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The Structure of the Covalent Adduct Formed by the Interaction of 3-Dimethylamino-1-propyne and the Flavine of Mitochondrial Amine Oxidase[†]

A. L. Maycock,[‡] R. H. Abeles,* J. I. Salach, and T. P. Singer

ABSTRACT: 3-Dimethylamino-1-propyne irreversibly inactivates mitochondrial monoamine oxidase from bovine liver. The inactivation results in the loss of absorption in the 450–500-nm region of the flavine spectrum and a concomitant increase in absorbance at 410 nm. For the enzyme-bound adduct $\epsilon_{410} = 28000$. The spectral properties of the adduct of the liver enzyme with 3-dimethylamino-1-propyne are similar to those observed when the pig kidney enzyme is inactivated with pargyline (Chuang et al. (1974), *J.*

Biol. Chem. 249, 2381). From a proteolytic digest of the enzyme inactivated with labeled inhibitor a flavine peptide has been isolated which contains 1 mol of inactivator/mol of flavine. The chemical and spectral properties of the adduct are those of compounds containing the structure $—N—CH=CH—CH=N^+<$. It was concluded that the flavine-inhibitor adduct is a N-5 substituted dihydroflavine and its structure has been determined.

Monoamine oxidase (MAO)¹ [EC 1.4.3.4], an enzyme containing covalently bound FAD (Nara et al., 1966; Erwin and Hellerman, 1967; Kearney et al., 1971), has been a favorite inhibitor target since 1952 when the inhibition of its activity was correlated with antidepressant activity in patients following the administration of iproniazid (Selikoff et

al., 1952). Literally hundreds of inhibitors have been described (Ho, 1972), many of which have been used in vivo to help elucidate the physiological and psychological roles of the neurohormones. Several of these compounds have been used clinically as either antidepressive or antihypertensive agents. Little is known, however, about the mechanism of action of these inhibitors.

Pargyline, an acetylenic amine, has been more extensively investigated than other inhibitors. In 1968 it was shown that inhibition of bovine kidney MAO by pargyline is irreversible (Hellerman and Erwin, 1968), but can be protected against by benzylamine; that the inhibitor combines with the oxidized form of the enzyme; and that inhibition results in the formation of a 1:1 covalent adduct between the enzyme and the inhibitor. Inactivation of MAO from pig liver mitochondria by pargyline has been studied by Orelund et al. (1973). Recently, Chuang et al. (1974) reported that the inhibition of kidney MAO by pargyline is accompanied by disappearance of the 455-nm band of the flavoquinone and the appearance of a new band at 410 nm. This fact and the observation that [7-¹⁴C]pargyline is recovered in the flavine peptide fraction after proteolytic digestion were taken as ev-

[†] From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154 (A.L.M. and R.H.A.), the Molecular Biology Division, Veterans Administration Hospital, San Francisco, California 94121, and the Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143 (J.I.S. and T.P.S.). Received September 8, 1975. This work was supported by the National Institutes of Health Grant No. GM 12633 (to R.H.A.); National Institutes of Health Special Fellowship 5F02 GM 40063-02 (to A.L.M.); and National Science Foundation Grant No. GB 60570X (to T.P.S.). This is publication No. 1056 from the Graduate Department of Biochemistry, Brandeis University.

[‡] Present address: Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065.

¹ Abbreviations used are: MAO, monoamine oxidase; pargyline, *N*-methyl-*N*-benzyl-2-propynylamine; [¹⁴C]CH₂ inactivator, 3-dimethylamino[3-¹⁴C]-1-propyne; [¹⁴C]CH₃ inactivator, 3-(*N,N*-[¹⁴C]dimethylamino)-1-propyne.

idence that the covalent adduct involves the FAD moiety and the inhibitor. Isolation of the pure adduct from proteolytic digests was not attempted.

Although the evidence cited above that covalent adducts are formed between the flavine of MAO and acetylenic inhibitors is persuasive, no adduct has ever been purified and, therefore, their detailed chemical structure is unknown. Structures for the flavine-inhibitor adduct have been proposed, however, on the basis of the similarity between the spectra of the inactivated enzyme and of compounds derived from the photochemical interaction between acetylenic substrates and 3-methyllumiflavine (Zeller et al., 1972; Gärtner and Hemmerich, 1975). Identification of enzyme-bound adducts based on spectral similarities is tenuous at best. We feel that the reliable determination of a structure requires the separation of the adduct from the apoprotein and chemical characterization of the purified adduct.

The structure of flavine-inhibitor adducts can provide information concerning the mechanism of action of these inactivators, as well as the mechanism of the interaction between flavine and substrate. In particular, it may have bearing on two central questions regarding the mechanism of action of flavines: at what point of the flavine does the substrate interact with the flavine and is there a covalent flavine-substrate intermediate (Bruice, 1975)?²

In order to definitely establish the structure of a flavine-acetylenic inactivator adduct, we have isolated and purified the modified flavine obtained from the inactivation of MAO by 3-dimethylamino-1-propyne. Since the FAD moiety of mitochondrial MAO has been shown to be covalently bound to the apoenzyme via a cysteine residue (Walker et al., 1971), the adduct could be isolated from the inactivated enzyme as a flavine peptide derivative by existing procedures (Kearney et al., 1971a). In this report we describe the chemical and spectral properties of this adduct and propose a structure for it. The mechanistic implications of this structure are also discussed.

Experimental Procedure

Materials. Dextran, type A, clinical grade was from Schwarz/Mann Division of Becton, Dickenson and Co., and ficol was from Pharmacia Fine Chemicals, Inc. Poly(ethylene glycol) 6000 was a gift from Union Carbide Corp., Chemicals Division, and Triton X-100 was purchased from Rohm and Haas. Triethanolamine, *N*-methylaniline, and dimethyl sulfate were obtained from Eastman Organic Chemicals. Florisil and formaldehyde were purchased from Fisher Scientific Co.; acid-washed Florisil (Supelcosil-ATF-061) was obtained from Supelco, Inc., and cellulose phosphate was from H. Reeve Angel & Co., Inc. The following chemicals were obtained from Aldrich Chemical Co., Inc.: dimethylamine hydrochloride, 3-dimethylamino-1-propyne, propargyl bromide, and propargyl alcohol.

[¹⁴C]Formaldehyde and [¹⁴C]dimethylamine hydrochloride were purchased from ICN Corp. Ozone was generated from oxygen with a Wellsbach Ozone Generator, Model T-23. Charcoal was Darco-G-60 from Fisher. 3-Methyl-5-(dimethylimino-1-propenyl)-1,5-dihydrolumiflavine (IV) and 3-methyl-4a,5-(3-hydroxy-1-propeno)-1a,5-dihydrolumiflavine (VI) (Zeller et al., 1972) were the gifts of P. Hemmerich and co-workers, and 3-methyl-4a,5-propano-4a,5-dihydrolumiflavine (VII) (Ghisla and Massey, 1974)

² For reviews of current work regarding these questions and the mechanism of action of flavines in general see Singer (1976).

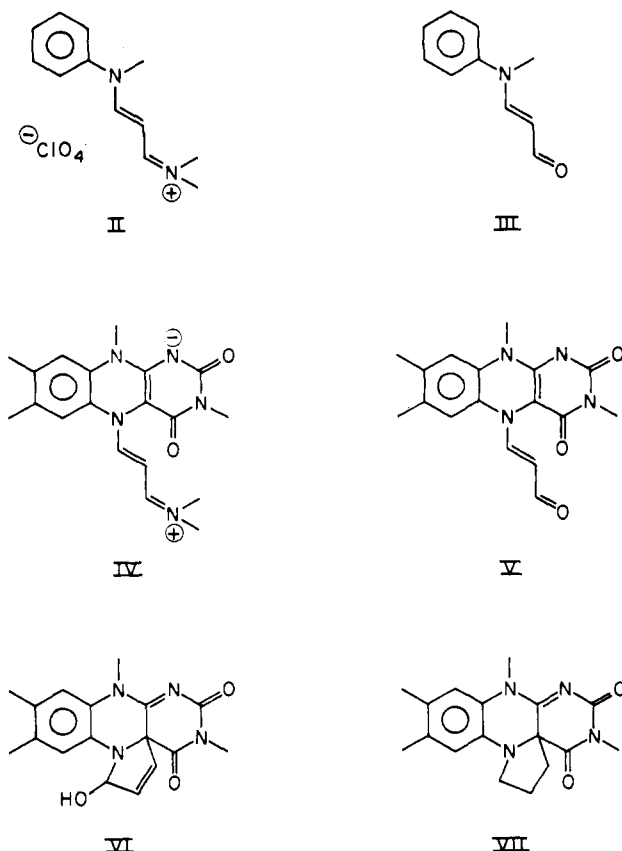


FIGURE 1: Structures of model compounds.

was donated by Dr. S. Ghisla. The structures of these compounds are shown in Figure 1. All other materials were either readily available commercial products, usually reagent grade, or were prepared as described below.

