed against four changes (100 mL each) of 8 M urea in 50 mM potassium phosphate, pH 7.2 buffer. Aliquots were counted, and the protein was normalized by Lowry assay. The amount of active MAO was determined by titration with [^{14}C]pargyline (Chuang et al., 1974).

Sodium Boro[^3H]hydride Treatment of N-(3-Oxo-3-phenylpropyl)piperidine. One milliliter of the same sodium boro[^3H]hydride solution used above was added to 12 mg of *N*-(3-oxo-3-phenylpropyl)piperidine hydrochloride. After this solution was allowed to stand for 15 h at room temperature, the white crystals that had formed were collected, washed with water, and suction dried. The product (sp act. 0.11 mCi/mmol) proved to be radiopure as well as chemically pure by TLC (system F, R_f 0.38); all of the radioactivity comigrated with carrier *N*-(3-hydroxy-3-phenylpropyl)piperidine.

Baeyer–Villiger Oxidation of N-(3-Oxo-3-phenylpropyl)-piperidine. A solution of peroxytrifluoroacetic acid was prepared by the addition of 30% hydrogen peroxide (1 mL) to trifluoroacetic acid (3 mL) containing ethanol (0.5 mL). After this was stirred at room temperature for 1.5 h and cooled to 0 °C, *N*-(3-oxo-3-phenylpropyl)piperidine hydrochloride (350 mg, 1.4 mmol) was added. The solution was stirred at 0 °C for 2 h and then at 4 °C for 15 h. The solution was made strongly basic by the addition of concentrated sodium hydroxide and heated at 85 °C for 16 h. After being cooled to room temperature, the solution was acidified with concentrated HCl, and the solvents were removed by rotary evaporation, leaving a tan solid. Concentrated HCl (8 mL) was added, and the mixture was extracted with ether (3 × 10 mL). The combined extracts were washed with sodium bicarbonate (2 × 15 mL) and brine (2 × 15 mL), dried ($MgSO_4$), and filtered, and the solvent was removed by rotary evaporation, leaving 72 mg (55%) of a yellow semisolid that was identified as phenol by TLC (system A, R_f 0.47; system B, R_f 0.34).

Baeyer–Villiger Oxidation of 1-[phenyl-^{14}C]PCPA-Inactivated MAO. The procedure for the preparation of the flavin peptide from 1-[^{14}C]PCPA-inactivated MAO (see Isolation of Flavin Peptide from 1-[*phenyl*-^{14}C]PCPA-inactivated MAO) was modified by omitting the sodium borohydride reduction of the adduct prior to trichloroacetic acid precipitation of the labeled enzyme. The remainder of the procedure was carried out as described. Cold peroxytrifluoroacetic acid solution prepared as above (1 mL) was added to 0.5 mL of this nonreduced isolated flavin peptide adduct solution. After 16 h at 4 °C, the solution was warmed to room temperature and made basic with concentrated sodium hydroxide. The solution was then heated at 85 °C for 14 h and rotary evaporated to a white solid. After the addition of phenol (20 mg) as carrier, the solid was dissolved in a minimum amount of concentrated HCl (0.5 mL) and extracted with ether (2 × 1 mL) and ethyl acetate (1 mL). Aliquots of the acidic solution and the extracts were removed for scintillation counting. Following concentration of the combined extracts to a smaller volume (0.5 mL), the residue was analyzed by

TLC (system A, R_f 0.47; system B, R_f 0.34).

Release of Radioactivity from 1-[phenyl-^{14}C]PCPA-Inactivated MAO with Base. MAO inactivated with radiolabeled 1-PCPA was prepared as described above (see Formation of the Irreversible Adduct of MAO with 1-PCPA) and further dialyzed against 8 M urea in 50 mM potassium phosphate, pH 7.2 buffer (3 × 80 mL, 1.5 h each, room temperature).

Three portions (50 μL each) of the dialyzed solution were removed and diluted 1:1 with 0.2 N sodium borate, pH 9.5 buffer. Sodium borohydride (1 mg) was added to one portion. After 1 h at room temperature, 0.5 N sodium hydroxide (800 μL) was added to this portion and to one other portion; sodium chloride (0.5 M, 800 μL) was added to the third portion (control). All three samples were then incubated in the dark at 37 °C.

After 1 h, aliquots (250 μL) of each sample were removed; the two basic aliquots were neutralized with 1 N hydrochloric acid (110 μL); water (110 μL) was added to the sodium chloride control. One aliquot of each solution was removed for scintillation counting and another aliquot (100 μL) was placed onto Dowex 1-X8 (200–400 mesh, OH^- form) columns (0.5 × 5.0 cm) equilibrated with deionized water. The columns were eluted initially with deionized water (7.5 mL) and then with 3 M sodium chloride (7.5 mL), collecting 1.5-mL fractions. The remaining portions of the neutralized aliquots were dialyzed against 8 M urea in 50 mM potassium phosphate, pH 7.2 buffer (2 times 80 mL, 1.5 h, room temperature). Aliquots were counted, and the protein was normalized by Lowry assay. The same procedure was repeated after 4 and 22 h.

Release of Radioactivity from 1-[^{14}C]PCPA-Inactivated MAO during Pronase Digestion. The procedure described under Isolation of a Flavin Peptide from 1-[*phenyl*-^{14}C]-PCPA-Inactivated MAO (see below), except with the omission of the sodium borohydride treatment, was followed to the Pronase step. The Pronase was added, and after 1, 4, and 11 days, aliquots were removed and applied to Dowex 1 columns, eluted, and counted as described under Release of Radioactivity from 1-[^{14}C]PCPA-Inactivated MAO with Base.

Isolation of a Flavin Peptide from 1-[phenyl-^{14}C]PCPA-Inactivated MAO. MAO inactivated with radiolabeled 1-PCPA was prepared as described above (see Formation of the Irreversible Adduct of MAO with 1-PCPA); then, sodium borohydride (3 mg) in 0.2 N sodium borate, pH 9.5 buffer (500 μL) was added, and the solution was incubated at room temperature in the dark for 15 h. The solution was dialyzed against potassium phosphate, pH 7.2 buffer (3 × 125 mL; 1.5 h each change); then, the protein was denatured by the addition of trichloroacetic acid (50 mg). The denatured enzyme was pelleted by centrifugation in a Beckman Microfuge B for 1 min, redispersed in 5% trichloroacetic acid (750 μL), and pelleted in the same manner. The pellet was then washed successively with methanol (750 μL), chloroform (2 × 750 μL), methanol (750 μL), 50 mM potassium phosphate, pH 7.9 (2 × 750 μL). All supernatants were placed into scintillation vials and counted. The washed protein was dispersed in 750 μL of 50 mM potassium phosphate, pH 7.9, and digested with Pronase (1.5 mg) at 37 °C for 16 h. Additional Pronase (1.5 mg) was added, and the digestion was continued for another 24 h. After centrifugation to remove a small white pellet, the supernatant solution was placed onto a Sephadex G-25 column (1 × 36 cm) swollen in deionized water and eluted with deionized water at a flow rate of 1.2 mL/min, collecting 1.5-mL fractions. Each fraction was assayed for radioactivity, and the absorbances of each fraction at 250, 350, and 450 nm were recorded. The fractions containing radioactivity with high A_{450}/A_{250} ratios were pooled, and the water was removed in vacuo. The resulting yellow semisolid residue was dissolved in water (1 mL) and applied to a Sephadex G-10 column (1 × 45 cm) and eluted with deionized water at a flow rate of 1 mL/min, collecting 1.5-mL fractions. The fractions were assayed and pooled in the same manner as above. Evaporation in vacuo yielded a small amount of yellow semicrystalline material, which was dissolved in deionized water (0.5 mL). The radioactive small molecule fractions from Sephadex G-10 were pooled, the water was evaporated, and TLC (system D) was performed on the residue.

