Sci. U.S.A. 70, 3531-3535.
Schejter, A., and Aviram, I. (1970), *J. Biol. Chem. 145,* 1552-1557.
Schejter, A., and George, P. (1965), *Nature (London) 206,* 1150-1151.
Stellwagen, E. (1966), *Biochem. Biophys. Res. Commun. 23,* 29-33.
Stellwagen, E. (1968), *Biochemistry 7,* 2496-2501.
Tsai, H. J., and Williams, G. R. (1965), *Can. J. Biochem. 43,* 1409-1415.
Wüthrich, K., Aviram, I., and Schejter, A. (1971), *Biochim. Biophys. Acta 253,* 98-103.

Affinity Labeling of Catechol *O*-Methyltransferase by *N*-Haloacetyl Derivatives of 3,5-Dimethoxy-4-hydroxyphenylethylamine and 3,4-Dimethoxy-5-hydroxyphenylethylamine. Kinetics of Inactivation†

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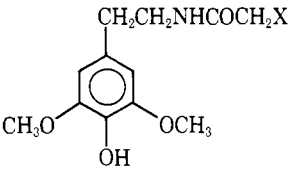
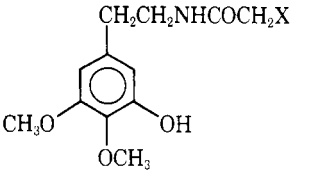
ABSTRACT: In an attempt to elucidate the relationship between the chemical structure and the catalytic function of catechol *O*-methyltransferase (COMT), several classes of affinity labeling reagents have been synthesized and their interaction with COMT has been studied. Earlier studies have shown that various *N*-haloacetyl derivatives of 3,5-dimethoxy-4-hydroxyphenylethylamine were effective affinity labeling reagents for this enzyme. In this report we have shown that *N*-haloacetyl derivatives of the isomeric 3,4-dimethoxy-5-hydroxyphenylethylamine also rapidly and irreversibly inactivate COMT and they satisfy many of the criteria established for affinity labeling reagents. This latter group of agents appear to modify a nucleophilic residue at the active site of COMT different from that modified by the 3,5-dimethoxy-4-hydroxyphenylethylamine series. Evidence to support this conclusion has been obtained by comparing the kinetics of COMT inactivation and the substrate protection profiles for these two classes of affinity labeling reagents.

The extraneuronal inactivation of catecholamines and the detoxification of many xenobiotic catechols is dependent upon the enzyme catechol *O*-methyltransferase (COMT)[1] (EC 2.1.1.6). COMT is a soluble, magnesium-requiring enzyme which transfers a methyl group from *S*-adenosylmethionine (AdoMet) to a catechol acceptor resulting in the formation of the meta and para O-methylated products (Axelrod and Tomchick, 1958; Molinoff and Axelrod, 1971; Flohe, 1974). In an effort to elucidate the relationship between the chemical structure and catalytic function of COMT we have synthesized various affinity labeling reagents for this enzyme. In earlier reports from this laboratory (Borchardt and Thakker, 1973, 1975) we have shown that *N*-iodoacetyl- and *N*-bromoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamines (**1a** and **b**) produce rapid inactivation of COMT and satisfy many of the criteria established for affinity labeling reagents. Since these derivatives of 3,5-dimethoxy-4-hydroxyphenylethylamine (**1a** and **b**) resemble meta-methoxylated products, it was of interest in our laboratory to synthesize and evaluate as affinity labeling reagents the corresponding derivatives of 3,4-dimethoxy-5-hydroxyphenylethylamine (**2a-c**), which resemble para methoxylated products. If both classes of inhibitors bind to the active site of COMT through the *o*-methoxy phenol functionality as proposed in Scheme I, then the re-

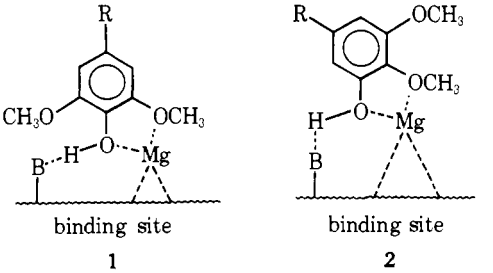


1a, X = I
b, X = Br



2a, X = H
b, X = I
c, X = Br

Scheme I



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[1] Abbreviations used are: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; COMT, catechol *O*-methyltransferase; DHA, 3,4-dihydroxyacetophenone; DCC, *N,N'*-dicyclohexylcarbodiimide.

spective side chains of these molecules should be exposed to slightly different environments on the enzyme surface and, thereby, serve as useful reagents to map the topography surrounding the catalytic site. From kinetic studies Coward and coworkers (1973) have proposed similar modes of binding for metanephrine and paranephrine, the products of methylation of epinephrine.

Therefore, we have synthesized and evaluated as affinity labeling reagents of COMT, the *N*-haloacetyl derivatives of 3,4-dimethoxy-5-hydroxyphenylethylamine **2b–c.** This report provides evidence that reagents **2b–c** satisfy the criteria established for affinity labeling reagents and in addition they appear to modify an amino acid residue on COMT different from that modified by the *N*-haloacetyl-3,5-dimethoxy-4-hydroxyphenylethylamines **1a–b.**

Materials and Methods

Melting points were obtained on a calibrated Thomas-Hoover Unit-melt and were corrected. Microanalyses were conducted on a F&M Model 185 C,H,N analyzer, The University of Kansas, Lawrence, Kan., and the Microanalytical Laboratory, National Institutes of Health, Bethesda, Md. Unless otherwise stated, the ir, nuclear magnetic resonance (NMR), and uv data were consistent with the assigned structures. Ir data were recorded on a Beckman IR-33 spectrophotometer, NMR data on a Varian Associates Model T-60 spectrophotometer (Me_4Si) and uv data on a Cary Model 14 spectrophotometer. Thick-layer chromatography was carried out using Analtech silica gel GF (1000μ). Spots were detected by visual examination under uv light. Scintillation counting was done on a Beckman LS-150 scintillation counter.

AdoMet-$^{14}CH_3$ (New England Nuclear, 55.0 Ci/mol) was diluted to a concentration of 10 μCi/ml and stored at −20°F. AdoMet chloride (Sigma) was stored as 0.01 *M* aqueous stock solution. The following compounds were commercially available from the indicated sources: AdoHcy (Sigma); 3,4-dihydroxybenzoic acid (Aldrich); [1-^{14}C]bromoacetic acid (specific activity = 54 Ci/mol), NCS tissue solubilizer (Amersham/Searle). 3,4-Dihydroxyacetophenone (Smissman and Borchardt, 1971), *N*-iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1a**), and *N*-bromoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1b**) (Borchardt and Thakker, 1975) were prepared by previously described procedures.

3,4-Dimethoxy-5-benzyloxybenzaldehyde. 3,4-Dimethoxy-5-hydroxybenzaldehyde (Mauthner, 1926) was treated with benzyl chloride according to the general procedure previously described (Borchardt and Thakker, 1975). Recrystallization from EtOAc–hexane afforded the aldehyde as colorless crystals: mp 67–68°; NMR ($CDCl_3$) δ 9.76 (s, 1 H, CHO), 7.43 (m, 5 H, aromatic), 7.18 (m, 2 H, aromatic), 5.18 (s, 2 H, OCH_2Ph), 3.94, 3.88 (2 singlets, 6 H, OCH_3). Anal. Calcd for $C_{16}H_{16}O_4$: C, 70.57; H, 5.92. Found: C, 70.76; H, 5.91.