Phospholipase A used was prepared by chromatography on Sephadex (Cremona and Kearney, 1964); the enzyme was assayed and activity units were defined as previously described (Salach et al., 1971). Phospholipase C was purchased from Sigma Chemical Co. and was stated to contain 5 units/mg of protein. Crystallized trypsin and chymotrypsin were obtained from Worthington Biochemical Corp., Pronase, B grade, from Sigma Chemical Co., and aminopeptidase M from Rohm and Haas, GmbH, Darmstadt.

Analytical Procedures. MAO activity was measured as previously reported (Tabor et al., 1954) but activity was expressed as micromoles of benzaldehyde formed per minute, using the molar absorptivity of benzaldehyde ($\epsilon_{250\text{ nm}}$ 12080). Radioactivity was determined in a Packard Tri-Carb liquid scintillation counter, a Beckman Model LS-150 counter, or a Nuclear Chicago gas flow counter. Samples containing very little radioactivity were counted until at least 2000 counts had been recorded. Usually these samples were counted several times. Errors are expressed as standard deviations. Melting points were determined on a Thomas-Hoover Uni-Melt and are uncorrected. Infrared spectra were determined on a Perkin-Elmer 137 Infracord, optical spectra on a Cary 14, 15, or 118 recording spectrophotometer, and nuclear magnetic resonance spectra on a Varian Model A-60 or a Bruker 90 Fourier transform NMR spectrometer. High pressure liquid chromatography was performed on a Waters Associates Model ALC-202 liquid chromatograph with a 2-ml loop injector for preparative separations. A Tracerlab 4 π scanner was used to locate ra-

dioactivity on paper chromatograms. Protein was measured by the biuret method (Gornall et al., 1949). Glyoxal and glycolaldehyde were determined by the carbonyl assay (Böhme and Winkler, 1954) at 510 and 590 nm (Wells, 1966), respectively, and 3-dimethylamino-1-propyne was determined by the mercuric acetate method (Siggia and Stahl, 1963).

Determination of Cysteinylflavine. The cysteinylflavine content of enzyme samples was determined by the fluorometric procedure for histidylflavine analysis (Singer et al., 1971) with the following modifications (Walker et al., 1971). Enzyme samples (1–3 mg of protein) were heat denatured (95°, 3 min), cooled, and treated with 0.2 volume of 55% (w/v) trichloroacetic acid; the sedimented protein was washed twice with 5% (w/v) trichloroacetic acid, once with acetone–6 *N* HCl (100:0.8, v/v), and twice with 1% (w/v) trichloroacetic acid. The protein was adjusted to pH 8 and digested overnight at 38° with 0.1 mg each of trypsin and chymotrypsin per mg of protein. The digest was lyophilized and dissolved in 0.1 ml of formic acid and 5 μ l of performic acid mixture³ (Walker et al., 1971) was added. Oxidation of the thioether was complete in 2 hr at 0°. Formic and performic acids were removed in vacuo and the flavine peptide residue, dissolved in 1 ml of 1% (w/v) trichloroacetic acid, was hydrolyzed at 38° overnight to the FMN level. Aliquots were then adjusted to pH 3.4 and to 7.0 and their fluorescence was measured before and after reduction with dithionite. The histidylflavine content of the sample is calculated from the difference in fluorescence units at pH 3.4 and 7.0, divided by 0.9 (Singer et al., 1971). The cysteinylflavine content is then calculated from the fluorescence at pH 3.4 (expressed as riboflavine) minus the histidylflavine content, divided by 0.65. Cysteinylflavine peptides yielded, after performic acid oxidation, on the average 65% of the molar fluorescence of an equimolar solution of riboflavine.

Enzyme Preparations. Two types of MAO preparations from bovine liver were used in this study. Type 1 was isolated from outer membrane by the method previously described (Kearney et al., 1971b); specific activity was 0.5–0.8 μ mol per min per mg. Preparations of type 2, used in later experiments, were isolated by an unpublished method based on the following steps. Bovine liver mitochondria were homogenized in cold distilled water and the insoluble residue, collected by centrifugation, was resuspended in 0.1 *M* triethanolamine buffer (pH 7.2) at room temperature. This suspension was made 0.025 *M* with respect to calcium chloride and digested with phospholipase A (1 mg/300 mg of mitochondria) and phospholipase C (1 mg/500 mg of mitochondria) for 120 min at 30°. Solubilized proteins were removed by centrifugation at room temperature and the residue was resuspended in 0.1 *M* triethanolamine buffer. The enzyme was extracted by the addition of Triton X-100 (0.21 mg/mg of protein) and the insoluble residue was removed by centrifugation at 40000g for 15 min. The extracted enzyme was partitioned by the technique of Albertsson (1971) in a three-phase system consisting of 7.3% (w/v) Dextran A, 8% (w/v) ficol, and 5.3% (w/v) poly(ethylene glycol) 6000. The MAO, found in the interface between the middle and lower phases, was collected, freed of the adjacent liquid phases, suspended in 0.1 *M* triethanolamine buffer (pH 7.2), and centrifuged. The enzyme remained in

the supernatant phase and was collected by centrifugation at 250000g for 60 min. The resulting bright yellow pellet contained about 40% of the total MAO of the mitochondria and was purified about 80-fold at this stage. Specific activities were of the order of 2.0 μ mol per min per mg of protein.

The preparation of 3-dimethylamino[3-¹⁴C]-1-propyne ([¹⁴C]CH₂ inactivator) was adapted from that previously described (Reppe, 1955). In a thick-walled test tube were placed 4.4 ml of aqueous [¹⁴C]formaldehyde (9.3 $\times$ 10⁸ dpm, 44.3 mCi/mmol), 85 μ l (ca. 1 mmol) of 37% formaldehyde, 107 mg (1.3 mmol) of dimethylamine hydrochloride, and a Teflon boiling chip. The mixture was shaken occasionally for 1 hr then treated with 4 mg of CuCl and 25 μ l of 10 *M* KOH (resulting pH $\sim$ 8). The tube was shaken for 16 hr under 15 psi of acetylene. The reaction mixture was acidified with 0.5 ml of concentrated HNO₃, taken up in 125 ml of H₂O, and extracted with three 15-ml portions of CHCl₃. The chloroform layer was discarded. The aqueous solution was treated with concentrated KOH to pH >11, treated with 50 ml of saturated NaCl, and extracted several times with CHCl₃. The combined organic layers were dried over anhydrous K₂CO₃, filtered, treated with 5 ml of concentrated HCl, and evaporated to dryness. The residue was dissolved in ca. 100 μ l of H₂O, and made just basic with concentrated KOH. The mixture was made up to 1.5 ml with high-pressure liquid chromatography solvent (0.1% (w/v) ammonium carbonate–acetonitrile, 1:1) and was chromatographed over a 2 ft $\times$ $\frac{3}{8}$ in. Corasil C₁₈ column with the same solvent at 2 ml/min. Fractions of 0.4 ml were collected, acidified with HCl, evaporated to near dryness under reduced pressure, and taken up in ca. 0.5 ml of D₂O. Fractions containing the pure product, as judged by nuclear magnetic resonance (NMR), were combined, concentrated to near dryness, and diluted to 5 ml with H₂O. Yield corresponded to 5.2 mg (6%) of the free base, chemically pure by NMR and containing 3.6 $\times$ 10⁷ dpm (specific activity 0.26 mCi/mmol). That the compound was radiochemically pure was verified by diluting a small sample with the unlabeled compound and recrystallizing the oxalate derivative (Campbell et al., 1952) from ethanol to constant specific activity.

The preparation of 3-[methyl-¹⁴C]dimethylamino-1-propyne ([¹⁴C]CH₃ inactivator) was adapted from that previously reported (Guermont, 1955). [¹⁴C]Dimethylamine hydrochloride (0.1 mCi, 49.6 mCi/mmol) in 0.13 ml of 20% ethanol was added to 0.3 ml of an ethanol solution containing 54 mg (1.2 mmol) of dimethylamine in a thick-walled test tube placed in an ice bath. The volume was made up to 1 ml with ethanol and 45 μ l (ca. 0.60 mmol) of propargyl bromide was added. The tube was sealed, heated to 100° for 4 hr, cooled in ice, and opened, and its contents was acidified with 2.5 ml of 3 *N* HCl. Ethanol was removed in a rotary evaporator. The material was washed with three 1-ml portions of ether, treated with 1 ml of methylene chloride, and made basic with 40% (w/v) NaOH. The aqueous layer was separated, saturated with NaCl, and extracted three times with 1-ml portions of ether. The combined organic layers were washed with two 1-ml portions of saturated NaCl, water was added, and the organic phase was carefully removed under reduced pressure. The product contained substantial amounts of radioactive impurities at this stage. Forty percent of the product was purified by high-pressure liquid chromatography as described above. The yield corresponded to 4.5 mg (9%) of the free base, pure by NMR and containing 1.02 $\times$ 10⁷ dpm (specific activity =

³ Performic acid was prepared by treating 9 ml of formic acid with 1 ml of 30% H₂O₂ for 1 hr at room temperature.