Rate of Reactivation of MAO Reversibly Inhibited by 1-PCPA. 1-PCPA (6 mM) in 50 mM potassium phosphate, pH 7.2 buffer (500 μL) was added to 8 μM MAO in the same buffer (1 mL) and then was incubated at 25 °C for 90 min. Three aliquots (200 μL) were removed, applied to Sephadex G-25 (0.7 × 10 cm) equilibrated with 20 mM potassium phosphate, pH 7.2 buffer, and eluted with the same buffer at a flow rate of 0.5 mL/min. A 0.5-mL fraction containing the majority of the enzyme was collected for each aliquot. One aliquot was diluted with an equal volume of 20 mM potassium phosphate, pH 7.2 buffer, another aliquot was diluted with an equal volume of the same buffer containing 10 mM β-mercaptoethanol, and the third aliquot was diluted with an equal volume of the buffer containing 10 mM β-mercaptoethanol and 2 mM benzylamine. Duplicates of each sample were run. The samples were incubated at 25 °C, and aliquots were assayed periodically for enzyme activity.

Identification and Quantification of Nonbound Oxidation Products. The outer portion of the microdialysis of MAO inactivated by radiolabeled 1-PCPA (described above) was applied to Dowex 50-X8 (200–400 mesh; H^+ form) (0.5 × 6.0 cm) equilibrated with deionized water. The columns were eluted initially with deionized water (25 mL) and then with 1.5 N HCl (25 mL), at a flow rate of approximately 1.5 mL/min. The aqueous fractions containing radioactivity were combined and extracted with ether (3 × 15 mL); the ether was removed by rotary evaporation to near dryness. Combined radioactive fractions from the acid wash were made alkaline with 6 N sodium hydroxide before extraction. The non-amine samples were analyzed by TLC in solvent systems C and D with acrylophenone (R_f^C 0.69, R_f^D 0.25), propiophenone (R_f^C 0.88, R_f^D 0.81), and 1-oxo-1-phenyl-3-hydroxy-2-propene (R_f^C 0.40, R_f^D 0.47) as standards. TLC (system C) of the combined amine fractions was performed with 1-PCPA (R_f 0.55) and β-aminopropiophenone (R_f 0.48) as standards. The developed chromatograms were cut into 5-mm strips for scintillation counting. The same procedure was performed with the outer portion of the microdialyses from the non-enzyme control. Part of the radioactive non-amine fraction was treated with 2,4-dinitrophenylhydrazine reagent (Shriner et al., 1980), extracted with ethyl acetate, and chromatographed (TLC system A) against the 2,4-dinitrophenylhydrazones of acrylophenone (R_f 0.59), propiophenone (R_f 0.68), and 1-oxo-1-phenyl-3-hydroxy-2-propene (R_f 0.55). The R_f (system A) of 2,4-dinitrophenylhydrazine was 0.27.

Change with Time in Optical Spectrum during Denaturation of MAO Reversibly Inhibited by 1-PCPA. 1-PCPA (10 mM) in 50 mM potassium phosphate, pH 7.2 buffer (200 μL) was added to 160 μM MAO in the same buffer (150 μL) and then incubated at 25 °C for 90 min. An aliquot (175 μL) was removed and applied onto Sephadex G-25 (0.7 × 12 cm) as described under Rate of Reactivation of MAO Reversibly

Inhibited by 1-PCPA, collecting a 750-μL fraction of the enzyme. A control sample also was prepared as above excluding inactivator. The optical spectrum of the inhibited enzyme vs. the control was recorded. Urea (360 mg, 8 M final concentration) was added to the inhibited and control enzyme, and the absorbance difference at 450 nm (Peach et al., 1980) was recorded with time. After 20 min, sodium dithionite was added to each sample separately to show that each was oxidized.

Release of Radioactivity during Denaturation of MAO Reversibly Inhibited by 1-[phenyl-^{14}C]PCPA. MAO was inhibited, gel filtered, and treated with urea as described under Change with Time in Optical Spectrum during Denaturation of MAO Reversibly Inhibited by 1-PCPA except that 1-[*phenyl*-^{14}C]PCPA was used as the inhibitor. Aliquots (85 μL) were removed at 0, 0.25, 0.5, 1.0, 1.5, 2.5, 4, and 22 h and applied to Sephadex G-25 (0.5 × 6.5 cm), eluting with water at a flow rate of 0.5 mL/min. Five 0.5-mL fractions were collected, and both the radioactivity released from the enzyme (small molecules) and the radioactivity remaining on the enzyme were determined.

Effect of Sodium Borohydride Treatment on Release of Radioactivity during Denaturation of MAO Reversibly Inhibited by 1-[phenyl-^{14}C]PCPA. 1-[*phenyl*-^{14}C]PCPA (10 mM) in 50 mM potassium phosphate, pH 7.2 buffer (100 μL) was added to 160 μM MAO in the same buffer (75 μL) and then was incubated at 25 °C for 90 min. Sodium borohydride (2 mg) in 0.2 N sodium borate, pH 9.5 (50 μL) was added and incubated at 25 °C for 2 h. The solution was dialyzed against four 80-mL portions of 20 mM potassium phosphate, pH 7.2 buffer for 30 min each. The enzyme solution then was treated in the same way as the unreduced sample described under Release of Radioactivity during Denaturation from MAO Reversibly Inhibited by 1-[*phenyl*-^{14}C]PCPA.