3,4-Dimethoxy-5-benzyloxy-β-nitrostyrene. To 3,4-dimethoxy-5-benzyloxybenzaldehyde (2.0 g, 7.35 mmol) dissolved in 75 ml of MeOH, was added nitromethane (0.5 g, 8.25 mmol). The reaction mixture was cooled to 10–15° and NaOH (0.33 g, 8.25 mmol) dissolved in 2 ml of H_2O was added dropwise. When the addition was complete the reaction mixture was poured into 7.5 ml of 6 *N* HCl. The precipitated product was collected by filtration, washed with cold MeOH, and crystallized from EtOAc–MeOH to afford 2.1 g (91%) of pure product: mp 102–103°; NMR ($CDCl_3$) δ 7.90 (d, 1 H, PhC*H*=CH, *J* = 13 Hz), 7.45 (d, 1 H, PhCH=C*H*, *J* = 13 Hz), 7.40 (m, 5 H, aromatic), 6.75 (m, 2 H, aromatic), 5.15 (s, 2 H, OCH_2Ph), 3.93, 3.88 (2 singlets, 6 H, OCH_3). *Anal.* Calcd for $C_{17}H_{17}NO_5$: C, 64.75; H, 5.43; N, 4.44. Found: C, 64.54; H, 5.34; N, 4.25.

3,4-Dimethoxy-5-hydroxyphenylethylamine Hydrochloride. A general method (Ramirez and Burger, 1950; Borchardt and Thakker, 1975) for the reduction of β-nitrostyrenes to the corresponding phenylethylamines with $LiAlH_4$ was used in the synthesis of this amine. To $LiAlH_4$ (1.685 g, 43.0 mmol) in 50 ml of Et_2O was added, over a period of 1 hr, a solution of 3,4-dimethoxy-5-benzyloxy-β-nitrostyrene (2.6 g, 8.25 mmol) dissolved in 250 ml of dry Et_2O containing 5–10 ml of dry tetrahydrofuran. The reaction mixture was refluxed for 3 hr, then cooled in ice bath and the excess $LiAlH_4$ was decomposed by adding "wet" Et_2O followed by H_2O. The precipitates were filtered and extracted with $CHCl_3$ to recover any occluded product. The filtrate and the $CHCl_3$ extracts were combined and concentrated under reduced pressure to yield a yellow oil. The oil was dissolved in Et_2O and the Et_2O solution dried ($MgSO_4$) and filtered. Dry HCl gas was bubbled into the cooled Et_2O solution and the resulting precipitate collected by filtration to afford 1.24 g (47%) of the intermediate 3,4-dimethoxy-5-benzyloxyphenylethylamine hydrochloride. Recrystallization from MeOH–Et_2O afforded pure product: mp 154–155°; NMR ($CDCl_3$) δ 7.85 (m, 3 H, NH_3^+), 7.40 (m, 5 H, aromatic), 6.50 (m, 2 H, aromatic), 5.10 (s, 2 H, OCH_2Ph), 3.80 (s, 6 H, OCH_3), 3.1 (m, 4 H, $ArCH_2CH_2$). Anal. Calcd for $C_{17}H_{22}NO_3Cl$: C, 63.06; H, 6.85; N, 4.33. Found: C, 63.14; H, 6.77; N, 4.00.

To a solution of 3,4-dimethoxy-5-benzyloxyphenylethylamine hydrochloride (1.01 g, 3.12 mmol) in 25 ml of MeOH was added 5% palladium on carbon (0.1 g) and the reaction mixture was hydrogenated at 25° under 2 atm pressure for 4 hr. The catalyst was then removed by filtration and MeOH removed under reduced pressure. The residue was crystallized from acetonitrile to yield 0.66 g (90.4%) of the desired 3,4-dimethoxy-5-hydroxyphenylethylamine hydrochloride: mp 186–188°; NMR (Me_2SO-d_6) δ 6.38 (s, 2 H, aromatic), 3.70, 3.58 (2 singlets, 6 H, OCH_3) 2.10–2.82 (m, 4 H, $ArCH_2CH_2$–). Anal. Calcd for $C_{10}H_{16}NO_3Cl$: C, 51.40; H, 6.90; N, 5.99. Found: C, 51.53; H, 6.90; N, 5.93.

N-Iodoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine (**2b**). To a solution of 3,4-dimethoxy-5-hydroxyphenylethylamine (0.32 g, 1.63 mmol) and *N,N'*-dicyclohexylcarbodiimide (DCC) (0.363 g, 1.63 mmol) in 250–300 ml of acetonitrile, was added iodoacetic acid (0.303 g, 1.63 mmol). The solution was stirred for 72 hr at ambient temperature, after which the reaction mixture was filtered and the solvent removed under reduced pressure. The product was purified using thick-layer chromatography on silica gel (EtOH–$CHCl_3$, 1:9). The product with an R_f 0.42 was extracted from the silica gel with EtOAc and the solvent was removed under reduced pressure. The residual oil was crystallized from $CHCl_3$–hexane to yield 0.22 g (37%) of desired *N*-iodoacetyl derivative **2b**: mp 109–111°; NMR ($CDCl_3$) δ 6.45 (d, 1 H, aromatic, *J* = 2 Hz), 6.35 (d, 1 H, aromatic, *J* = 2 Hz), 6.30 (m, 1 H, –NHCO–), 6.00 (s, 1 H, phenol), 3.95 (s, 6 H, OCH_3), 3.65 (s, 2 H, $COCH_2I$), 3.55 (apparent q, 2 H, $-CH_2CH_2NH-$, *J* = 7 Hz), 2.75 (t, 2 H, $ArCH_2CH_2$, *J* = 7 Hz); ir (KBr) 3260 (br) and 1630 cm^{-1}. Anal. Calcd for $C_{12}H_{16}NO_4I$: C, 39.47; H, 4.42; N, 3.84. Found: C, 39.59; H, 4.36; N, 3.61.

N-Bromoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine (**2c**). 3,4-Dimethoxy-5-hydroxyphenylethylamine (0.252 g, 1.28 mmol) was treated with bromoacetic acid (0.196 g, 1.41 mmol), and DCC (0.291 g, 1.41 mmol) in acetonitrile under conditions similar to those described for the preparation of *N*-iodoacetyl derivative **2b.** The crude product was purified using thick-layer chromatography on silica gel (EtOH–$CHCl_3$, 1:9). The product with an R_f 0.44 was extracted from silica gel with EtOAc and the solvent was removed under reduced pressure. The residual oil was crystallized from $CHCl_3$–hexane to yield 0.06 g (19.7%) of **2c**: mp 105–107°; NMR ($CDCl_3$) δ 6.45 (d, 1 H, aromatic, J = 2 Hz), 6.32 (d, 1 H, aromatic, J = 2 Hz), 5.90 (m, 1 H, phenol), 5.90 (m, 1 H, –NHCO–), 3.90 (2 singlets, 8 H, OCH_3 and $COCH_2Br$), 3.50 (apparent q, 2 H, $-CH_2CH_2NH-$, J = 7 Hz), 2.70 (t, 2 H, $ArCH_2CH_2$, J = 7 Hz); ir (KBr) 3350, 3250, and 1650 cm^{-1}. Anal. Calcd for $C_{12}H_{16}NO_4Br$: C, 45.32; H, 5.06; N, 4.40. Found: C, 45.40; H, 5.02; N, 4.17.

N-Acetyl-3,4-dimethoxy-5-hydroxyphenylethylamine (**2a**). 3,4-Dimethoxy-5-hydroxyphenylethylamine (0.088 g, 0.45 mmol) was dissolved in 5 ml of CH_3OH. Acetic anhydride (0.07 g, 0.68 mmol) was added and the reaction mixture was stirred at ambient temperature for 2 hr. The solvent and excess acetic anhydride were removed under reduced pressure and the residual oil was crystallized from EtOAc–hexane to yield 0.055 g (51.2%) of the *N*-acetyl derivative **2a**: mp 101–102°; NMR ($CDCl_3$) δ 6.45 (d, 1 H, aromatic, J = 2 Hz), 6.30 (d, 1 H, aromatic, J = 2 Hz), 5.75 (m, 2 H, phenol and –NHCO–), 3.90 (2 singlets, 6 H, OCH_3), 3.50 (apparent q, 2 H, $-CH_2CH_2NH-$, J = 7 Hz), 2.75 (t, 2 H, $ArCH_2CH_2$, J = 7 Hz), 2.00 (s, 3 H, $COCH_3$); ir (KBr) 3340, 2940 (br), and 1630 cm^{-1}. Anal. Calcd for $C_{12}H_{17}NO_4$: C, 60.24; H, 7.16, N, 5.85. Found: C, 60.59; H, 7.23; N, 5.90.