0.086 mCi/mmol). The rest of the product was purified as described below.

The aqueous solution was acidified with HCl, concentrated, and chromatographed on paper (Whatman 3MM, ascending, with butanol-acetic acid-H₂O, 6:3:1). The desired compound (R_f 0.4) was located by scanning a small strip for radioactivity and spraying it with basic permanganate. The desired material was eluted with 0.1 *N* HCl, concentrated, and diluted to 5 ml. The yield was 7.0 mg (14%) of the free base, containing 1.58×10^7 dpm, specific activity = 0.085 mCi/mmol). Again the oxalate derivative was used to verify radiopurity of these two samples.

Isolation of Cysteinyflavine Peptides Containing Labeled Inactivator: General Methods. The method used for purification of flavine peptides depended on the type of enzyme preparation. In the case of the enzyme-inhibitor adduct of the type 1 enzyme, digestion and purification of the peptides followed the method described earlier (Kearney et al., 1971a) with the following modification. Prior to cellulose phosphate chromatography the flavine peptides were chromatographed on an acid-washed Florisil column (2 × 15 cm). This was washed with 5% (v/v) acetic acid and water, and eluted with 5% (v/v) pyridine as in chromatography on untreated Florisil. In the preparation of the flavine peptides labeled with [¹⁴C]CH₂ inactivator the initial chromatography on untreated Florisil was omitted.

The labeled flavine peptides eluted with 5% pyridine from acid-washed Florisil were lyophilized and applied to cellulose phosphate in 0.02 *M* pyridinium acetate (pH 4.0) as described (Kearney et al., 1971a). The eluted flavine-adduct peptides from this chromatography were then further purified, as follows. The lyophilized material was dissolved in 2.0 ml of 60 mM phosphate (pH 7.0) and digested at 37° with 2.0 mg of aminopeptidase M for 16 hr. Following digestion, the pH was adjusted to 4.0 and the material was chromatographed on a second cellulose phosphate column (0.9 × 11 cm) as before. The radioactive band was lyophilized, placed on Whatman No. 1 paper, and subjected to high voltage electrophoresis at pH 1.6 in 8% (v/v) formic acid, 50 V/cm. The location of the radiolabel was determined by radio-autography and the band eluted from the paper. High voltage electrophoresis was repeated in 10% pyridine (v/v), which had been adjusted to pH 6.5 with acetic acid, again at 50 V/cm. In the preparation of the adduct with [¹⁴C]CH₂ inactivator, the electrophoresis step at pH 1.6 was omitted.

In type 2 preparations, purification of the flavine peptide-inactivator adduct was considerably less extensive. Following the trypsin-chymotrypsin digestion step, Pronase was added (1 mg/20 mg of protein) and the digestion continued an additional 24 hr at 38° in the presence of toluene as a bacteriostatic agent. The chilled digest was precipitated with 5% (w/v) trichloroacetic acid and the flavine peptides in the supernatant liquid hydrolyzed to the mononucleotide level by heating to 110° for 3 hr in a sealed tube. The cooled hydrolysate was chromatographed on an acid-washed Florisil column (2 × 30 cm) in the manner just described for type 1 preparations. The majority of the radioisotope applied (67%) appeared in the 5% pyridine eluate which was lyophilized and used without further purification for chemical degradation studies.

Isolation of Cysteinyflavine Peptides Containing Labeled Inactivator: Specific Experiments. In experiment 1, 243 mg of type 1 enzyme preparation (specific activity = 0.54 μmol per min per mg of protein) was incubated with 41

Table I: Isolation of Cysteinyflavine-Inactivator Adduct.

Step	[¹⁴ C]CH ₃ Inactivator	[¹⁴ C]CH ₂ Inactivator	
	Expt 1 (nCi)	Expt 2 (nCi)	Expt 3 (nCi)
Proteolytic digest	26.8	100	294 ^a
Cl ₃ CCOOH extract of digest	23.4	81	
Standard Florisil chromatography	9.1		
Acid-washed Florisil	8.4	57	30
First cellulose phosphate column	7.3	60	
Second cellulose phosphate column	2.5	37	
High voltage electrophoresis			
At pH 1.6	1.8		
At pH 6.5	0.41	35	
Repeat acid-washed Florisil			
Acetic acid eluate		24 (24%)	
0.5–20% pyridine fractions		6.2 (6%)	

^a Expected 73–97 nCi based on flavine.

μmol of [¹⁴C]CH₃ inactivator in 44 ml of P_i buffer (pH 6.8) at 30° in the dark. Inhibition was 99% by 30 min. The reaction mixture was chilled in ice and 0.1 volume of 55% (w/v) trichloroacetic acid was added. The flavine peptide adduct was then isolated as described above. (Data are summarized in Table I, experiment 1.)

In experiment 2, 263 mg of type 1 enzyme preparation (specific activity = 0.66) was incubated with 3.75 μmol of [¹⁴C]CH₂ inactivator in 15.3 ml under the conditions of experiment 1. The reaction was halted after 60 min (~90% inhibition) as above. Purification of the adduct is summarized in Table I, experiment 2.

In experiment 3, 453 mg of type 2 enzyme (specific activity = 1.94 μmol per min per mg) was incubated with 3.4 μmol of inhibitor in 17.4 ml under the conditions described below for experiment 4. The difference spectrum (inhibited enzyme vs. untreated enzyme) at this point is shown in Figure 2. The adduct, designated adduct 3, was only partially purified from the proteolytic digest in order to minimize losses (Table I, experiment 3).

In experiment 4, aliquots of the same enzyme preparation were treated with both [¹⁴C]CH₃ inactivator and [¹⁴C]CH₂ inactivator and the adducts were isolated side by side (Table II). The adducts were formed by incubating 8 mg of type 2 enzyme (specific activity = 1.7 μmol per min per mg of protein) in 2.2–2.3 ml of 0.1 *M* triethanolamine buffer (pH 7.2) at 30° in the dark with 3.7 μmol of inhibitor. Inhibition was complete in 20 min with both samples. The resulting enzyme adduct was precipitated, washed, and digested with trypsin and chymotrypsin as described (Kearney et al., 1971a) with the following modification. A gas train containing 6 *N* HCl in each of two traps for each digest and moist nitrogen as a carrier gas was present during digestion in order to collect volatile amines. Digests were precipitated with 5% (w/v) trichloroacetic acid and digestion was repeated on the insoluble portions using half the original amounts of proteases. The fractions soluble in trichloroacetic acid were combined and the two radiolabeled flavine adducts were submitted to chromatography on regular (untreated) Florisil columns (1 × 20 cm). The ¹⁴C-labeled flavines were very strongly retained and were only partially eluted by 0.05 *M* NH₄OH. These eluates were kept in ice and were immediately evaporated to dryness in a

Table II: Effect of the Location of ^{14}C in the Inactivator on the Recovery of ^{14}C in the Adduct.

Step	$[^{14}\text{C}]\text{CH}_3$ Inactivator		$[^{14}\text{C}]\text{CH}_2$ Inactivator	
	nmols of ^{14}C	%	nmols of ^{14}C	%
Proteolytic digest of Cl_3CCOOH precipitate	106	(100)	48	(100)
Cl_3CCOOH soluble part of above	85.2	80	47.0	99
Cl_3CCOOH insoluble part of above	20.7	20	0.55	1
HCl vapor trap	0.8	<1	0.3	<1
Standard Florisil chromatography				
Acetic acid and water eluates	0		0	
0.05 M NH_4OH eluate	46.3	44	27.7	58
Concentration on rotary evaporator				
Nonvolatile	24.4	23	26.7	56
Volatile	13.8	13	0.8	2

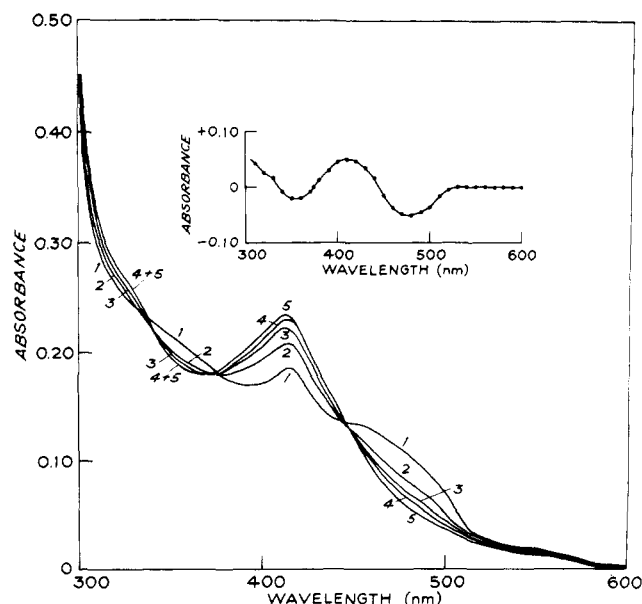


FIGURE 2: Spectral changes accompanying inhibition of MAO by 3-dimethylamino-1-propyne. MAO, 0.38 mg (specific activity 2.81), equivalent to 2.3 nmol of cysteinylflavine, was incubated with 2.4 μmol of inhibitor (5.23 mM final) in 0.455 ml of 50 mM NaP_i (pH 7.2) containing 1.1% (w/v) Triton X-100 at 22°. Spectra were taken at intervals of 10 min against a reference containing all additions except enzyme. Curve 1, native enzyme; curves 2, 3, 4, and 5: 10, 20, 30, and 40 min after addition of inhibitor. After 40 min the enzyme was 88% inhibited. Inset: difference spectrum of native enzyme vs. inhibited enzyme after 50-min incubation.

rotary evaporator. H_2SO_4 (6 M) was added to the vapor trap. The contents of the acid trap were concentrated in the same evaporator to a small volume, carefully adjusted to pH 3.8 at room temperature, and further concentrated. Aliquots of each trap were then counted together with the flavine adduct which remained.