Results and Discussion

1-PCPA satisfies the usual criteria for a mechanism-based inactivator. Inactivation of MAO at different concentrations of 1-PCPA gave pseudo-first-order rates of inactivation for at least the first three half-lives, after which an upward deviation from linearity was observed. The rate of inactivation by 1-PCPA was slower in the presence of substrate (benzylamine) than in its absence (Figure 1). From a plot of 1/[I] vs. $t_{1/2}$ for the initial rates (Kitz & Wilson, 1962), the K_i for 1-PCPA was determined to be 0.20 mM, and the k_{inac} was 2.00 min^{-1} (insert of Figure 1). Dialysis of 1-PCPA-inactivated MAO against 50 mM potassium phosphate, pH 7.2 buffer resulted in recovery of some enzyme activity. The percentage of activity restored was found to be dependent upon the concentration of 1-PCPA used and the length of the incubation period. After dialysis, further incubation with benzylamine did not restore any additional enzyme activity. When MAO was treated with 1-[*phenyl*-^{14}C]PCPA, 1.06–1.40 equiv of radioactivity remained bound to the enzyme, after dialysis, relative to the amount of [^{14}C]pargyline incorporated (Chuang et al., 1974). Denaturation of 1-[^{14}C]PCPA-inactivated MAO in 6 M urea did not release a significant amount of radioactivity, and following trichloroacetic acid precipitation and $CHCl_3$/MeOH extractions of the labeled enzyme, 1.0 equiv of radioactivity remained bound (corrected for any remaining MAO activity). The cyclopropyl ring is important to inactivation since α,α-dimethylbenzylamine, the acyclic analogue of 1-PCPA, is neither an inactivator nor a substrate (2 mM, 76 h).

The interaction of 1-PCPA with MAO to produce this stable adduct is not a simple one; it is reminiscent of the inactivation

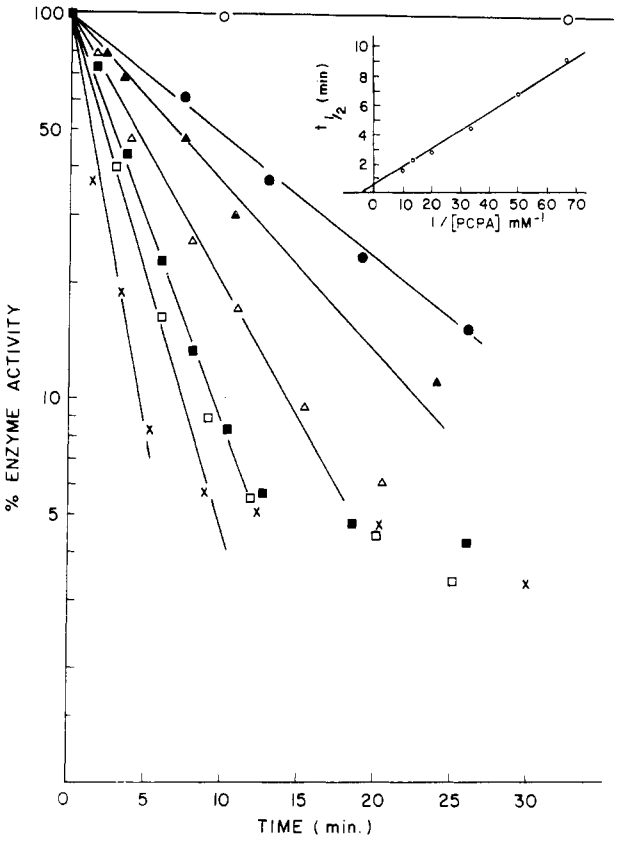
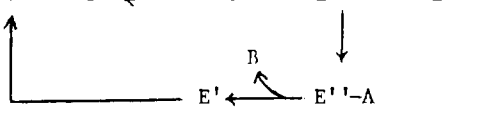


FIGURE 1: Time-dependent inactivation of MAO by 1-PCPA. MAO (7.5 μM) was allowed to incubate with 100 (×), 75 (□), 50 (■), 30 (△), 20 (▲), 15 (●), and 0 μM (○) 1-PCPA. Aliquots (15 μL) were removed at various times and diluted with 485 μL of assay solution. When 200 μM benzylamine was present with 50 μM 1-PCPA, the inactivation rate was the same as that of 15 μM 1-PCPA alone.

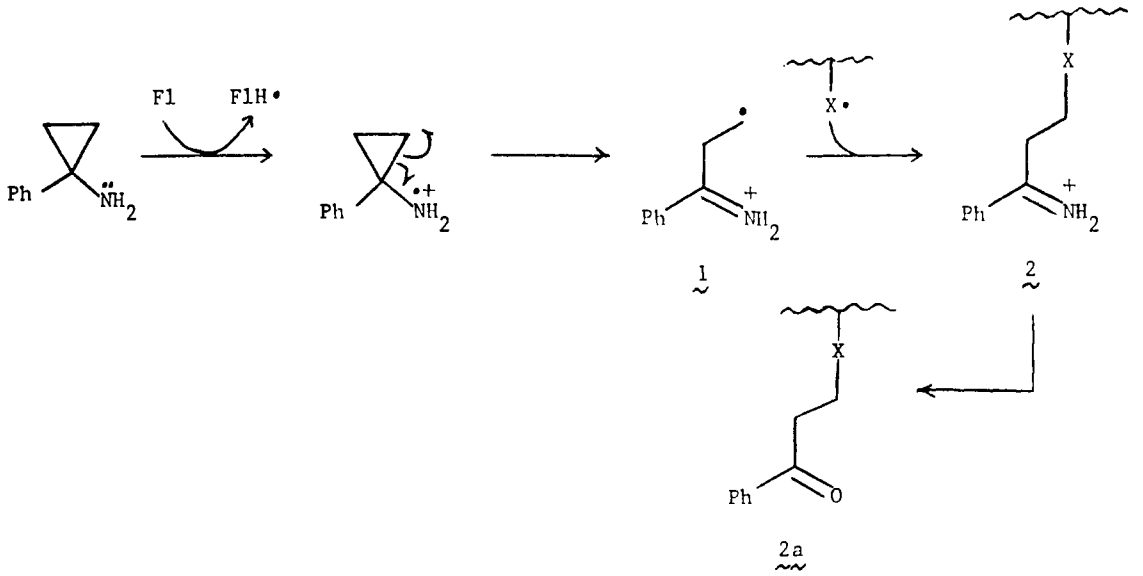
Scheme I: General Kinetic Scheme for Inactivation of MAO by 1-PCPA



of RTEM β-lactamase by certain β-lactams (Fisher et al., 1978; Brenner et al., 1981; Easton & Knowles, 1982). When MAO is allowed to incubate with molar ratios of 1-PCPA to enzyme between 1.0 and 10 over an extended period of time, the rapid partial loss of enzyme activity gradually reverses and rises to a plateau activity level of less than 100% of the control (Figure 2). A plot of enzyme activity following dialysis at the plateau level vs. the number of equivalents of 1-PCPA used (Figure 3) shows that it takes eight 1-PCPA molecules to produce complete inactivation of MAO, which then results in the attachment of one molecule (1.06–1.40 equiv) of 1-[*phenyl*-^{14}C]PCPA to the enzyme. This suggests that seven molecules of 1-PCPA are being metabolized in this process or, in other words, the partition ratio (the ratio of product formation to inactivation) is 7. A generalized reaction scheme consistent with these data is shown in Scheme I. The pathway leading to irreversible covalent inactivation (E′–A) will be discussed first, then the reversible one (E″–A) will be considered.