N-[1-^{14}C]Bromoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1b**). This ^{14}C-labeled material was prepared using a procedure identical with that used in the preparation of the unlabeled **1b** (Borchardt and Thakker, 1975). 3,5-Dimethoxy-4-hydroxyphenylethylamine (Borchardt and Thakker, 1975) (0.03 g, 0.152 mmol) was condensed with [1-^{14}C]bromoacetic acid (0.032 g, 0.23 mmol, 1.09 Ci/mol) using DCC (0.046 g, 0.23 mmol) in acetonitrile. The radioactive product was purified by thick-layer chromatography on silica gel (hexane–2-propanol–$CHCl_3$, 1:75:91.5) and recrystallized ($CHCl_3$–hexane–EtOAc) to a constant specific activity of 1.062 Ci/mol; mp 104–105° (lit. [Borchardt and Thakker, 1975] mp 104–105°).

COMT Isolation and Assay. COMT was purified from rat liver (male, Sprague-Dawley, 180–200 g) using previously published procedures (Borchardt et al., 1975). The enzyme used in the kinetic inactivation experiments was purified through the calcium phosphate step resulting in a 47.8-fold purification from the crude supernatant. This enzyme had a specific activity of 49.6 nmol of product per mg of protein per min using 3,4-dihydroxybenzoic acid as a substrate. This form of the enzyme was very stable so that under the conditions of the inactivation experiments no loss of activity was observed in control samples. The enzyme used in the incorporation studies was further purified by affinity chromatography and Sephadex G-100 chromatography resulting in a 910-fold purification from the crude supernatant (Borchardt et al., 1975). The specific activity of this enzyme was about 950 nmol of product per mg of protein per min. This highly purified enzyme was stable under the conditions used for the incorporation experiments (25°, 0–30 min) but was unstable at higher temperatures and longer incubation times. The enzyme activity was determined using *S*-adenosylmethionine-$^{14}CH_3$ (AdoMet-$^{14}CH_3$) and 3,4-dihydroxybenzoate as substrates according to a previously described radiochemical assay (Borchardt, 1973).

COMT Inactivation Experiments. (a) With *N*-Haloacetyl-3,5-dimethoxy-4-hydroxyphenylethylamines (**1a** and **b**). The COMT inactivation experiments using derivatives **1a** and **b** were carried out using procedures and conditions identical with those described earlier from our laboratory (Borchardt and Thakker, 1975). The pseudo-first-order rate constants of inactivation, k_{app}, were calculated from the slopes of the plots of log of percent activity remaining vs. time (Kitz and Wilson, 1962; Petra, 1971; Borchardt and Thakker, 1973, 1975).

(b) With *N*-Haloacetyl-3,4-dimethoxy-5-hydroxyphenylethylamines (**2a–c**). Inactivation studies using *N*-haloacetyl-3,4-dimethoxy-5-hydroxyphenylethylamines (**2a–c**) presented several experimental problems, since these compounds were found to be very potent reversible inhibitors of COMT. As a result, after inactivation experiments were completed, the preincubation samples had to be diluted or dialyzed in order to minimize the reversible inhibition so that enzyme activity could be accurately determined. Therefore, inactivation of COMT by the *N*-haloacetyl-3,4-dimethoxy-5-hydroxyphenylethylamines **2a–c** was determined by either of the following procedures.

Method 1. A preincubation mixture was prepared consisting of the following components (in μmoles): water, so that the final volume was 0.8 ml; magnesium chloride (1.20); phosphate buffer, pH 7.6 (100); inhibitor (variable); and purified enzyme preparation (500 μg). The preincubation step was started by addition of enzyme and after the appropriate incubation time at 37°, the sample was placed in an ice bath (0–2°). The resulting reaction mixture was placed in dialysis tubing and dialyzed against 300 volumes of pH 7.6 phosphate buffer (50 mM) over a 24-hr period at 4°. After dialysis an aliquot (0.20 ml) of the reaction mixture was assayed for COMT activity by addition of 0.05 μCi of AdoMet-$^{14}CH_3$, AdoMet (0.25 μmol), and 3,4-dihydroxybenzoic acid (0.50 μmol) to a final volume of 0.25 ml. The assay mixtures were incubated for 12 min at 37° and the methylated product isolated as described earlier (Borchardt and Thakker, 1975). If no inhibitor was added to the preincubation mixture, no substantial loss in enzyme activity was observed using this dialysis technique.

Method 2. A preincubation mixture was prepared consisting of the following components (in micromoles): water, so that the final volume was 0.1 ml; magnesium chloride (0.30); phosphate buffer, pH 7.6 (10.0); inhibitor (variable); and purified enzyme preparation (400 μg). The preincubation step was started by addition of enzyme and after the appropriate incubation time at 37°, the reaction was stopped by diluting the reaction mixture with 0.9 ml of 50 m*M* phosphate buffer (pH 7.6) (1–4°). Aliquots (0.20 ml) of the reaction mixture were assayed for residual COMT activity using the procedures described above. The kinetic rate constants for COMT inactivation produced by derivatives **2a–c** were comparable using either Method 1 or 2.

Incorporation of Radioactivity. Reaction mixtures for the incorporation experiments consisted of the following components (in micromoles): H_2O so that the final volume

was 0.15 ml; magnesium chloride (0.15), TES buffer (pH 7.6) (5.0); *N*-[1-^{14}C]bromoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1b**) (specific activity = 1.062 Ci/mol) (variable); and purified COMT (35–50 μg). The incubations were carried out at 25°. After the appropriate incubation times, the reaction mixtures were transferred to an ice bath and then filtered through a Millipore filter (Type HAMK, 0.25 mm) under suction. The filter was washed with 20 ml of distilled water and dried and the extent of radioactivity incorporated was determined by placing the filter in a vial containing 10 ml of toluene-based scintillation fluid and counting for radioactivity.

For the electrophoretic studies, 200 μg of COMT was labeled with *N*-[1-^{14}C]bromoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine as described above. The excess inhibitor was removed by exhaustive dialysis against distilled water, after which the reaction mixture was lyophilized. The lyophilized protein was dissolved in 50 μl of 0.2 *M* phosphate buffer (pH 7.0) which contained 0.2% sodium dodecyl sulfate. To the dissolved protein was added 5 μl of 0.05% Bromophenol Blue in MeOH, 1 drop of glycerol, and 10 μl of 2-mercaptoethanol, and the resulting solution was then subjected to electrophoresis on polyacrylamide gels (10% acrylamide and 0.27% bisacrylamide cross-linked, pH 7.0) (Weber and Osborn, 1969; Borchardt et al., 1975). The gels were fixed and stained by incubation for 2 hr at room temperature in a solution of 0.25% Coomassie Blue in MeOH–acetic acid–H_2O (45:9:46). The gels were subjected to densitometric scanning at 550 nm before slicing in order to estimate the relative amount of COMT. Each gel was then cut into 3-mm slices and each slice extracted with 1.5 ml of a mixture of NCS tissue solubilizer and H_2O (9:1) by heating in a sealed scintillation vial at 50° for 12 hr. Toluene-based scintillation fluid (10 ml) was then added to each vial and the radioactivity measured after equilibrating the vials at ambient temperature for 12 hr.