Adduct pK_a : From the large number of optical spectra taken at different pH values it was possible to estimate the pK to be between 4 and 5.

Ozonolysis of Adduct with Glyoxal Carrier. One milliliter of adduct 3, containing 2146 cpm, was diluted to 2 ml with water (final pH 4.5–5.0), treated with 1.23 μmol of glyoxal, and cooled in an ice bath. Excess ozone ($\sim 80 \mu\text{mol}$) was passed through the solution over a 2-min period. The solution was kept in ice an additional 2 min, then purged with a moderate stream of N_2 for 2 min to remove excess O_3 . 2,4-Dinitrophenylhydrazine ($\sim 10 \mu\text{mol}$ in 1.0 ml of 2 N HCl) was added and the solution was refrigerated overnight after standing at room temperature for 2 hr. The

precipitate was collected by centrifugation and its specific activity was determined. The crude material was then purified to constant specific activity by chromatography first over Celite (Schwartz, 1962), then over silica gel G (Ronkainen, 1967). A control sample was treated in exactly the same manner, except that O_2 was substituted for O_3 . The results are presented in Table III.

In a similar experiment 901 cpm of the same adduct sample plus 50 μl of acetic acid and 0.087 mmol of glyoxal was diluted to 2.9 ml with water and treated with excess ozone at 0°. After 5-min incubation in an ice bath, it was further treated with 10 mg of zinc dust and allowed to stand at room temperature for 1 hr. The zinc was centrifuged down, the supernatant liquid removed, and the latter treated with 200 μl of 20% trichloroacetic acid,⁴ then diluted to 4.9 ml. Semicarbazide hydrochloride (100 mg) was added to the stirred solution; a white precipitate formed which was centrifuged after 15 min, washed twice with 5-ml portions of H_2O and washed twice with 5-ml portions of ethanol, and dried. The specific activity of the glyoxal bissemicarbazone was determined and it was converted to the bis-2,4-dinitrophenylhydrazone derivative (Wagner et al., 1966) by stirring it overnight with a filtered solution prepared from 2 g of 2,4-dinitrophenylhydrazine and 200 ml of 2 N HCl. The orange precipitate was collected and washed extensively with ethanol and water, and its specific activity was determined (Table III).

Adduct with Base. A 50- μl sample of adduct 3 was added to ca. 0.7 ml of 0.05 M sodium pyrophosphate (pH 9.0). The visible spectrum was recorded at 10, 50, and 135 min. No change was observed. After 150 min sodium dithionite was added. Again, no spectral change was noted.

A 0.20-ml sample of adduct 3 was added to 0.70 ml of water and the visible spectrum was recorded. One microliter of 5 N NaOH was added and the spectrum was again recorded. Then 100 μl of 5 N NaOH was added (final pH 12.7) and the spectrum was recorded after 5, 35, and after 77 min. After 90 min sodium dithionite was added, the spectral change was recorded, and O_2 was passed through the solution. The subsequent slow decay of absorption in the 450-nm region was monitored for 6 hr. An aliquot of the final solution was counted; the remaining solution was treated with 10 mg of charcoal and another aliquot was counted. The data appear in Table IV.

Preparation of *N*-Phenyl-*N*-methyl-3-aminopropenal (III) (Makin et al., 1971). To a stirred solution of 100 ml

⁴ Trichloroacetic acid was added only to duplicate the conditions previously used to prepare the semicarbazide (Wagner et al., 1966).

Table III: Ozonolysis of the Adduct from [^{14}C]CH₂ Inactivator.

Reagent	Carrier (nmol)	Adduct (cpm)	Cpm after Derivatizing the Carrier		Specific Activity of Purified Derivative (cpm/ μmol)	Yield of Compound (%)
			In Derivative	In Supernatant Liquid		
O ₂	Glyoxal (1.23)	2146	93	1660	7 $\pm$ 2 ^a	<1
O ₃	Glyoxal (1.23)	2146	1232	444	1018 $\pm$ 40 ^b	55
O ₃	Glyoxal (86.6)	901		152	6.8 $\pm$ 0.3 ^c	65

^a Bis-2,4-dinitrophenylhydrazones (2,4-Dnp) after chromatography over Celite. ^b 2,4-Dnp purified to constant specific activity by chromatography over Celite, then over silica gel G. ^c 2,4-Dnp had same specific activity as the bissemicarbazone from which it was prepared.

Table IV: Adsorption of Radioactivity onto Charcoal Following Treatment of the Adduct under a Variety of Conditions.^a

Reagent	Cpm in Solution after Reaction		% of Radioactivity Not Adsorbed
	Before Charcoal Treatment	After Charcoal Treatment	
None		10	5
pH 1.3, 11 hr	187	29	16
pH 9, 2.5 hr	79	-2	0
pH 12.9, 2.5 hr	193	116	60
pH 12.7, 20 hr	296	302	102

^a After the adduct was treated under the conditions described, an aliquote of the solution was counted. The remaining solution was treated with 10 mg of charcoal and centrifuged, and an aliquot of the supernatant liquid was counted.

of benzene (dried over anhydrous sodium sulfate), 3.7 g (66 mmol) of propargyl alcohol (97%), 8.4 g (79 mmol) of freshly distilled *N*-methylaniline, and 33.0 g of manganese dioxide (Attenburrow et al., 1952) were added in ten approximately equal portions at 30-min intervals. The mixture was stirred under a drying tube for 18 hr, filtered, and concentrated in the rotary evaporator. The brown oil was distilled to give 3.1 g (29%) of viscous yellow oil: bp 30–32° (<1 mm) [lit. (Makin, 1971) bp 145–147° (3 mm)]; NMR (D₂O) 3.4 (s, 3 H), 5.5 (broad m, 1 H, disappears in D⁺), 7.2–7.6 (m, 5 H), 7.7 (d, *J* = 12 Hz, 1 H, collapses to singlet in D⁺), and 8.9 ppm (d, *J* = 9 Hz, 1 H, collapses to singlet in D⁺); uv λ_{max} (H₂O) 306 (ϵ 32500) (lit. Makin et al., 1971) λ_{max} (EtOH) 302 (ϵ 32000).

The preparation of *N*-(3-dimethylaminoprop-2-en-1-ylidene)-*N*-methylaniline perchlorate (II) was adapted from known procedures (Brederick et al., 1968; Arnold and Holý, 1963). To compound III (743 mg, 4.6 mmol), stirred in 5 ml of benzene in a flask protected from moisture, 0.5 ml of dimethyl sulfate was added all at once. The solution was stirred for 21 hr during which time a red oil separated. Chloroform (25 ml) passed over basic alumina was added; the solution was cooled to below -40° and 0.5–0.6 ml of condensed dimethylamine was evaporated into the stirred solution. The solution was then stirred 1.5 hr at room temperature. The solvent was removed in the rotary evaporator; the residue was suspended in 5–6 ml of H₂O and 8 ml of 50% sodium perchlorate monohydrate was added. The solid was filtered off and taken up in hot H₂O. The hot solution was filtered rapidly and set aside to cool. Crude product was 185 mg (14%) of yellow crystals, mp 146–148.5°. A small amount was recrystallized from H₂O to give pale yellow plates: mp 148–149° [lit. (Brederick et al., 1968) mp 143–146°]; NMR (D₂O) 3.27 (3 H, s), 3.42 (3 H, s), 3.56 (3 H, s), 5.62 (1 H, t, *J* = 12 Hz), 7.24–7.67 (5 H, m), and

7.73–8.02 ppm (2 H, distorted doublet of doublets). In addition three small peaks of equal intensity appeared at 2.9–3.6 ppm. These were always in the same ratio to the NCH₃ peaks and could not be removed by repeated crystallization. They may be due to the presence of a conformational isomer, uv λ_{max} (H₂O) 327 (ϵ 45000).