The steady-state rate for the irreversible component of the inactivation by 1-PCPA was determined. A half-life of 10 h at a concentration of 2.0 mM inactivator (which is 10 times the K_i and is assumed to represent saturation) indicates that the k_{inac} is 0.0012 min^{-1}. This rate is 0.0006 times that of the

Scheme II: Proposed Mechanism for Inactivation of MAO by 1-PCPA



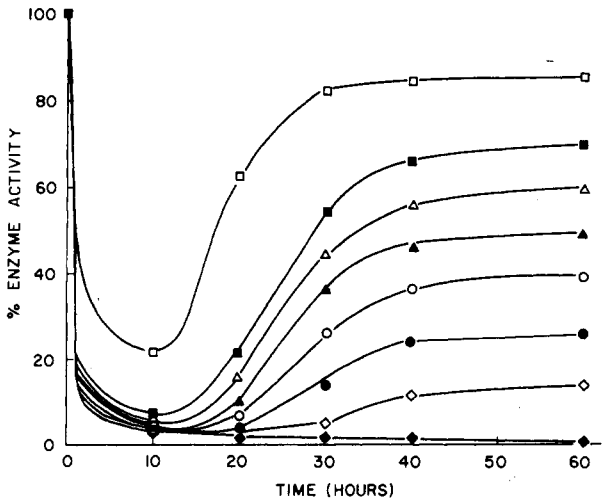


FIGURE 2: MAO activity vs. time at different 1-PCPA concentrations. MAO (7.5 μM) containing various concentrations of 1-PCPA in 50 mM potassium phosphate, pH 7.2 buffer (250 μL total volume) was incubated at 25 °C in the dark. At times indicated, a 20-μL aliquot was removed and assayed for enzyme activity, 1-PCPA/MAO ratios of 1 (□), 2 (■), 3 (△), 4 (▲), 5 (○), 6 (●), 7 (◇), and 10 (◆) were used.

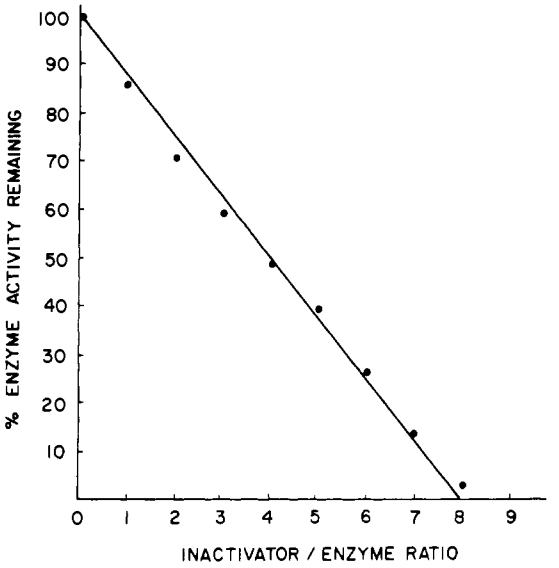


FIGURE 3: Determination of turnover number during inactivation of MAO by 1-PCPA. The plateau enzyme activity values in Figure 2 were replotted vs. the 1-PCPA to MAO ratio.

initial inactivation rate (2.0 min^{-1}) and accounts for the excessively long time (8–10 days) required for complete irreversible inactivation.

On the basis of the reported mechanism of inactivation of MAO by *trans*-2-phenylcyclopropylamine (Silverman, 1983) and by *N*-(1-methylcyclopropyl)benzylamine (Silverman & Yamasaki, 1984), a reasonable pathway for irreversible inactivation of MAO by 1-PCPA is shown in Scheme II. One-electron oxidation of 1-PCPA by the flavin would produce the amine radical cation, which, upon homolytic cyclopropyl ring opening, would lead to a reactive radical (**1**). This radical could be captured by an active site radical to give the adduct (**2**).[2] Three organic reactions were carried out on the inactivated enzyme to confirm that the structure of the adduct was that shown in Scheme II. Treatment of the adduct with sodium boro[^{3}H]hydride resulted in the incorporation of 0.73 equiv of hydride per molecule of inactivated enzyme after the amount incorporated into native enzyme was subtracted (2.34 equiv of tritium per active site vs. 1.61 equiv for the noninactivated control). The amount of hydride incorporation was determined by assuming a tritium isotope effect for this reaction of 0.31, which was the tritium isotope effect observed during a model reaction of *N*-(3-oxo-3-phenylpropyl)piperidine with sodium boro[^{3}H]hydride. This hydride incorporation result is consistent with an adduct that contains a keto group.

Phenyl alkyl ketones undergo a Baeyer–Villiger reaction (Hassall, 1957) with peroxytrifluoroacetic acid to give phenyl esters almost exclusively; saponification of the phenyl esters yields phenol. These reactions were carried out on *N*-(3-oxo-3-phenylpropyl)piperidine as a model for inactivated MAO, and a 55% yield of phenol was obtained. When MAO, which was irreversibly inactivated with excess 1-[*phenyl*-^{14}C]PCPA, dialyzed, and Pronase digested to assure solution chemistry, was treated under the identical conditions as the model reactions, [^{14}C]phenol (82% of the theoretical 5500 dpm) was isolated. TLC showed that 97% of this radioactivity comigrated with phenol. This indicates that the enzyme adduct is a phenyl ketone.

[2] Recently, Guengerich et al. (1984) showed that a series of heteroatom-substituted cyclopropanes inactivated cytochrome P-450 at rates that paralleled the single-electron oxidation potentials of the compounds. These experiments suggest a one-electron oxidation mechanism for cytochrome P-450 and indicate the feasibility of the mechanism for MAO.

Table I: Release of Radioactivity from 1-[^{14}C]PCPA-Inactivated MAO with Base[a]

	% radioactivity released with time after Dowex 1 chromatography[b]					
	0.5 h		4 h		22 h	
sample	H_2O wash[c]	NaCl wash[d]	H_2O wash[c]	NaCl wash[d]	H_2O wash[c]	NaCl wash[d]
NaCl control	0	0	1	0	7	0
NaOH ($NaBH_4$ reduced)	0	1	1	5	3	31
NaOH	9	1	11	5	46	31

[a] See Materials and Methods. [b] Relative to amount bound to enzyme before sample treatment. [c] Percent radioactivity eluted through Dowex 1 with H_2O. [d] Percent radioactivity eluted through Dowex 1 with NaCl.