Results

Chemistry. The synthetic routes used for the synthesis of the *N*-acyl-3,4-dimethoxy-5-hydroxyphenylethylamines **2a–c** were modeled after the procedures used for the preparation of the corresponding *N*-acyl-3,5-dimethoxy-4-hydroxyphenylethylamines **1a** and **b** (Borchardt and Thakker, 1975). 3,4-Dimethoxy-5-benzyloxybenzaldehyde was treated with nitromethane to yield 3,4-dimethoxy-5-benzyloxy-β-nitrostyrene. Reduction of this nitrostyrene derivative with $LiAlH_4$ afforded the intermediate 3,4-dimethoxy-5-benzyloxyphenylethylamine, which was followed by catalytic hydrogenation to yield the desired 3,4-dimethoxy-5-hydroxyphenylethylamine. The 3,4-dimethoxy-5-hydroxyphenylethylamine was converted to the desired *N*-haloacetyl derivatives **2b** and **c** by condensation with the appropriate acids in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC). The *N*-acetyl derivative **2a** was prepared by direct condensation of 3,4-dimethoxy-5-hydroxyphenylethylamine with acetic anhydride in methanol. The desired *N*-acyl derivatives **2a** and **b** and their synthetic intermediates were characterized by their spectral properties, chromatographic properties, and elemental analyses.

N-[1-^{14}C]Bromoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1b**) was prepared using a procedure identical with that used for the synthesis of the corresponding unlabeled compound (Borchardt and Thakker, 1975). This procedure involved the DCC-catalyzed condensation of 3,5-dimethoxy-4-hydroxyphenylethylamine with [1-^{14}C]bromoacetic acid (1.09 Ci/mol) to yield the desired ^{14}C-labeled inhibitor **1b** with a specific activity of 1.062 Ci/mol.

COMT Inactivation Studies. In an effort to establish whether the *N*-acyl-3,4-dimethoxy-5-hydroxyphenylethylamines **2a–c** have inherent affinities for the active site of COMT, we have determined the kinetic patterns for the reversible inhibition of this enzyme by the *N*-acetyl derivative **2a**. This analog does not have a chemically reactive moiety; therefore, its interaction with the enzyme should be of a reversible nature. When 3,4-dihydroxybenzoate was the variable substrate and compound **2a** the inhibitor, a noncompetitive pattern of inhibition of COMT was observed (K_i = 3.0 ± 0.3 μM). When AdoMet was the variable substrate, an uncompetitive pattern of inhibition was observed (K_i = 58 ± 2.4 μM). These kinetic patterns are similar to those observed previously for the 3,5-dimethoxy-4-hydroxyphenylethylamine series of affinity labeling reagents (Borchardt and Thakker, 1975).

In order to evaluate the potential of the *N*-haloacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine series as affinity labeling reagents, COMT was preincubated with the *N*-iodoacetyl derivative **2b** and *N*-bromoacetyl derivative **2c**. With both reagents a dramatic decrease in enzymatic activity as a function of increasing preincubation time was observed. This inactivation of COMT was completely irreversible, since enzyme activity cannot be recovered after dialysis or gel filtration on Sephadex G-25. In an effort to evaluate the relative reactivity of these reagents with COMT, the time courses for inactivation of this enzyme by the various *N*-acyl-3,4-dimethoxy-5-hydroxyphenylethylamines **2a–c** were determined. Similar to our earlier observations with the *N*-haloacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine series (Borchardt and Thakker, 1973, 1975), linear relationships between the log of the percent activities remaining vs. preincubation times were observed when COMT was preincubated with the *N*-iodoacetyl derivative **2b** or the *N*-bromoacetyl derivative **2c**. This linearity is characteristic of first-order processes with respect to the active enzyme remaining, which can be defined kinetically by an apparent first-order rate constant, k_{app}. Since iodide is a better leaving group than bromide in a nucleophilic reaction, the increased activity of the *N*-iodoacetyl derivative **2b** (k_{app} = 0.02 ± 0.001 min^{-1} (0.25 mM)) relative to the *N*-bromoacetyl derivative **2c** (k_{app} = 0.011 ± 0.0006 min^{-1} (0.25 mM)) was predicted. The *N*-acetyl derivative **2a** produced no inactivation of this enzyme as would be expected. Incubation of the enzyme alone under these conditions also produced no loss of enzyme activity.

In order to provide additional lines of evidence that the irreversible inactivation of COMT by reagents **2b** and **2c** proceeds via unimolecular reactions within dissociable complexes rather than by nonspecific bimolecular reactions, the rates of enzyme inactivation as a function of inhibitor concentrations were investigated. Kinetic evidence for the existence of such rate-limiting steps during the inactivation of COMT by affinity labeling reagents has been demonstrated previously (Borchardt and Thakker, 1973, 1975; Borchardt, 1975). The model for this type of inactivation is shown in eq 1 and 2, where E·I is the reversible complex, E–I the inactive enzyme, k_2 the first-order rate constant, and K_I the steady-state constant of inactivation $K_I = [E][I]/[E{\cdot}I]$. (See Kitz and Wilson (1962) and Petra (1971) for the derivation of these equations.)

$$E + I \overset{K_I}{\rightleftharpoons} E{\cdot}I \overset{k_2}{\rightarrow} E\text{–}I \qquad (1)$$

$$\frac{1}{k_{app}} = \frac{K_I}{k_2[I]} + \frac{1}{k_2} \quad (2)$$

For various concentrations of the *N*-iodoacetyl derivative **2b**, the time courses for COMT inactivation were determined and, as predicted, the inhibition was pseudo first order in enzyme concentration in all cases. Therefore, for each inhibitor concentration an apparent first-order rate constant (k_{app}) could be calculated. A plot of the reciprocals of the apparent first-order rate constants ($1/k_{app}$) vs. the reciprocals of the concentrations of the inhibitor **2b** ($1/[I]$), produced a nonlinear relationship as is shown in Figure 1. This nonlinear relationship observed for the *N*-iodoacetyl derivative **2b** is in sharp contrast to the linear relationship observed previously for *N*-iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1a**) (Borchardt and Thakker, 1973, 1975). For the sake of comparison, data for the *N*-iodoacetyl derivative **1a** is also plotted in Figure 1. If the kinetic data for **2b** are plotted as k_{app} vs. inhibitor concentration, the resulting curve is distinctly sigmoidal suggesting a positive cooperativity in this inactivation process.[2]

In an effort to further define the mechanism by which **2b** inactivates COMT, the kinetic order of the enzyme–inhibitor reaction was determined using the approach first described by Levy et al. (1963). The magnitude of the pseudo-first-order rate constant, k_{app}, depends upon the concentration of inhibitor as described in eq 3, where k' is a first-order constant, and n is a number equal to the average order of the reaction with respect to the concentration of the inhibitor ([I]). Taking the logarithms of both sides leads to eq 4 from which the order of the reaction can be experimentally estimated by plotting the log k_{app} against log [I] with the slope equal to n, the kinetic order of the reaction. In Figure 2 are shown plots of log k_{app} vs. log [I] for inactivation of COMT by *N*-iodoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine (**2b**) and *N*-iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1a**). In both cases linear relationships were observed with the slopes equal to 1.78 ± 0.089 for the derivative **2b** and 0.86 ± 0.14 for the derivative **1a**. The slope of approximate unity for **1a** suggests that one molecule of the inhibitor is bound to one molecule of COMT when inactivation occurs. In contrast, the slope of approximately two for **2b** indicates that two molecules of the inhibitor are bound to COMT when inactivation occurs,