Hydrolysis of III in Acid. Compound III was hydrolyzed on a preparative scale to identify the major basic product. Compound III (48.8 mg, 0.303 mmol) was dissolved in 50 ml of ethanol, and 2 ml of concentrated HCl was added. After 13 hr 18% of the original 306-nm band remained. The mixture was concentrated to ca. 10 ml in the rotary evaporator and diluted to ca. 50 ml with H₂O. The basic materials, isolated by standard acid–base extraction procedures, were dissolved in CDCl₃, and the NMR spectrum was recorded. Only *N*-methylaniline was present. A known amount of pure *N*-methylaniline was added and the spectrum recorded again. The enhanced peak intensity showed that the original reaction mixture had 32.5 mg (62%) of *N*-methylaniline as the only basic product detectable by NMR.

Hydrolysis of III in Base. To compound III (54.4 mg, 0.34 mmol) in 100 ml of 50% ethanol 10 g of NaOH was added with good stirring. No further reaction took place after 1.5 hr, as judged by the absorption spectrum. The solution was cooled in ice and slowly acidified to pH <1 with concentrated HCl. The solution was concentrated to ca. 30 ml, decanted from the solid which formed and worked up, as described above for the acid-catalyzed hydrolysis of III. The yield was 24.5 mg (68%) of *N*-methylaniline as the only basic product detectable by NMR.

Ozonolysis of IV. Small samples of 3-methyl-5-(dimethylimino-1-propenyl)-1,5-dihydroflavine (IV, Figure 1) were ozonized to less than completion (as judged by uv spectra) and dimethylformamide was detected in the mixtures by NMR. Selective enhancement of the proper peaks occurred when authentic dimethylformamide was added to the mixtures.

Another sample was treated successively with excess ozone, zinc dust, and 2,4-dinitrophenylhydrazine. The precipitate was removed by centrifugation, washed extensively with warm 50% ethanol, and examined by TLC in a system which separates bis-2,4-dinitrophenylhydrazones efficiently (Ronkainen, 1967). The major product was identical with the derivative of glyoxal.

Results

Nature of the Inactivation Process. It is known (McEwen et al., 1969; Hellerman and Erwin, 1968) that MAO of human liver and bovine kidney is inhibited by 3-amino-1-propyne and related compounds by dual mechanisms: there is an immediate competitive inhibition (Figure 3) followed

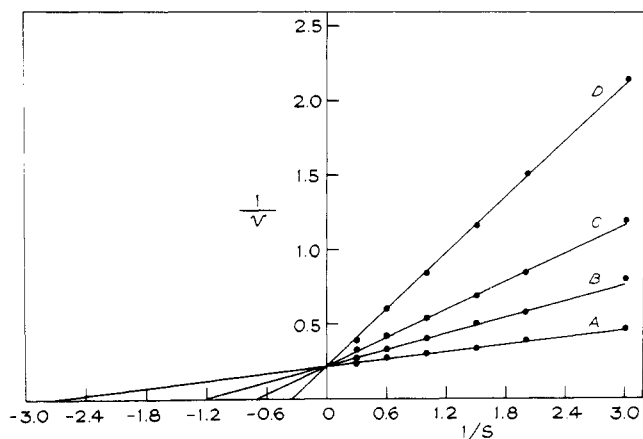


FIGURE 3: Lineweaver-Burk plots indicating apparent competitive inhibition of MAO by 3-dimethylamino-1-propyne. Substrate, in amounts indicated, together with inhibitor in 50 mM NaP_i (pH 7.2) was brought to 30° and the reaction initiated by addition of 0.024 mg of enzyme (specific activity = 2.59 μ mol per min per mg). Final pH was 7.1 in 3.0 ml total volume. (A) In absence of inhibitor; (B) in presence of 1.76 mM inhibitor; (C) 3.33 mM inhibitor; and (D) 6.70 mM inhibitor. Abscissa, reciprocal concentration of benzylamine (mM); ordinate, reciprocal activity, expressed as μ mol of substrate oxidized per min per ml.

Table V: Irreversibility of Inactivation and of Inhibitor Binding to MAO by Gel Filtration and Dialysis.

Expt	Treatment	Enzyme Activity (μ mol/min per mg of Protein)	Radioactive Inhibitor Bound (nmol/mg)
I	Untreated enzyme	3.11	
	After incubation with inhibitor	0	
	Same, after exclusion on Sephadex G-50	0.02	8.97
	Same, after precipitation with trichloroacetic acid and proteolytic digestion		7.65
II	Untreated enzyme	2.96	
	After incubation with inhibitor	0.006	
	After dialysis	0.003	
III	Untreated enzyme	3.11	
	After incubation with inhibitor	0.06	
	After gel exclusion on Sephadex G-25, precipitation, and proteolysis		6.32

by a time-dependent, irreversible inhibition (inactivation) (Figure 4). As will be shown below, this is also true of the inhibition of bovine liver MAO by 3-dimethylamino-1-propyne ($(\text{CH}_3)_2\text{NCH}_2\text{C}\equiv\text{CH}$ (I)) the inhibitor used in the present study.

In initial rate measurements (30 sec to 3 min assay period, depending on the inhibitor concentration) compound I was found to be a pure competitive inhibitor. The K_i values varied somewhat with the concentration of inhibitor. The K_i values, with inhibitor concentrations in parentheses, were: 1.4 mM (1.8 mM), 1.2 mM (3.3 mM), and 1.0 mM, while the K_m for benzylamine was 0.37 mM.

Inactivation by compound I is a first-order process but occurs more slowly than in the case of pargyline (Heller-

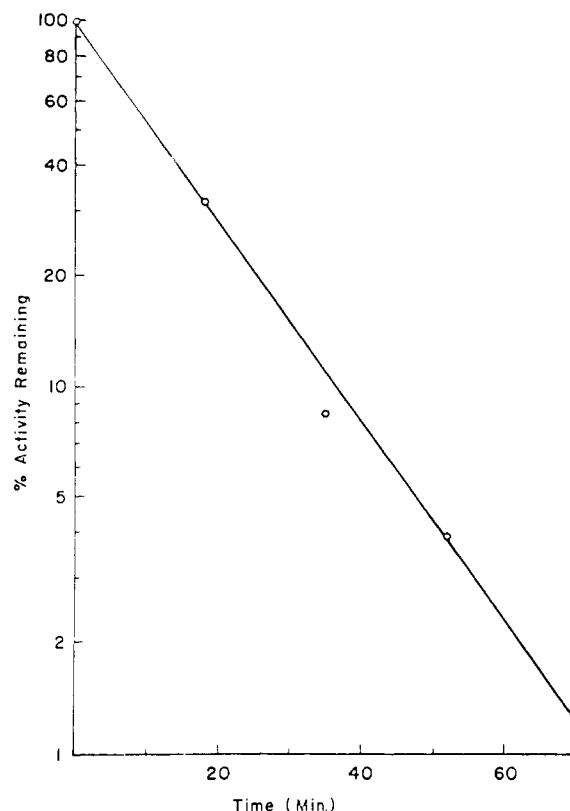


FIGURE 4: Inactivation of MAO with 3-dimethylamino-1-propyne. Enzyme (1.7 ml; 4.2 mg/ml; specific activity = 0.26 μ mol per min per mg) was mixed with 0.85 ml of 0.2 M sodium phosphate (pH 7.8) and treated with 50 μ l of a 0.017 M solution of the inactivator (final concentration 3.3×10^{-4} M). Aliquots were assayed until the enzyme was 99% inactivated.

man and Erwin, 1968). Since benzylamine protects the enzyme against the action of compound I, the inactivation process must require combination of the inhibitor at the substrate site. Once irreversible inactivation occurs, neither extensive dialysis nor gel exclusion on Sephadex reactivates the enzyme (Table V). When MAO is inactivated with compound I labeled either in the methyl group, [^{14}C]CH₃ inactivator, or in the methylene group, [^{14}C]CH₂ inactivator, radioactivity becomes tightly bound to the protein, as shown by the retention of radioactivity in protein fractions recovered from gel exclusion on Sephadex or from precipitation with trichloroacetic acid (Table V).

In previous studies with [7- ^{14}C]pargyline (Chuang et al., 1974) it was found that exactly 1 mol of labeled inhibitor became bound to MAO on complete inactivation. With the inhibitor used in the present study a considerably higher ratio of inhibitor binding per mole of enzyme was observed. Thus, when 1250 nmol of [^{14}C]CH₂ inactivator was reacted with 12.5 nmol (based on cysteinylflavine content) of highly purified MAO and the reaction mixture was passed through either Sephadex G-25 or G-50, and the excluded fraction precipitated with trichloroacetic acid then digested with trypsin-chymotrypsin, the resulting crude flavine peptide extract contained 29 (Sephadex G-25 experiment) to 35 nmol (Sephadex G-50 experiment) of radioactivity. This corresponds to a ratio of 2.3–2.8 mol of inhibitor per mol of cysteinylflavine. Although the determination of flavine in the study of Chuang et al. (1974) was an approximation based on bleaching of the enzyme with dithionite and an assumed molar absorptivity, rather than analysis for cysteinylflavine as in the present study, this would not account

for the discrepancy in the amount of incorporated label. A more plausible explanation is that the slow oxidation of compound I gives rise to product(s) which combine nonspecifically with MAO itself, as well as with protein impurities. Hence, only a part of the protein-bound radioactivity in proteolytic digests of MAO inactivated by [^{14}C]CH₂ inactivator represents specific binding to the flavine. This situation emphasizes the importance of purifying the adduct by a procedure selective for cysteinylflavine peptides, in order to assure that the radioactivity in the final purified adduct is all cysteinylflavine bound (see below).