The third organic reaction used to characterize the structure of the active site adduct (**2**, Scheme II) was a base-catalyzed retro-Michael reaction (Bergmann et al., 1959). The product of a Michael reaction (the addition of a nucleophile to the β-position of an α,β-unsaturated carbonyl compound) can be converted back to the nucleophile and the α,β-unsaturated carbonyl compound in the presence of base. Structure **2a** (Scheme II) is a Michael adduct (even though it, presumably, was not formed by a Michael reaction) and, therefore, is a candidate for a base-catalyzed retro-Michael reaction. Treatment with base of MAO that was labeled with 1-[*phenyl*-^{14}C]PCPA resulted in a time-dependent release of radioactivity that was shown by TLC to be [*phenyl*-^{14}C]acrylophenone (Table I). When the labeled MAO was reduced with sodium borohydride prior to base treatment, essentially no acrylophenone was released (Table I). Reduction of the ketone to an alcohol would prohibit the retro-Michael reaction by rendering the α-proton much less acidic. Released radioactivity that was retarded on Dowex 1 until eluted with NaCl presumably still contained a flavin-derived fragment and probably resulted from β-elimination of the flavinyl adduct from the protein (Silverman & Yamasaki, 1984). Incubation of labeled MAO at pH 7.9 during Pronase digestion also gave release of acrylophenone, but at a much slower rate than did hydroxide treatment; after 1, 4, and 11 days only 7%, 12%, and 28%, respectively, of the radioactivity bound to the enzyme was released as acrylophenone. The isolation of acrylophenone from this base reaction, in addition to the results of the Baeyer–Villiger oxidation and borohydride reduction, is consistent with active site adduct **2** (Scheme II).

The identity of X in Scheme II was determined in two ways. First, the change in the optical spectrum of the FAD cofactor during enzyme inactivation was monitored; oxidized flavin was converted to reduced flavin in a time-dependent process that corresponded to the decrease in enzyme activity (Figure 4A). Concomitant with partial return of enzyme activity as a result of the use of an insufficient amount of inactivator, an insufficient length of incubation time, dilution, or denaturation was a partial return of the flavin spectrum from that of reduced to the oxidized form (Figure 4B). When the enzyme was totally inactivated, essentially no reversion of reduced flavin to oxidized flavin occurred even under denaturing conditions. Addition of dithionite to the completely inactivated enzyme did not change the spectrum significantly. This suggests that, at least, the irreversible component of the inactivation involves covalent bond formation to the flavin cofactor. The second method for determining the identity of X involved Pronase digestion of the radioactively labeled enzyme. The FAD is known (Walker et al., 1971) to be covalently bonded at the 8α-position to a cysteine residue of MAO. Consequently, if complete digestion of the protein were to occur, an alkylated cysteinyl $FADH_2$ fragment with a M_r in the range of 1000–1100 would result. Since acrylophenone was shown to be released slowly from 1-[*phenyl*-^{14}C]PCPA-inactivated MAO by Pronase treatment at pH 7.9 (vide supra), the adduct

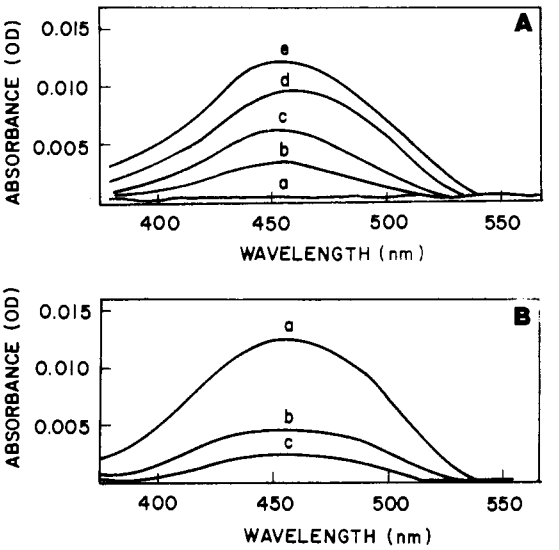


FIGURE 4: (A) Different spectrum for inactivation of MAO by 1-PCPA at various times: a (0 min), b (15 min), c (30 min), d (60 min), and e (120 min). The reference cell contained MAO (8 μM) in 50 mM potassium phosphate, pH 7.2 buffer; the sample cell also contained 15 μM 1-PCPA. (B) MAO was incubated with 50 μM 1-PCPA for 90 min relative to untreated enzyme (a). Each solution was diluted 1:2 with 50 mM potassium phosphate, pH 7.2 buffer, and the difference spectrum was recorded after 15 min (b). Each solution was then diluted 1:2 with 12 M urea in the same buffer (final urea concentration 8 M), and the difference spectrum was recorded after 15 min (c).

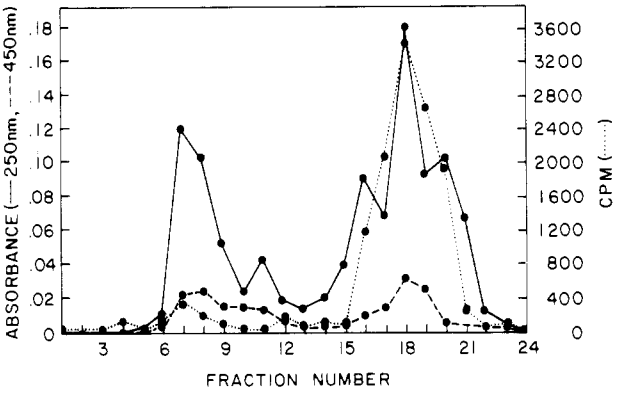


FIGURE 5: Sephadex G-25 chromatography of Pronase-digested 1-[*phenyl*-^{14}C]PCPA-inactivated MAO. See Materials and Methods.

was initially reduced with sodium borohydride prior to Pronase treatment. Chromatography on Sephadex G-25 permitted separation of the larger, partially digested fragments from an alkylated cysteinyl $FADH_2$ fragment (Figure 5); further chromatography on Sephadex G-10 (Figure 6) permitted separation of the desired fragment from the small molecules (mostly amino acids and small peptides). On both gel filtration columns the radioactivity comigrated with the absorbance at 450 nm, and this corresponded to the flavin-containing fragment.

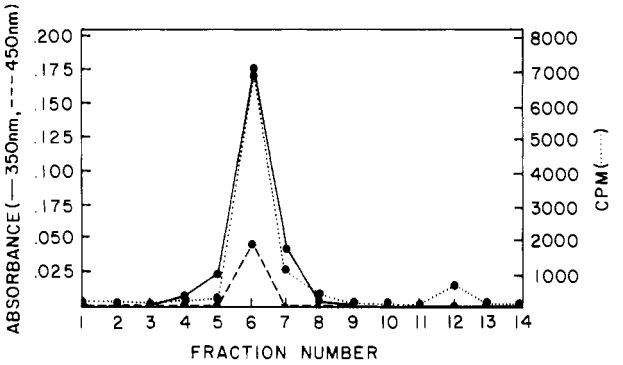


FIGURE 6: Sephadex G-10 chromatography of radioactive fractions from the Sephadex G-25 column. See Materials and Methods.