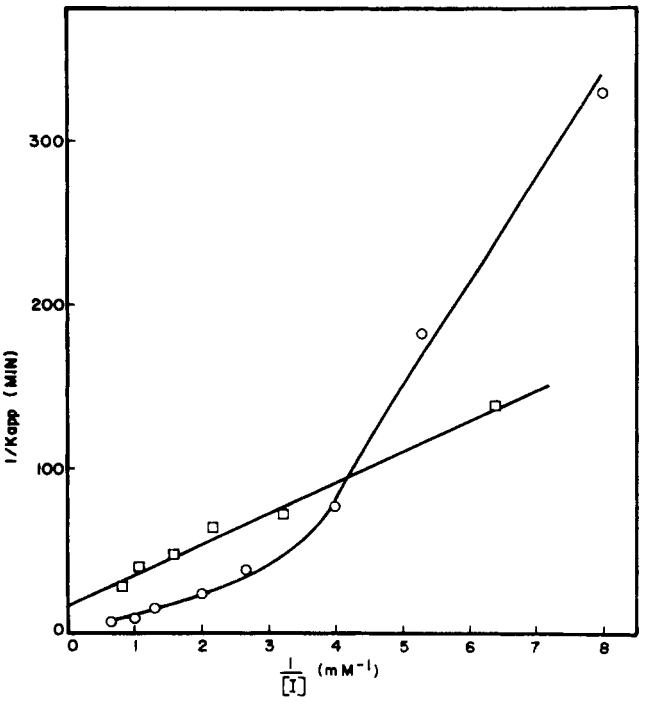

FIGURE 1: Effect of varying concentrations of *N*-iodoacetyl derivatives **1a** and **2b** on the rate of inactivation of COMT. A double reciprocal plot of k_{app}'s vs. inhibitor concentrations: (○) *N*-iodoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine (**2b**); (□) *N*-iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1a**). The k_{app}'s for **1a** and **2b** were determined by the procedures outlined in the Experimental Section. Enzyme purified through the calcium phosphate step (Borchardt et al., 1975) was used in these experiments.

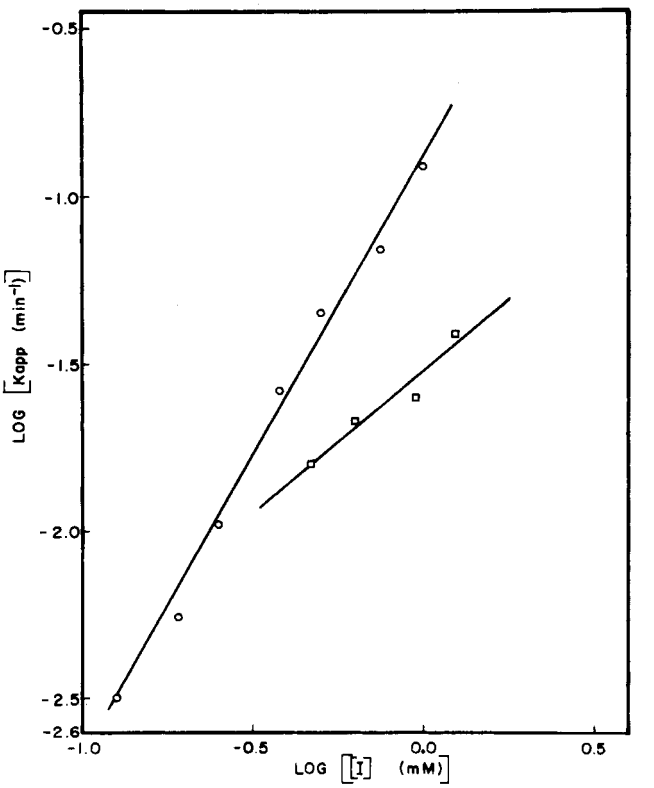

FIGURE 2: A plot of the logarithm of the pseudo-first-order rate constants of inactivation, k_{app}, vs. the logarithm of the concentration of the inhibitors: (○) *N*-iodoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine (**2b**); (□) *N*-iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1a**). The data used to construct this plot are that shown in Figure 1.

[2] Evidence has been obtained to explain the kinetics of the inactivation of COMT by **2b** using the following model system:

$$E + 2I \underset{}{\overset{k_{II}}{\rightleftharpoons}} EI_2 \xrightarrow{k_2} \text{inactive enzyme}$$

where E is the free enzyme, I the inhibitor (*N*-iodoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine (**2b**)), EI_2 the ternary complex formed from one molecule of the enzyme and two molecules of the inhibitor, K_{II} the steady-state constant ($K_{II} = [E][I]^2/[EI_2]$), and k_2 the first-order rate constant. The following equation can be derived to describe this model:

$$\frac{1}{k_{app}} = \frac{K_{II}}{k_2}\frac{1}{[I]^2} + \frac{1}{k_2}$$

For a system to fit this model reciprocal plots of $1/k_{app}$ vs. $1/[I]^2$ should be linear with the y intercept = $1/k_2$ and the slope = K_{II}/k_2. When the data for COMT inactivation by **2b** were plotted as reciprocals of k_{app} vs. $[I]^2$ a linear relationship was observed ($R = 0.99$). This observation provided further evidence that the inactivation of COMT by **2b** occurred by a mechanism involving positive cooperativity in which two molecules of the inhibitor are bound to the enzyme at the time of inactivation.

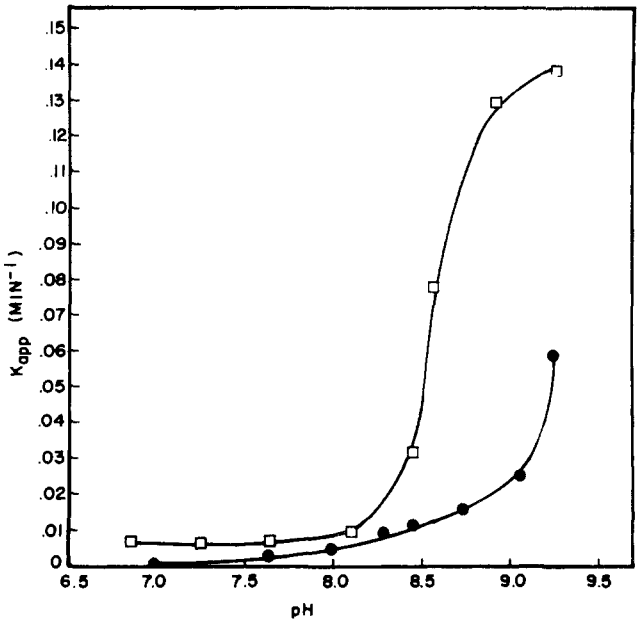

FIGURE 3: Effect of pH on the rate of inactivation of COMT by fixed concentrations of *N*-iodoacetyl derivatives **1a** and **2b**. (□) *N*-Iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1a**) (concentration 156 μM); (●) *N*-iodoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine (**2b**) (concentration 50 μM). The pseudo-first-order rate constants of inactivation, k_{app}, were determined as described in the Experimental Section.

further supporting the proposed positive cooperativity for inactivation of COMT by this reagent.

$$k_{app} = k'[I]^n \quad (3)$$

$$\log k_{app} = \log k' + n \log [I] \quad (4)$$

The physicochemical properties of the nucleophile(s) on COMT being modified by these affinity labeling reagents were explored by studying the effect of pH on the rate of inactivation of this enzyme. Shown in Figure 3 are plots of the apparent first-order rate constants, k_{app}, vs. the pH's of the preincubation mixtures in which the inactivation of COMT by the reagents **1a** and **2b** was studied. The approximately 20-fold increase in the rate of inactivation observed in the pH profile for *N*-iodoacetyl derivative **1a** would suggest the ionization of a nucleophile on the enzyme with a pK_a of approximately 8.5–8.6. In contrast the profile for the *N*-iodoacetyl derivative **2b** shows that k_{app} changes less dramatically with pH. The rise in k_{app} observed for derivative **2b** in the higher pH range may indicate the ionization of a nucleophile on the enzyme with a pK_a > 9.0. Changes in the binding constants of the ligand to the enzyme with changes in pH could also give rise to the type of pH profiles shown in Figure 3. This possibility as the causitive factor for the inflection points observed in Figure 3 was ruled out when it was observed that the magnitude of the reversible binding of *N*-acetyl-3,5-dimethoxy-4-hydroxyphenylethylamine and *N*-acetyl-3,4-dimethoxy-5-hydroxyphenylethylamine (**2a**) to COMT were not dramatically affected by such pH changes.