Figure 2 shows the spectral changes occurring during the inactivation of MAO with compound I. As in the case of pargyline (Chuang et al., 1974), concurrently with disappearance of the long wavelength absorption of the enzyme in the 450–500-nm region, absorbance at 410 nm rises as inactivation develops. Based on cysteinylflavine content, the molar absorptivity of the inhibited species is ϵ_{410} 28000 (average value from three experiments). These values may be compared with the optical properties of the kidney holoenzyme–pargyline adduct, λ_{max} 410 nm (ϵ 32300), calculated from Figure 1 of Chuang et al., (1974), and of a proteolytic digest of that adduct, λ_{max} 398 nm (ϵ 29900), pH unstated.

Purification and Physical Properties of the Adduct. The adduct was purified from tryptic–chymotryptic digests by a procedure (Table I) similar to that previously reported for the isolation of a cysteinylflavine pentapeptide from uninhibited MAO (Kearney et al., 1971a). As a result, the highly purified adduct was expected to be at the phosphorylation level of FMN and to contain a short peptide bound to the 8 α position of the flavine nucleus by a thioether bond.

Since on inactivation of MAO with [^{14}C]CH₂ inactivator nearly twice as much radioactivity becomes spuriously bound to protein as is specifically bound to the flavine moiety itself (above), one would expect substantial losses of radioactivity as the adduct is purified. In fact, all spurious radioactivity must have been removed by the end of the first chromatography on Florisil (Table I) since the subsequent yields of radioactivity are those expected from comparable yields of the cysteinylflavine peptide during its isolation from uninhibited MAO. Furthermore, in subsequent purification steps the ratio of absorbance to ^{14}C content of the adduct remains essentially constant.

If oxidation of [^{14}C]CH₂ inactivator to the aldehyde and subsequent combination of the latter with protein accounts for incorporation of some of the spuriously bound radioactivity, then less unspecifically bound radioactivity would be expected in the adduct derived from [^{14}C]CH₃ inactivator and hence less radioactivity should be lost upon purification. Contrary to this expectation, most of the radioactivity was lost during purification of this adduct (Table I, experiment 1). At no time, however, was any significant change in the optical spectrum of the adduct observed. Most notably an absorbance increase in the 450-nm region, which would have indicated the formation of oxidized flavine, did not occur. The only reasonable way to account for the large loss of radioactivity from the [^{14}C]CH₃ inactivator adduct, compared to that from the [^{14}C]CH₂ inactivator adduct, is by the release of dimethylamine. Some effort was made to determine under what conditions dimethylamine was most likely to be expelled. The experiments in Table I were carried out under similar but not identical conditions so that direct comparison of the losses of radioactivity cannot be made. In the experiments of Table II the same enzyme preparation was used with the two types of labeled inhibitor

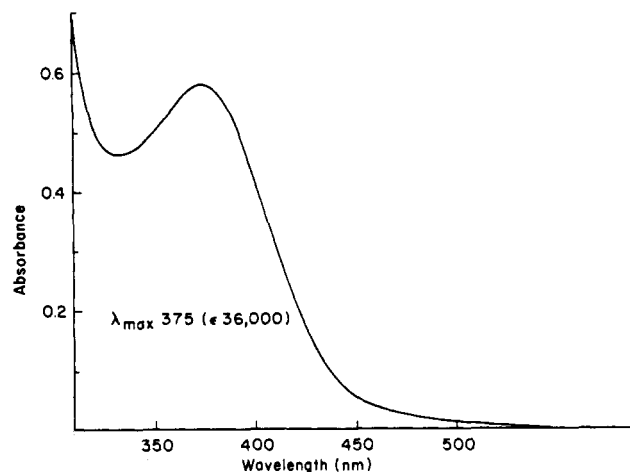


FIGURE 5: Absorption spectrum of cysteinylflavine peptide adduct isolated from beef liver MAO inhibited with 3-dimethylamino[3- ^{14}C]-1-propyne. The sample is the final acetic acid eluate, Table II, experiment II, dissolved in water.

and the isolation of the adducts was performed side-by-side by identical procedures, representing the early steps of the isolation method summarized in Table I. These experiments show that radioactivity, presumably dimethylamine, is lost much more readily under basic conditions (50 mM NH₄OH) than under acidic ones. It has been reported (Oreland et al., 1973) that MAO adducts obtained from [7- ^{14}C]pargyline also lose radioactivity under strongly basic conditions.

The purity of the adduct isolated and characterized below is based on the following considerations. After the initial Florisil step (Table I) at each stage radioactivity coincided with the yellow color and no counts could be detected elsewhere. Electrophoresis at two pH values (Table I, experiment 1) gave a single band of radioactivity. Further, the molar absorptivity (ϵ 36000) calculated from the ^{14}C content of the purified adduct from experiment 2 (Table I) agreed satisfactorily with that of the holoenzyme–inhibitor adduct (ϵ_{410} 28000) based on cysteinylflavine content and with similar data reported in the literature for the adduct formed with pargyline (Chuang et al., 1974). These data also suggest the absence of any significant amounts of non-specific label in the purified adduct.

The highly purified adduct migrates to the anodes at pH 6.5 but only half as fast as does FMN. The material does not fluoresce significantly but does show an intense band in the visible (Figure 5). The absorption is shifted from that of the holoenzyme–inactivator adduct and is pH dependent: pH 8, λ_{max} 380 nm (ϵ 33500); pH 2, λ_{max} 365 nm (ϵ 35600). The molar absorptivities were calculated from the specific activity of [^{14}C]CH₂ inactivator by assuming a 1:1 ratio of cysteinylflavine peptide to inactivator and that all spurious radioactivity had been removed. The similarity of the molar absorptivity of the adducts at the flavine peptide stage in the present study and in the report of Chuang et al. (1974) implies that the assumption of a 1:1 stoichiometry in our purified adduct is justified. The pH dependence of the maximum suggests an approximate pK_a of 4–5.

A sample of adduct from [^{14}C]CH₂ inactivator which had been partially purified was used in subsequent chemical degradation procedures. One would expect this sample to contain the dimethylamino group because it was not subjected to strongly basic conditions at any time (Table V, experiment III). A number of experiments described below

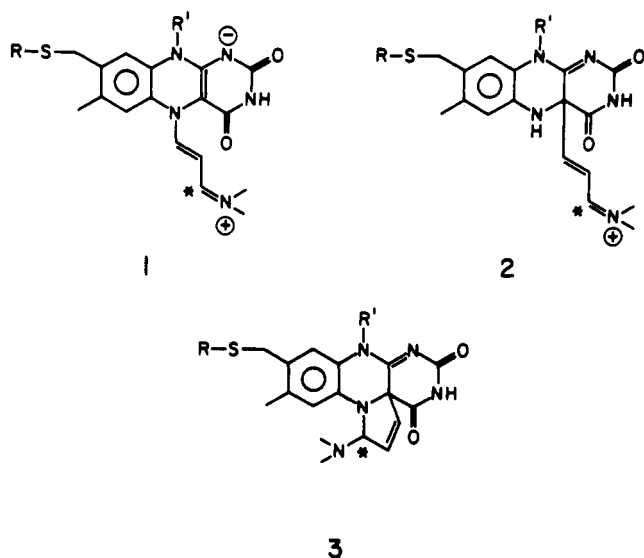


FIGURE 6: Possible structures for the adduct based on the isolation of radioactive glyoxal after ozonolysis. R is the peptide linked through the SH of cysteine. R' is the ribityl phosphate residue. Asterisks indicate possible location of ^{14}C . See text for assumptions made in generating structures.

corroborate this expectation. Furthermore, it is shown below that this sample is not seriously contaminated with nonspecifically bound radioactivity.

Chemical Studies of the Adduct. Initially the stability of the flavine-inhibitor adduct to acid and base was examined by observing spectral changes and by determining the amount of radioactivity from the adduct which could be adsorbed on charcoal. The results are summarized in Table IV. Essentially all of the radioactivity in the untreated adduct can be adsorbed onto charcoal. After the adduct is held at pH 1.3 for 11 hr no spectral change is observed and most of the radioactivity (84%) can still be adsorbed onto charcoal. The fact that some radioactivity remains in solution suggests either that the original adduct was a mixture containing an acid labile minor component, that some of the adduct had spontaneously decomposed to acid labile material over a period of several weeks, or that some nonspecifically bound label had been released. The fact that most of the radioactivity is absorbed by charcoal and that there are no spectral changes means that the adduct can contain no acid-sensitive ketal-type functions which would hydrolyze either to release a ^{14}C -containing fragment or to produce spectral changes.