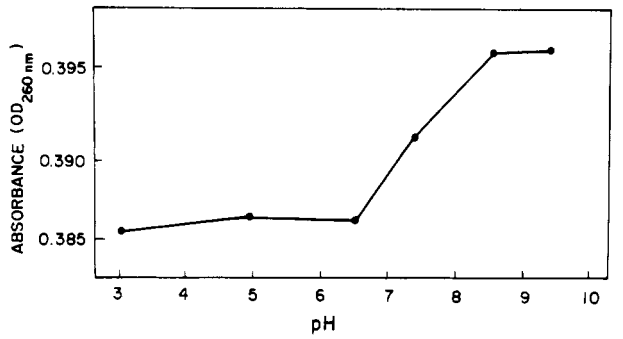
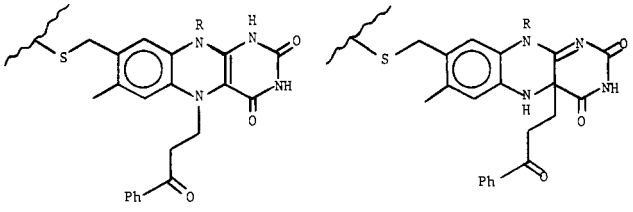


FIGURE 7: Titration of the flavin peptide isolated from 1-[*phenyl*-^{14}C]PCPA-inactivated MAO. The flavin peptide was prepared as described under Materials and Methods. The change in absorbance at 260 nm for pH values between 3 and 9.5, as measured directly with a microelectrode, was monitored.

Once it was established that the irreversible adduct was a flavin adduct, attempts were made to determine the site on the flavin to which the inactivator was bonded. The N_5 (Maycock et al., 1976) and the C_{4a} (Nagy et al., 1979) positions are the most likely sites of attachment. These two types of adducts can be differentiated because in an N_5 adduct (**3**)



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4

the N_1 proton has a pK_a between 6 and 7.5 and can be titrated, but in a C_{4a} adduct (**4**), the N_5 proton has a much higher pK_a (no resonance stabilization), and no pK_a between 2 and 10 is exhibited (Porter et al., 1973; Maycock et al., 1976). Generally, this titration test for an N_5 vs. a C_{4a} adduct is carried out on enzymes where the flavin is noncovalently bound and easily removed. However, in MAO the flavin cofactor is covalently bound to the protein where there are numerous protons possessing pK_a values in the 6–7.5 range and considerable interference at 260 nm in the optical spectrum where this titration is monitored. Consequently, the inactivated enzyme that was borohydride reduced, Pronase treated, and purified as described above was used. The borohydride reduction of the phenyl ketone also had the advantage of preventing interference of that moiety in the 260-nm range of the optical spectrum. Titration reproducibly revealed a pK_a between pH 7.1 and 7.5 (Figure 7 shows the results of one experiment). This is consistent with N_5 addition; N^5-

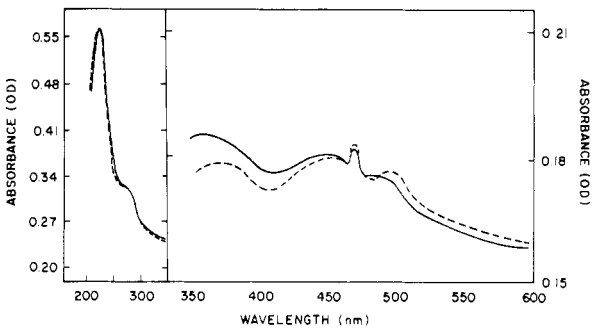


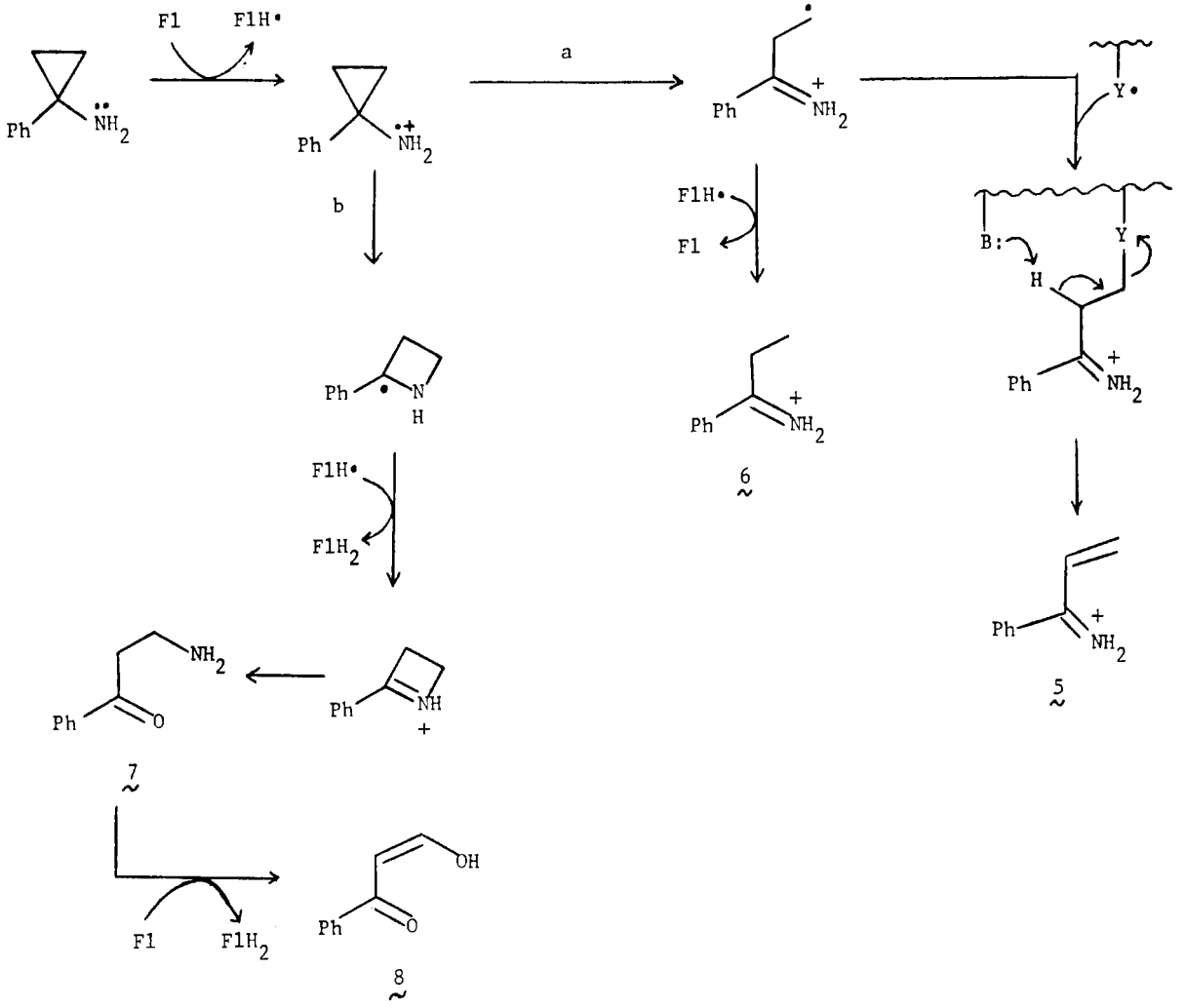
FIGURE 8: Absorption spectrum of flavin peptide isolated from 1-[*phenyl*-^{14}C]PCPA-inactivated MAO at pH 9 (—) and 2 (– –). The flavin peptide was prepared as described under Materials and Methods.