In an attempt to further elucidate the nature of the interaction between these affinity labeling agents and the active site of COMT, more extensive substrate protection studies were carried out. Since both classes of affinity labeling reagents would be expected to bind at the catechol binding site on COMT, catechol substrates should protect the enzyme from inactivation by these agents. Table I shows that 3,4-dihydroxyacetophenone (DHA), a substrate for COMT, significantly protects the enzyme from inactivation by *N*-iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1a**). Other catechol substrates such as D,L-norepinephrine and 3,4-dihydroxybenzoic acid also afford similar protection to the enzyme from inactivation by this reagent. Although DHA alone in high concentrations produces only partial protection of the enzyme, if AdoMet or *S*-adenosylhomocysteine (AdoHcy) are included in the preincubation mixture with DHA, the enzyme is completely protected from inactivation. This observation is of particular interest because AdoMet or AdoHcy by themselves in the preincubation mixture produced an enhanced rate of inactivation by **1a** rather than protection. If the inactivation of COMT by the *N*-iodoacetyl derivative **1a** was carried out in the presence of various concentrations of AdoHcy, the rate of the inactivation (k_{app}) was found to increase with increasing AdoHcy concentration. For example, a concentration of 0.49 mM of reagent **1a** produced the following rates of COMT inactivation: [AdoHcy]= 0, k_{app} = 0.016 ± 0.0007 min^{-1}; [AdoHcy] = 1 μM, k_{app} = 0.022 ± 0.0003 min^{-1}; [AdoHcy] = 10 μM, k_{app} = 0.034 ± 0.002 min^{-1}; and [AdoHcy] = 100 μM, k_{app} = 0.038 ± 0.001 min^{-1}. This rate enhancement phenomenon appears to conform to saturation kinetics, since concentrations of AdoHcy above 100 μM produced no additional rate enhancement.

Table I: Substrate Protection of COMT from Inactivation by *N*-Iodoacetyl-3,5-dimethoxy-4-hydroxyphenylamine (**1a**).

Reaction Mixture	Additions [a] (mM) DNA	AdoMet	AdoHcy	% Residual Activity after 60 min, 37° [b]
1				22
2	20			66
3	20	0.016		91
4	20	0.033		101
5	20		0.016	91
6	20		0.83	106

[a] The standard preincubation mixture consisted of the *N*-iodoacetyl derivative **1a** (0.83 mM); magnesium chloride (1.00 mM); TES buffer, pH 7.6 (33 mM); purified enzyme preparation (100 μg); and the indicated additions. The preincubations were carried out for 0 or 60 min at 37° after which the samples were assayed as described in the text. The enzyme used in this study was purified through the calcium phosphate step (Borchardt et al., 1975). [b] Residual activity after 60 min was calculated relative to the activity of the control samples preincubated for 0 min.

In Table II are shown the results of similar protection studies using *N*-iodoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine (**2b**) as the affinity labeling reagent. Again a significant degree of protection of COMT from inactivation by **2b** was produced when a catechol substrate such as DHA was included in the preincubation mixture. The amount of protection could be increased significantly if AdoMet or AdoHcy were included with DHA in the preincubation mix. Complete protection of the enzyme was not observed even in the presence of high concentrations of DHA and AdoHcy. This was probably a result of the experimental design, since prior to the assay for residual enzyme activity, a dialysis step was required to remove excess inhibitor. These results for **2b** are similar to the data shown in Table I for the *N*-iodoacetyl derivative **1a**. However, in contrast to the results obtained for **1a**, if AdoMet or AdoHcy

Table II: Substrate Protection of COMT from Inactivation by *N*-Iodoacetyl-3, 4-dimethoxy-5-hydroxyphenylethylamine (**2b**).

Reaction Mixture	Additions[a] (m*M*)			% Residual Activity after 60 min, 37°[b]
	DHA	AdoMet	AdoHcy	
1				17
2	10			56
3		10		49
4			0.01	37
5	10	0.1		66
6	10	10		85
7	10		0.01	64
8	10		1.0	85

[a] The standard preincubation mixture consisted of the *N*-iodoacetyl derivative **2b** (0.33 m*M*); magnesium chloride (1.6 m*M*); phosphate buffer (pH 7.6) (45 m*M*); purified enzyme preparation (400 μg), and the indicated additions in a total volume of 0.75 ml. The preincubation was carried out for 0 or 60 min at 37° after which the samples were dialyzed and assayed as described in the text. The enzyme used in this study was purified through the calcium phosphate step (Borchardt et al., 1975). [b] Residual activity was calculated with respect to the activity of samples incubated for 0 min.

alone are included in the preincubation mixture containing **2b,** significant protection of COMT from inactivation was observed. This is in marked contrast to the enhanced rates of inactivation of COMT observed when AdoMet or AdoHcy were present with **1a.**

Incorporation of Radioactivity. To provide further evidence that these reagents are indeed inactivating COMT by affinity labeling of the active site of this enzyme, the ^{14}C-labeled derivative of *N*-bromoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (**1b**) was prepared and its incorporation into the enzyme was studied. For the incorporation studies enzyme purified through the Sephadex G-100 chromatographic step was used (Borchardt et al., 1975). This stage of purification resulted in a 910-fold increase in specific activity as compared to the crude liver supernatant. From Coomassie Blue stained gels it was estimated (Borchardt et al., 1975) that COMT represents 60–70% of the protein present in this preparation. Homogeneous enzyme has been prepared (Borchardt et al., 1975); however, because of its extreme instability this highly purified COMT could not be used for the incorporation studies described here.

In Figure 4 is shown a comparison of the rate of COMT inactivation produced by the *N*-[^{14}C]bromoacetyl derivative **1b** vs. the amount of radioactivity incorporated into the protein. The radioactivity incorporation appears to parallel quite closely the loss in enzymatic activity. The extent of incorporation of the ligand, when total inactivation of COMT was achieved, was calculated to be approximately one molecule of reagent per molecules of enzyme using a molecular weight for COMT of 23,000 (Borchardt et al., 1975; Bohuon and Assicott, 1973). These data provide further evidence in support of the specific modification of the active site of COMT by these reagents. Further evidence in support of this conclusion comes from the results of the experiment shown in Figure 5. A COMT preparation, which had been completely inactivated by the *N*-[^{14}C]bromoacetyl derivative **1b,** was subjected to dodecyl sulfate polyacrylamide disc gel electrophoresis. In Figure 5 is shown the resulting Coomassie Blue stained gel and the corresponding plot of the radioactivity present in each slice of the gel. The major-