At pH 9 the adduct is stable to spectral changes and to the release of radioactivity for several hours. If the pH is raised to 12.7, however, an immediate spectral change occurs with a band appearing in the region 420–520 nm, characteristic of oxidized flavine. This band reaches a maximum after ca. 1 hr, then decreases slowly over a period of several hours. It is bleached by the addition of sodium dithionite but is completely regenerated when oxygen is passed through the solution. If the oxidized flavine has a molar absorptivity of 12000 at 450 nm (Ghisla and Hemmerich, 1971), the band bleached by dithionite represents the production of ca. 1.6 nmol (67%) of oxidized flavine from 2.4 nmol of adduct by this alkaline treatment. Furthermore, after the adduct had been in base for 20 hr most of the radioactivity could no longer be adsorbed onto charcoal. The fact that oxidized flavine can be recovered from the adduct constitutes evidence that the flavine nucleus remains intact

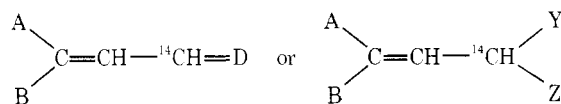
Table VI: Spectral and Acid-Base Properties of Substituted Dihydroflavines and Model Compounds.^a

Substitution or Compound	λ_{max} (H_2O) (nm)	ϵ	pK_a
N-5	296–355	5000–10000	6–7
C-4a	360–370	5000–9000	None 2–10
N-5, C-4a	325–360	5000–10000	
II	327	45000	
IV	375 (pH 2)	26000	5.3
	390 (pH 7)	24000	
Adduct	365 (pH 2)	35600	4–5
	380 (pH 8)	33500	

^a See Ghisla et al., 1973; Porter et al., 1973; Hevesi and Bruce, 1973; Brustlein and Hemmerich, 1968; Zeller et al., 1972; Gärtner and Hemmerich, 1975.

when MAO is inactivated by acetylenic amines. These data also show that at least 67% of the radioactivity in this sample is associated with the modified flavine, i.e., is specifically bound.

Treatment of the $[^{14}\text{C}]\text{CH}_2$ inactivator adduct with excess ozone at pH 4–5 produces $[^{14}\text{C}]\text{glyoxal}$ in yields up to 65% (Table III). If the carbon chain of the inactivator remains intact during adduct formation, then the adduct must contain a structural unit of the type:



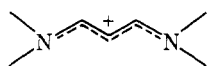
to account for the production of $[^{14}\text{C}]\text{glyoxal}$. At least one of the substituents must be bound to the flavine nucleus and both Y and Z must be heteroatoms. Since alkyl-substituted propargylamines, i.e., $\text{RC}\equiv\text{CCH}_2\text{NR}'\text{R}''$, also function as enzyme inhibitors (Swett et al., 1963), the proton ($\text{R}=\text{H}$) of a terminal acetylenic amine is probably not involved in adduct formation. Therefore substituent A or B is hydrogen. It has been suggested that any biological reaction involving covalent substrate-flavine intermediates will probably take place at N-5 or C-4a of the nucleus (Bruce, 1975). Assuming that, as a substrate analogue, the inactivator is most likely to interact with these same positions, we will consider C-4a and N-5 as the most reasonable sites for attachment of the flavine to the inactivator. At this point several structures might be considered for the adduct. However, the following chemical facts (discussed above) reveal that the initial adduct could have only structures 1, 2, or 3 (Figure 6): (1) the adduct is stable in acid; (2) the adduct after proteolytic digestion contains dimethylamine; (3) oxidized flavine can be released from the adduct upon base treatment; and (4) the adduct from $[^{14}\text{C}]\text{CH}_2$ inactivator yields $[^{14}\text{C}]\text{glyoxal}$ on ozonolysis.

In Table VI we compare the optical spectrum and pK_a of the adduct in regard to these possible structures and compare these with corresponding data for a number of substituted reduced flavines and model compounds. None of the substituted flavines has a long wavelength molar absorptivity (ϵ) exceeding 10000. The adduct, however, has a high ϵ value of ca. 35000, which suggests the presence of some unusual structural feature. Structures 2 and 3 should exhibit only "normal" spectra, as discussed above, whereas structure 1 has increased conjugation and might be expected to give an abnormal spectrum. C-5 monosubstituted reduced flavines often exhibit a pK between 6 and 7 for the ionization at N-1 and show a hypsochromic shift in λ_{max} upon de-

protonation (Walker et al., 1970; Porter et al., 1973). Reduced flavines substituted at C-4a ordinarily exhibit no pK between 2 and 10. The pK value of the adduct is more consistent with N-5 monosubstitution, although the observed bathochromic shift of $\lambda_{\max}$ upon deprotonation is opposite to that expected for simple N-5 derivatives. These results strongly suggest that structure 1 is most compatible with the data.

Studies of Model Compounds. We have obtained a number of compounds (Figure 1) which serve as models for possible structures of the adduct and we have compared their physical and chemical properties to those of the isolated adduct.

Compound II (a model for structure 1) has extremely intense absorption (ϵ 45000 at $\lambda_{\max}$ 327 nm), as expected for the



chromophore (Leonard and Adamcik, 1959). Compound II is spectrally stable for 6 hr in strong acid (pH 0.6) and in weak base (pH 9), but hydrolyzes in strong base. At pH 11.6 it is converted rapidly ($t_{1/2} \sim 5$ min) to III, $\lambda_{\max}$ 306 nm (ϵ 32500). Compound III is labile in both acid and base. It hydrolyzes with $t_{1/2} \sim 4$ hr in 0.1 *N* NaOH (Table VII). The only basic product which could be detected from both acid- and base-catalyzed hydrolysis of III is *N*-methylaniline. The fact that III is readily hydrolyzed at low pH, whereas the adduct is essentially stable, rules out β -amino- α,β -unsaturated carbonyl structures, such as III, for the adduct. This conclusion is consistent with our earlier expectation that the dimethylamino group is still present in the adduct. On the other hand, the hydrolytic behavior of II is consistent with that of the adduct.

A more satisfying model for structure 1 is compound IV, which was prepared by Zeller et al. (1972). Its structure has been recently determined (Gärtner and Hemmerich, 1975; Maycock, 1975). The visible spectrum of IV and its pK are compatible with those of the adduct (Table VI). The hydrolytic behavior of IV is similar to that of the enzymatically derived adduct. Although stable in strong acid (52 hr at pH 1.3) and at pH 9, IV is converted at pH 11.6 ($t_{1/2} \sim 20$ min) to a new compound [$\lambda_{\max}$ 358 nm (ϵ 18600)]. This new compound is probably V, as previously suggested by Zeller et al. (1972), and by analogy to the production of III from II. At higher pH values V slowly decomposes to unidentified products whereas in acid it is readily converted to 3-methylumiflavine, probably by hydrolytic cleavage of the N-5 side chain, followed by air oxidation of the reduced flavine so produced. Glyoxal is produced when IV is treated with ozone. These observations lend further support to structure 1 for the adduct.

Compound VI was also studied. As previously reported (Zeller et al., 1972), it displays a "normal" reduced flavine spectrum [$\lambda_{\max}$ 360 nm (ϵ 5000)] and it undergoes rapid decomposition in both acid (pH 2) and base (pH 11.5). Hence the properties of VI clearly exclude it as a model for the enzyme adduct.

Compounds II, III, IV, VI, and VII were treated with sodium borohydride under the conditions given in Table VII. Two distinct patterns emerged, as indicated. We suggest that the rapid reduction is due to the presence of a C-10a, N-1 double bond, which is found only in VI and VII. All the other reductions probably involve attack on side chains which are highly resonance stabilized, a process likely to be

Table VII: Reactivities of the Adduct and Model Compounds.^a

Compound	Reaction with		
	NaBH ₄ ^b	H ⁺ ^c	OH ^c
Adduct	Slow	None 11 hr at pH 1.3	$t_{1/2} < 1$ hr ^d at pH 12.7
II	Slow	None 6 hr at pH 0.6	$t_{1/2} \sim 5$ min at pH 11.9
III	Slow	$t_{1/2} = 1$ hr at pH 1.3	$t_{1/2} = 4$ hr in 0.1 <i>N</i> NaOH
IV	Slow	None 52 hr at pH 1.3	$t_{1/2} = 20$ min ^d at pH 11.6
V		$t_{1/2} \sim 2$ min ^d at pH 1.3	$t_{1/2}$ several hours at pH 13
VI	Rapid	Instantaneous at pH 2	Instantaneous at pH 11.5
VII	Rapid		

^a Determined by monitoring the optical spectra. ^b Compounds were treated in 1–2 ml of H₂O or 0.1–1.0 *M* sodium borate (pH 8.6–9.0) with two different quantities of sodium borohydride (less than 0.5 mg and more than 10 mg). Reactions completed in less than 1 min with the smaller amount of reductant are called rapid. Reactions completed only after several minutes with the larger amount are called slow. ^c Samples were dissolved in 1–2 ml of H₂O; spectra were recorded, and concentrated HCl or concentrated NaOH was added. pH values were determined at the conclusion of the reaction. ^d Oxidized flavine formed.