benzyllumiflavin, for example, has a pK_a of 7.2 (Walker et al., 1967). Another test for an N_5 vs. a C_{4a} adduct also was performed. The protonation of N_1 in N_5-substituted flavins causes a shift to shorter wavelengths for absorption maxima in the 200–260-nm region and a shift to longer wavelengths for absorption maxima in the 320–360-nm range. For example, the 255- and 320-nm maxima at pH 9 for 5-(cyanomethyl)dihydroflavin shift to 248 and 330 nm, respectively, at pH 2 (Porter et al., 1973). The isolated flavin peptide adduct in our work showed similar spectral changes; the absorption maxima at 208 and 352 nm at pH 9 shifted to 206 and 362 nm at pH 2 (Figure 8). All of these results suggest an N_5 adduct.

Also consistent with an N_5 adduct is its behavior in base. If the proton α to the phenyl ketone carbonyl (**3** or **4**) were removed by base, elimination of the flavinyl anion could occur, provided that the anion was an adequate leaving group. Elimination of an N_5-flavin anion (high pK_a conjugate acid) should be a poor reaction, whereas elimination of a C_{4a}-flavin anion (low pK_a conjugate acid) should be a good reaction. Also, in the case of the N_5 adduct (**3**), treatment with base would initially result in deprotonation at N_1 to form a delocalized carbanion, and this would further stabilize the adduct toward elimination. Since base is known to catalyze the cleavage of cystine residues in proteins by β-elimination of the thiomercaptide ion (Nashef et al., 1977), a competing β-elimination reaction would be the elimination of the flavin–inactivator adduct from the cysteine residue that connects the flavin at the 8α-position to the protein (Walker et al., 1971). Radioactivity that was still attached to the flavin, however, was shown to be retained on Dowex 1 ion exchange resin (and be removed by high salt concentration), whereas radioactive acrylophenone, released by β-elimination of the flavinyl anion, was unretarded. As shown in Table I, the rate of elimination of the flavin in base to give acrylophenone is quite slow; therefore, N_5 attachment seems more plausible than C_{4a} attachment.

As was mentioned above, it takes eight molecules of 1-PCPA to produce a stable 1:1 adduct with MAO; seven molecules, apparently, are metabolized in a time-dependent reaction to something that is released from the enzyme on standing or by dialysis (Figures 2 and 3). The reactivation of MAO reversibly inhibited by 1-PCPA followed unimolecular decomposition kinetics. The half-life for reactivation was 80 min, which corresponds to a rate constant for reactivation of 0.0087 min^{-1}. This rate constant is 7.25 times (0.0087/0.0012) that determined for the irreversible inactivation component (vide supra) and is consistent with the observed partition ratio of 7. The addition of benzylamine to the buffer had no effect on the rate of adduct release. This, therefore, is a different process than the reactivation of *N*-cyclopropylbenzylamine-inactivated

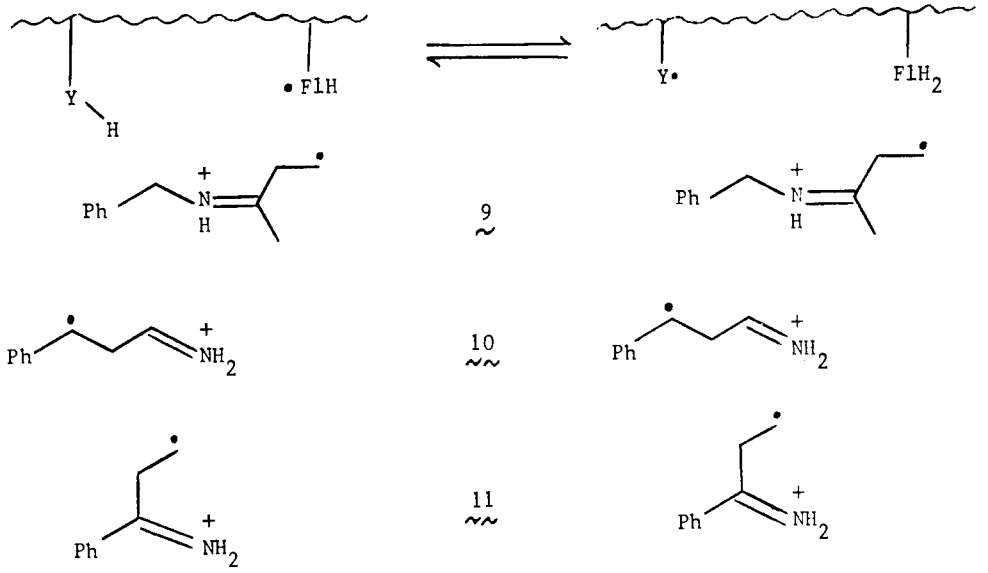
Scheme III: Possible Pathways for Metabolite Formation