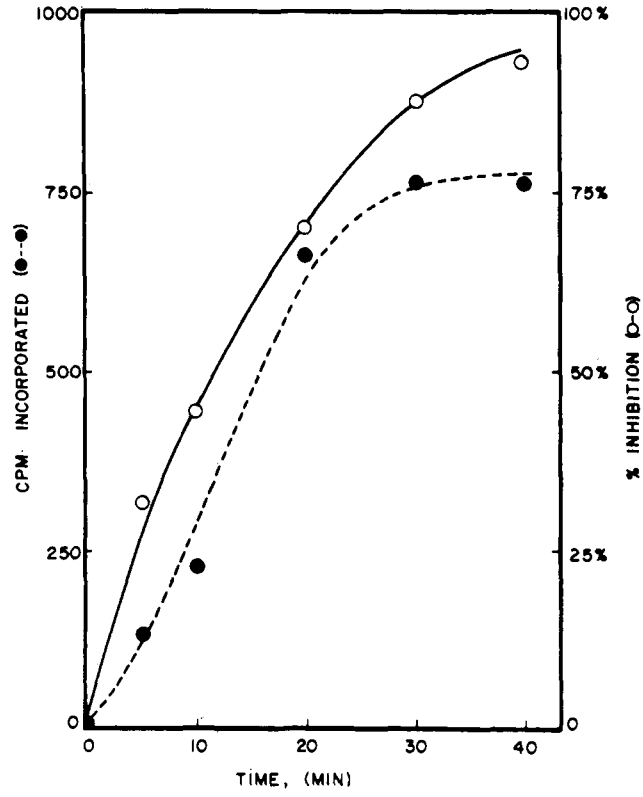

FIGURE 4: Extent of COMT inactivation produced by *N*-[^{14}C]bromoacetyl derivative **1b** as compared to cpm of radioactivity incorporated. Preincubations with COMT were carried out using *N*-[^{14}C]bromoacetyl derivative **1b** (0.33 m*M*) at 25°. Enzyme purified through Sephadex G-100 chromatography (Borchardt et al., 1975) was used in these studies. The COMT activity remaining and the radioactivity incorporated were determined as described in the Experimental Section.

ity of the radioactivity incorporated into the protein appears to be associated with the major peak having a molecular weight of 23,000 corresponding to COMT (Borchardt et al., 1975; Bohuon and Assicott, 1973). Some incorporation into the band having a molecular weight of approximately 40,000 was also observed. It is not clear whether this represents nonspecific incorporation or involves specific incorporation into an isozyme of COMT. Such isozymes of COMT have previously been reported (Bohuon and Assicott, 1973).

Discussion

In earlier publications from this laboratory it was shown that *N*-iodoacetyl- and *N*-bromoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamines **1a** and **b,** respectively, satisfy many of the criteria established for affinity labeling reagents (Borchardt and Thakker, 1973, 1975). The evidence included kinetic experiments in which it was shown that the inactivation of COMT by these reagents proceeds by unimolecular reactions within dissociable complexes rather than by nonspecific bimolecular reactions. In addition, preliminary experiments (Borchardt and Thakker, 1973) showed that the enzyme could be protected from inactivation by these reagents if catechol substrate were present. More extensive protection studies reported in this paper have shown that, indeed, catechol substrates will partially protect COMT from inactivation by the *N*-haloacetyl derivatives **1a** or **b.** However, complete protection could only be achieved when both catechol substrate and the methyl donor, AdoMet, or its product, AdoHcy, were present in the preincubation mixture. This enhanced protection in the

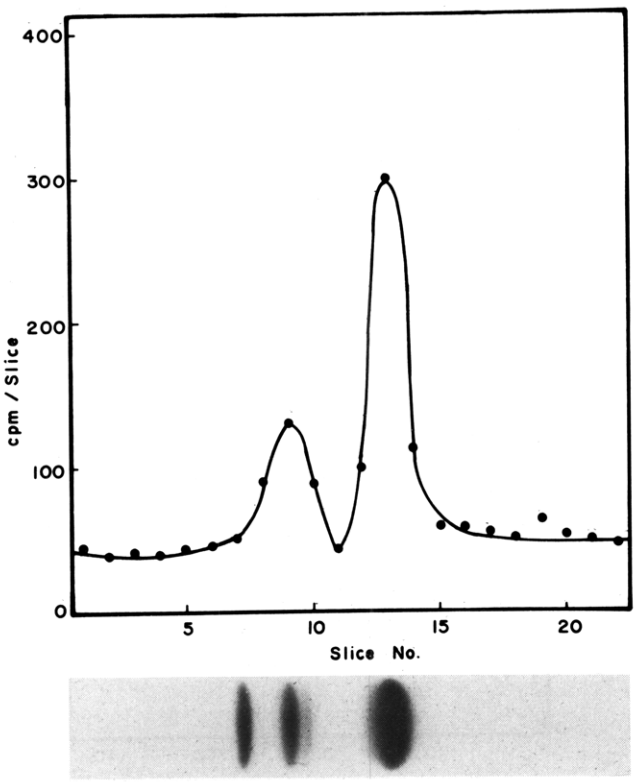

FIGURE 5: Sodium dodecyl sulfate polyacrylamide gel electrophoretic pattern of COMT inactivated by *N*-[^{14}C]bromoacetyl derivative **1b** and the corresponding plot of cpm of ^{14}C radioactivity per slice. COMT purified through the G-100 chromatographic step was used for these studies (Borchardt et al., 1975). Gels were stained with Coomassie Blue after which they were cut into 3-mm slices and each slice was extracted with NCS tissue solubilizer and the radioactivity determined as described in the Experimental Section.

presence of AdoMet or AdoHcy probably results from improved binding of the catechol substrate to the active site, thereby making the active site completely inaccessible to the affinity labeling reagent. Of particular interest was the observation that inclusion of AdoMet or AdoHcy in the preincubation mixture resulted in an enhanced rate of inactivation of COMT by reagent **1a.** The magnitude of this rate enhancement was dependent on AdoHcy or AdoMet concentration, but appeared to obey saturation kinetics. This increased rate of COMT inactivation could result from enhanced binding of the affinity labeling reagent **1a.** An alternative explanation would be the possibility that binding of AdoMet (or AdoHcy) to COMT produces a conformational change in the enzyme which results in increased accessibility of the active site nucleophilic residue which is being modified by reagent **1a.** Kinetic inactivation experiments for the *N*-iodoacetyl derivative **1a,** which were carried out in the absence and presence of AdoHcy, suggest that the presence of AdoHcy affects the steady-state constant, K_I, rather than the first-order rate constant, k_2. These results would support a mechanism involving the increased enzymatic binding of the reagents **1a** in the presence of AdoHcy as an explanation for the rate enhancement effect observed.

The effects of AdoMet (or AdoHcy) on the inactivation of COMT by reagent **1a** are also of particular interest, since they appear to be consistent with earlier kinetic observations of Coward et al. (1973). From inhibition kinetic experiments Coward et al. (1973) proposed that AdoMet affected the enzymatic binding of catecholamines and their products to COMT. Specifically they proposed that a conformational change occurred in the AdoMet-COMT complex prior to the binding of metanephrine, the meta-methoxylated product of epinephrine. It was proposed that this conformational change was required to accommodate the "abnormal" position of the ethanolamine side chain of metanephrine at the enzymatic binding site. Since reagent **1a** resembles a meta-methoxylated product, the nature of the binding of **1a** to COMT is probably similar to the binding of metanephrine. Therefore, the effects of AdoMet (or AdoHcy) on the inactivation of COMT by reagent **1a** and the proposed explanation involving effects on enzymatic binding of **1a** appear to be consistent with these earlier kinetic results (Coward et al., 1973).

The present studies suggest that the nucleophilic group being modified by reagent **1a** or **1b** is probably a cysteine residue with inactivation proceeding through an alkylation mechanism. The observation (Borchardt and Thakker, 1975) that the *N*-iodoacetyl derivative **1a** inactivates COMT at a faster rate than the *N*-bromoacetyl derivative **1b** would support such an alkylation mechanism, since this would be the order of reactivity expected in a nucleophilic displacement reaction. The observed incorporation of the *N*-[^{14}C]bromoacetyl derivative **1b** into COMT, which appears to correlate with loss of enzyme activity, would provide further evidence in support of this mechanism. The changes observed in the pseudo-first-order constants of inactivation, k_{app}, with pH for the reagent **1a** would strongly suggest that the moiety undergoing modification was a sulfhydryl group. The increased rate of inactivation observed on going from pH 8.0 to 9.0 is suggestive of ionization of a sulfhydryl group to a sulfhydryl anion, the latter being more nucleophilic. This increase in the rate of inactivation cannot be explained by an increase in the reversible binding of **1a,** since changes in pH had little effect on the reversible inhibition of COMT by the corresponding *N*-acetyl-3,5-dimethoxy-4-hydroxyphenylethylamine. Norris et al. (1973) have earlier provided evidence for the presence of two nucleophilic residues at the active site of this enzyme. Because of the reactivity of these nucleophilic groups to *N*-ethylmaleimide, Norris et al. (1973) proposed that the amino acid residues being modified were both cysteines. The data reported in this study would support the presence of at least one cysteine residue at the active site of COMT.