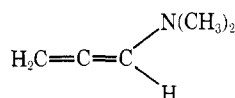
somewhat slow. The adduct itself behaves toward sodium borohydride as do model compounds II, III, and IV.

In summary, the chemical properties, spectral properties, and pK of the enzymatically derived adduct are consistent with those of the model compounds II and IV and inconsistent in certain important respects with the properties of model compounds III, V, VI, and VII.

Discussion

The most reasonable structure for the major inhibitor-flavine adduct derived from MAO is structure 1 (Figure 6). The close agreement derived from MAO is structure 1 (Figure 6). The close agreement of the molar absorptivity of the inactivated holoenzyme with that of the isolated purified adduct suggests that no other major adduct is formed with a significantly different molar absorptivity. A further point to be considered is that ozonolysis of the [¹⁴C]CH₂ inactivator adduct yields less than 100% of the expected [¹⁴C]glyoxal. This could be due to a variety of reasons. Since a partially purified adduct was used for chemical degradation, the adduct might have been contaminated with impurities which contained radioactivity not bound to flavine. However, such contamination would be slight since it was established earlier that most spurious radioactivity is removed early in the purification scheme. A more likely possibility is that ozonolysis did not proceed with 100% yield due to inherent difficulties with ozonolysis reacting. Conditions for ozonolysis could not be optimized due to the limited amount of material available.

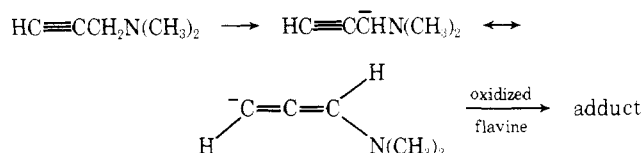
The prototype of suicide enzyme inactivation by acetylenic compounds is the system elegantly studied by Bloch and co-workers (Endo et al., 1970). They showed that the inactivation depended upon the enzymatic conversion of an acetylene to the isomeric (conjugated) allene, which functioned as the actual inactivator. Inactivation occurs by the irreversible addition of N-1 of a histidine residue to the central carbon of the allene moiety. In our case, the isomer allene



seems an unlikely intermediate for the following reasons. As a nucleophile, the allene could react through its N or C-2, neither of which would lead to the observed adduct. On the other hand, being unconjugated, the allene is not activated for addition of nucleophiles at any position.

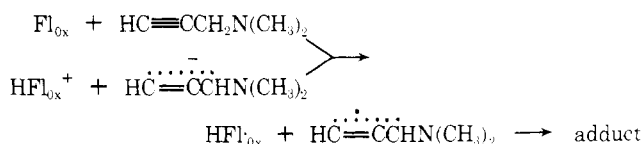
Recently, Bruce (1975) has critically reviewed the information available on the mechanisms of flavine reactions. In the context of his findings we can envision three possible mechanisms through which our adduct could have been formed: carbanion formation, radical formation, complete oxidation.

If the enzyme catalyzes removal of an α proton from the inactivator, the intermediate carbanion could add to N-5 of the oxidized coenzyme to give, after protonation, the observed adduct:



In this sequence, oxidized flavine reacts with *reduced* inactivator to form the stable covalent bond. The likelihood of a carbanion intermediate in this system is questionable. Cases in which carbanion intermediates have been implicated involve substrates, such as α -amino acids or α -hydroxy acids, which have activating groups adjacent to the site of proton removal.

For some flavine oxidations Bruce (1975) suggests the possible intermediate formation of a radical pair complex involving flavine and substrate. This complex could be formed either by hydrogen atom transfer from substrate to flavine or by electron transfer from an initially formed substrate carbanion to flavine. Collapse of this radical pair followed by protonation could explain formation of the adduct. In this scheme a *partially reduced* flavine reacts with a *partially oxidized* inactivator.



Complete oxidation of the inhibitor (by *any* mechanism) would produce reduced flavine and $\text{HC}\equiv\text{CCH}=\text{N(CH}_3)_2$, a highly reactive compound, expected to be an excellent acceptor in Michael addition reactions. Addition of N-5 of the reduced flavine across the triple bond would produce the observed adduct. Such addition is reasonable because model studies have demonstrated that N-5 (as well as C-4a) of 1,5-dihydroflavines is indeed nucleophilic (Ghisla et al., 1973). In this case the inactivation process involves reaction of *reduced* flavine with *oxidized* inactivator.

The three possible mechanisms presented differ primarily in the relative oxidation states of the reacting species. At present, we have no way of distinguishing among them.

The usual oxidation catalyzed by MAO occurs at C-1 of the substrate amine. A covalent adduct formed during the normal catalytic process could in principle involve either C-1 or the nitrogen atom of the substrate. In our adduct, however, the covalent attachment is through C-3, a position

which could not be available for adduct formation during normal substrate oxidation. Hence, the possibility of the formation of covalent adducts during the normal catalytic cycle remains unresolved. It should be pointed out that with the exception of one system (Porter et al., 1973), no covalent flavine intermediates have been detected when oxidation occurs on a substrate carbon, either in model flavine reactions or in enzyme-catalyzed reactions. In enzyme-reactions catalyzed by N-5 deazaflavoproteins, any covalent intermediates should be stable. Even in these cases, however, no such intermediates have been observed (Jorns and Hersh, 1974; Fisher and Walsh, 1974; Averill et al., 1975).

The fact that C-3 of the inactivator has added to N-5 suggests that the substrate or product is so oriented at the active site that the amino group of C-1 of the substrate is not near N-5. This, therefore, argues against intermediates in which the substrate forms an adduct with N-5 of the flavine.

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Purification and Properties of Gentamicin Acetyltransferase I[†]

Jeffrey W. Williams[‡] and Dexter B. Northrop*

ABSTRACT: Gentamicin acetyltransferase I is induced 13-fold in R factor resistant *Escherichia coli* by high concentrations (1 mg/ml) of gentamicin in the growth medium. The enzyme is maximally released from bacteria by osmotic shock in late-log phase, unlike previously studied periplasmic enzymes. Streptomycin sulfate and ammonium sulfate precipitations of shockate followed by affinity and ion-exchange chromatography recover 51% of the induced enzyme with a 360-fold increase in purity (12% of 4400-fold, uninduced). The purified enzyme appears homogeneous by six criteria, the first aminoglycoside inactivating enzyme so

purified. Sodium dodecyl sulfate electrophoresis, amino acid analysis, and sedimentation analyses indicate a tetrameric protein of 63000 molecular weight. The protein does not contain tryptophan. Kinetic analyses yield apparent values of: $V_{\max} = 3.4 \pm 0.2 \mu\text{mol per min mg at pH 8}$ (optimum), K_m (acetyl-CoA) = $3.9 \pm 0.2 \mu\text{M}$, K_m (gentamicin C_{1a}) = $0.3 \pm 0.08 \mu\text{M}$, K_i (gentamicin substrate inhibition) = $160 \pm 29 \mu\text{M}$. The activity of the enzyme is stable to a variety of conditions, including lyophilization and prolonged storage, and can be monitored by two convenient spectrophotometric assays.

Resistance to aminoglycoside antibiotics in many species of bacteria is determined by the presence of R factors which direct the synthesis of a new family of enzymes (Benveniste and Davies, 1973). These enzymes inactivate aminoglycoside antibiotics by three separate mechanisms: acetylation, adenylation and phosphorylation. A thorough understanding of the mechanisms of resistance is dependent upon characterization of these unusual enzymes, which in turn is

dependent upon substantial purification of the enzymes in a reasonable quantity. In addition, considerable interest exists in preparing stable forms of the antibiotic inactivating enzymes in quantity, for the purpose of developing rapid and specific clinical assays of antibiotics in body fluids (Williams et al., 1975; Smith and Smith, 1974; Holmes and Sanford, 1974; Haas and Davies, 1973). Despite differences between inactivating mechanisms, the enzymes show a number of similarities. Notable among these are extremely low yields of enzyme from cellular extracts and a limited stability, particularly upon exposure to cellulose and Sephadex column resins, properties which have greatly hindered purification (D. B. Northrop, J. Davies, and co-workers, unpublished results; Smith and Smith, 1974). This paper describes the purification of gentamicin acetyltransferase I to

[†] From the School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706. Received June 26, 1975. This investigation was supported by grants from the University of Wisconsin Graduate School and Research Corporation. A preliminary report of some of these findings has appeared (Williams and Northrop, 1975).

[‡] Graduate Fellow of the American Foundation for Pharmaceutical Education.