MAO, which is catalyzed by benzylamine.[3] Four metabolite possibilities were prepared on the basis of two general pathways considered (Scheme III). Pathway a is in accordance with the proposed mechanism for inactivation of MAO by *trans*-2-phenylcyclopropylamine (Silverman, 1983) and *N*-(1-methylcyclopropyl)benzylamine (Silverman & Yamasaki, 1984) and involves homolytic cyclopropyl ring cleavage. After covalent bond formation, base-catalyzed (either specific as shown or general base catalyzed) elimination of HX leads to the imine of acrylophenone (**5**), which would rapidly hydrolyze to acrylophenone. Alternatively, and much less likely, electron transfer back to the intermediate radical would give the imine of propiophenone (**6**), which would give propiophenone upon hydrolysis. Pathway b would involve cyclopropyl ring expansion to the azetidine radical followed by second electron oxidation. The 2-phenylazete that would form could spontaneously hydrolyze to β-aminopropiophenone (**7**) which, as a substrate for MAO, could be oxidized further to 1-oxo-1-phenyl-3-hydroxy-2-propene (**8**). Metabolites isolated by ion exchange chromatography revealed that 7 equiv of a radioactive non-amine was produced and no radioactive amine other than 1-PCPA was detected. Eighty-two percent of the non-amine radioactivity (2870 dpm) was identified by TLC as acrylophenone; 92% of the radioactivity was identified as acrylophenone by TLC of its 2,4-dinitrophenylhydrazone derivative. This suggests that pathway a leading to the immonium salt of acrylophenone (**5**) is most viable. This pathway, however, is essentially the same as the mechanism proposed for the irreversible inactivation component (Scheme II) and also involves covalent bond formation. Since gel filtration does not release the reversible adduct, a covalent bond is plausible. Although the mechanisms shown in Schemes II and III for the irreversible and reversible adducts, respectively, are the same, the product stabilities may be different if the groups indicated as X and Y in these schemes are different. The data presented above for irreversible inactivation indicated that X is the flavin and attachment is at the N_5 position. Therefore, Y either must be something other than the flavin or be a C_{4a}-flavin adduct. Peach et al. (1980) have shown that *trans*-2-phenylcyclopropylamine inactivates MAO and forms a covalent bond to a group other than the flavin; an active site cysteine residue was suggested as a possibility. Furthermore, denaturation of this enzyme adduct at neutral pH (Paech et al., 1980) is accompanied by an elimination of the adduct from the enzyme (Silverman, 1983). If Y (Scheme III) is a group other than an N_5-flavin, e.g., an active site cysteine or a C_{4a}-flavin, the rate of elimination of cysteine or the reduced C_{4a}-flavin anion (good leaving groups) may be considerably faster than the rate of elimination of a reduced N_5-flavin anion, as discussed above. Alternatively (or additionally), a specific base needed for removal of the α-proton of the adduct may be proximal only when attachment of the inactivator is at Y, not when it is attached at the N_5 position of the flavin. Experiments that parallel those of Paech et al. (1980) were

[3] R. B. Silverman and R. B. Yamasaki, unpublished results.

Scheme IV: Bond Formation as a Result of Active Site Geometry Relative to the Position of Inactivator Radicals



carried out in order to shed light on the nature of Y and to reject this alternative explanation that specific base-catalyzed elimination is involved. Upon denaturation of 1-[*phenyl*-^{14}C]PCPA-inactivated MAO, the release of radioactivity from the enzyme exhibited unimolecular decomposition kinetics for the first 3 h with a half-life of 65 min; this corresponds to a rate constant for radioactivity release of 0.0107 min^{-1}. Concomitant with release of radioactivity, there was oxidation of the flavin; however, all of the flavin returned to its oxidized form in 10 min at which time 90% of the radioactivity was still bound to the enzyme. This indicates that Y is *not* a flavin C_{4a} adduct; it must be an amino acid residue. Also, elimination is probably not specific base catalyzed since it occurs under denaturing conditions. Further evidence for the involvement of a retro-Michael reaction in the release of acrylophenone is the observation that sodium borohydride treatment of the reversible adduct essentially prevents the release of radioactivity; after 22 h, 90% of the radioactivity remained bound to the enzyme. This is similar to the results obtained with the irreversible adduct (vide supra).

Two questions, then, need to be answered: on the basis of the mechanism shown in Scheme III, how would it be possible for a group other than the flavin to become attached to the inactivator, and why does more than one adduct form? One-electron transfer from the amine to the flavin would generate a flavin semiquinone radical. It has been suggested that there is an active site cysteine (Singer & Barron, 1945; Gomes et al., 1969), and thiols are known to be excellent hydrogen atom donors (Knight, 1974). Therefore, in order to rationalize capture of the radical generated from cyclopropyl ring opening (Scheme II or III) by a thiyl radical, it need be suggested that the flavin radical abstracts a hydrogen atom from the thiol of an active site cysteine. If this hydrogen atom abstraction is an equilibrium process, then for any turnover, either a flavin radical or a thiyl radical could be present. From the data obtained, seven out of eight turnovers occur via capture by a radical other than flavin, and one occurs by flavin radical interception. This could occur if the thiyl radical were located closer to the inactivator radical than was the flavin radical or if steric constraints made flavin radical capture more difficult for some reason. The geometries of the active site and the incipient radicals may determine what active site moiety becomes attached to the inactivator. It was shown previously that *N*-(1-methylcyclopropyl)benzylamine (Silverman & Yamasaki, 1984) formed a covalent bond only to the flavin, whereas *trans*-2-phenylcyclopropylamine (Paech et al., 1980; Silverman, 1983) only attached to an amino acid residue. These results and the mixed-bonding results of 1-PCPA could be rationalized by geometry considerations. In Scheme IV the equilibrium between the flavin semiquinone (FlH·) and the unknown amino acid residue radical (Y·) is shown. Beneath these are shown the proposed intermediates for inactivation of MAO by *N*-(1-methylcyclopropyl)-benzylamine (**9**) (Silverman & Yamasaki, 1984), *trans*-2-phenylcyclopropylamine (**10**) (Silverman, 1983), and 1-PCPA (**11**) where the immonium nitrogen is the common anchor point for all three intermediates. Although this is only a hypothetical two-dimensional schematic, it is interesting that radical **9** would lie close to the FlH· (and only flavin attachment would occur), radical **10** would lie close to the Y· (and only attachment to Y would occur), and radical **11** would be in the middle (and susceptible to bond formation with both). Others have noted attachment of inactivators to more than one site on an enzyme. It was suggested that two different active site nucleophiles were involved in the inactivation of alanine racemase by difluoroalanine (Wang & Walsh, 1981); (methylenecyclopropyl)acetyl-CoA is believed to bond partially to the FAD cofactor and partially to active site protein residues of general acyl-CoA dehydrogenase (Wenz et al., 1981).

In summary, 1-PCPA is a mechanism-based inactivator of MAO that interacts with two different sites on the enzyme. Attachment to what is believed to be the N_5 position of the FAD cofactor produces a stable 1:1 adduct having structure **2** (Scheme II). Interaction with another site on the enzyme gives a labile adduct that may have the same general structure as that of **2**, but because Y is a good leaving group, it decomposes to acrylophenone and the free enzyme.

Registry No. MAO, 9001-66-5; 1-PCPA·HCl, 73930-39-9; 1-[*phenyl*-^{14}C]PCPA·HCl, 95216-83-4; *N*-(3-hydroxy-3-phenylpropyl)piperidine, 952-51-2; β-aminopropiophenone hydrochloride, 7495-58-1; 1-phenylcyclopropanecarbonitrile, 935-44-4; 1-phenylcyclopropanecarboxylic acid, 6120-95-2; [*ring*-U-^{14}C]benzyl cyanide, 95216-84-5; 1-[*phenyl*-^{14}C]phenylcyclopropanecarbonitrile, 95216-85-6; *N*-(3-oxo-3-phenylpropyl)piperidine hydrochloride, 886-06-6; benzoylacetonitrile, 614-16-4.

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