The results reported here for the *N*-haloacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine series **2b** and **c** would suggest that this class of affinity labeling reagents are modifying an amino acid residue on COMT different from that being modified by the *N*-haloacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine series **1a** and **b**. From the results of kinetic experiments, it was concluded that the 3,4-dimethoxy-5-hydroxyphenylethylamines **2b** and **c**, unlike the 3,5-dimethoxy-4-hydroxyphenylethylamines **1a** and **b**, are inactivating COMT by a mechanism more complex than a unimolecular reaction within a dissociable complex. The nonlinearity of the plot of the reciprocals of the pseudo-first-order rate constants ($1/k_{app}$) vs. the reciprocals of the concentrations of the inhibitor **2b** ($1/[I]$) and the observed kinetic order of two for the reaction of **2b** with COMT would both suggest a complex enzyme–ligand interaction. The sigmodial curve observed when k_{app} was plotted vs. inhibitor concentration would suggest a positive cooperativity during this inactivation process.[2] The observed kinetic order would further substantiate this hypothesis. In addition, there are dramatic differences between the two series of af-

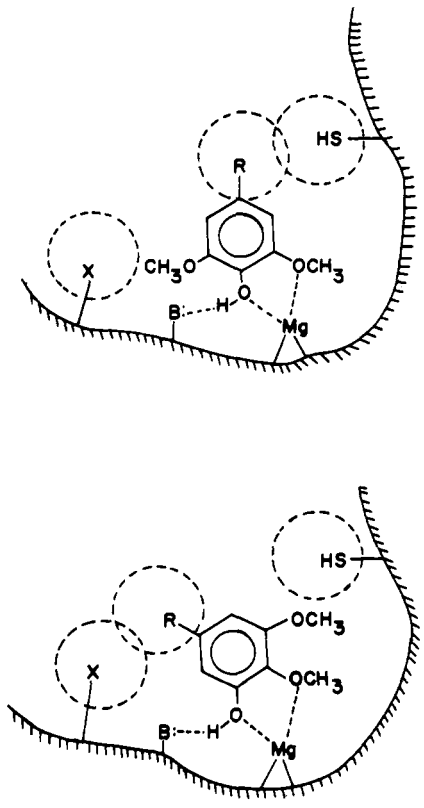

FIGURE 6: Proposed enzymatic binding of *N*-acyl-3,5-dimethoxy-4-hydroxyphenylethylamines (**1a** and **b**) and *N*-acyl-3,4-dimethoxy-5-hydroxyphenylethylamines (**2a–c**). Dotted circles are to denote proximity of the alkyl side chains of these reagents to the nucleophilic amino acid residues being modified.

finity labeling reagents with respect to their substrate protection profiles and with respect to the effect of changes in pH on their rates of inactivation of COMT.

Of particular interest was the observation that COMT could be partially protected from inactivation by compounds in the 3,4-dimethoxy-5-hydroxyphenylethylamine series (**2b** and **c**) when AdoMet or AdoHcy were included in the preincubation mixture. This result is in sharp contrast to the rate enhancement effects that AdoMet or AdoHcy have on the inactivation of COMT produced by the compounds in the 3,5-dimethoxy-4-hydroxyphenylethylamine series (**1a** and **b**). The protection of COMT by AdoMet or AdoHcy suggests that when these ligands bind to the enzyme, there is either a physical protection of the nucleophilic residue or a conformational change of the enzyme which makes the nucleophile less accessible to the affinity labeling reagent. Regardless of the mechanism, this protection of COMT by AdoHcy or AdoMet from inactivation by **2a** or **2b** is completely different from that observed for the other class of affinity labeling reagents. This basic difference in substrate protection would suggest that these two classes of affinity labeling reagents are modifying different nucleophilic residues on the enzyme. The observed pH profile for the inactivation of COMT by the *N*-iodoacetyl derivative **2b** would further support the hypothesis that a different nucleophilic residue was being modified by this class of affinity labeling reagents. The gradual increase in k_{app} in the pH range above 8.5 would suggest that the group being titrated is perhaps a terminal amino group of lysine with a $pK_a > 9.0$, rather than a cysteine residue which is the proposed group being modified by compounds in the 3,5-dimethoxy-4-hydroxyphenylethylamine series (**1a** and **b**).

In Figure 6 is depicted the different binding modes for the two series of affinity labeling reagents. For both classes of reagents the inhibitor is proposed to be binding initially to COMT through the *o*-methoxy phenol functionality. The modes of binding proposed in Figure 6 are similar to those suggested earlier by Coward et al. (1973) for the binding of metanephrine and paranephrine to the catechol binding site of COMT. The similarity in the modes of binding of these two classes of affinity labeling reagents would permit the side chains of these molecules to be exposed to slightly different environments on the enzyme active site, apparently permitting interaction with two different nucleophilic residues. The residue which is modified by the 3,5-dimethoxy-4-hydroxyphenylethylamine series may be the sulfhydryl group of a cysteine residue, whereas the group being modified by the 3,4-dimethoxy-5-hydroxyphenylethylamine series may be an amino group of a lysine residue. However, at this point the exact nature of either of these functional groups is only speculative. The identification of the specific amino acids being modified will have to await degradation of the inactivated protein and identification of the modified amino acids. Such studies are presently under investigation in our laboratory.

References

Axelrod, J., and Tomchick, R. (1958), *J. Biol. Chem. 233,* 702.

Bohuon, C., and Assicott, M. (1973), in Frontiers in Catecholamine Research, Usdin, E., and Snyder, S., Eds., New York, N.Y., Pergammon Press, p 107.

Borchardt, R. T. (1973), *J. Med. Chem. 16,* 377.

Borchardt, R. T. (1975), *Mol. Pharmacol. 11,* 436.

Borchardt, R. T., Cheng, C. F., and Thakker, D. R. (1975), *Biochem. Biophys. Res. Commun. 63,* 69.

Borchardt, R. T., and Thakker, D. (1973), *Biochem. Biophys. Res. Commun. 54,* 1233.

Borchardt, R. T., and Thakker, D. R. (1975), *J. Med. Chem. 18,* 152.

Coward, J. K., Slisz, E. P., and Wu, F. Y. (1973), *Biochemistry 12,* 2291.

Flohe, L. (1974), *Int. Pharmacopsychiatry 9,* 52.

Kitz, R., and Wilson, I. B. (1962), *J. Biol. Chem. 237,* 3245.

Levy, H. M., Leber, P. D., and Ryan, E. M. (1963), *J. Biol. Chem. 238,* 3654.

Mauthner, F. (1926), *Justus Liebigs Ann. Chem. 449,* 102.

Molinoff, P. B., and Axelrod, J. (1971), *Annu. Rev. Biochem. 40,* 465.

Norris, N. D., McNeal, F., and Creveling, C. R. (1973), Abstracts of the 8th Middle Atlantic Regional Meeting of the American Chemical Society, Washington, D.C., p 60.

Petra, P. H. (1971), *Biochemistry 10,* 3163.

Ramirez, F. A., and Burger, A. (1950), *J. Am. Chem. Soc. 72,* 2781.

Smissman, E. E., and Borchardt, R. T. (1971), *J. Med. Chem. 14,* 702.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.