Catechol *O*-Methyltransferase. 7. Affinity Labeling with the Oxidation Products of 6-Aminodopamine†

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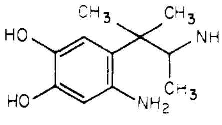
6-Aminodopamine (6-NH2DA) and various analogs of 6-NH2DA have been evaluated for their ability to inactivate purified catechol *O*-methyltransferase (COMT) in vitro. The inactivation of COMT by these agents could be prevented by including an antioxidant in the preincubation mixture or by excluding oxygen; however, catalase did not protect the enzyme from inactivation. Substrate protection studies and kinetic studies suggested that the loss of enzyme activity resulted from the alkylation of an amino acid residue at the active site of COMT by the quinoid type products which were generated upon air oxidation of 6-NH2DA. In addition, we have explored in more detail the reactivity toward COMT of specific intermediates in the oxidation pathway of 6-NH2DA by using various 6-NH2DA analogs. From the above studies we have concluded that 6-aminodopamine-*p*-quinone (6-NH2DAQ) is perhaps the most toxic species toward COMT. However, the aminochromes which are formed from 6-NH2DAQ are also effective in inactivating COMT. The results of these studies have provided a useful model system for observing the interaction of 6-NH2DA and its oxidation products with proteins; in addition, it has provided additional insight into the topography of the active site of COMT.

In recent years 6-hydroxydopamine (6-OHDA)[2a,b] and 6-aminodopamine (6-NH2DA)[2c,d] have become important pharmacological tools, since in vivo they produce fairly selective destruction of norepinephrine-containing nerve terminals. Despite the efforts of many investigators the molecular mechanisms by which these compounds produce degeneration of sympathetic neurons are unclear and remain the subject of much current research. There is good evidence to indicate that both 6-OHDA and 6-NH2DA are transported by the neuronal membrane pump and this appears to be a prerequisite to their degenerative effects.[2b-d,3] Molecular theories to explain the degenerative effects of these compounds center around two main ideas: (a) the formation of quinoid-like compounds (Scheme I, 6-OHDAQ, 6-NH2DAQ, aminochromes 1 and 2), which could react as alkylating agents with macromolecules;[4] or (b) formation of hydrogen peroxide and superoxide anions which are known to have toxic effects on proteins.[5]

Recently[6] in our laboratory it was observed that 6-OHDA rapidly and irreversibly inactivates the enzyme catechol *O*-methyltransferase (COMT, E.C. 2.1.1.6) in vitro. The mechanism of this inactivation appears to be a selective alteration of the active site of COMT, thereby making 6-OHDA a potentially useful chemical probe to explore the topography of the active site of this enzyme. In addition, this system could be utilized as a model to study the interaction of 6-OHDA with proteins and thus perhaps provide some insight into the mechanism of 6-OHDA's degenerative effects in vivo. From our earlier studies,[6] we were able to show that the inactivation of COMT by 6-OHDA resulted from the alkylation of a nucleophilic residue at the active site of this enzyme. The hydrogen peroxide which was produced upon oxidation of 6-OHDA did not alter the enzyme activity. Evidence was obtained to suggest that the alkylating species were probably the aminochromes 1 and 2 (Scheme I) which arise from intramolecular cyclization of 6-hydroxydopamine-*p*-quinone (6-OHDAQ). The 6-OHDAQ itself did not appear to be the active intermediate which caused the inactivation of COMT.

In an effort to further elucidate the mechanism of the inactivation of COMT, we have studied the interaction of this enzyme with 6-NH2DA and with various analogs of 6-NH2DA (**3–6**). The analogs were prepared in an attempt to answer specific questions concerning the inactivation process. The oxidation pathway for 6-NH2DA (Scheme I) is similar to that for 6-OHDA[7] and has been shown to involve the initial formation of 6-NH2DAQ, which then rapidly cyclizes to aminochrome 1.[7a] Rearrangement of aminochrome 1 to 5,6-dihydroxyindole (5,6-DHI) and its subsequent oxidation to aminochrome 2 are steps common to the oxidation pathways for both 6-OHDA and 6-NH2DA. One aspect of particular interest to us was the potential reactivity of 6-NH2DAQ toward COMT. Compounds **5** and **6** were synthesized in an attempt to answer this question. It was predicted that these analogs (**5**, **6**) would rapidly oxidize to quinoid systems related to 6-NH2DAQ, but cyclization to the corresponding aminochromes would be blocked because of the methyl substitution on the terminal nitrogen. In addition, compounds **3** and **4** were prepared to evaluate the reactivity of the intermediate aminochrome 1 in this oxidation pathway. It was anticipated that compounds **3** and **4** would rapidly oxidize and cyclize to compounds related to aminochrome 1; however, rearrangement of these to the corresponding 5,6-DHI's should be blocked because of the alkyl substituents adjacent to the aromatic ring. The results of these investigations are reported in this paper.



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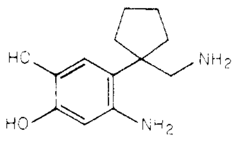

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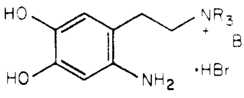

5, R = CH_3, CH_3, H
6, R = CH_3, CH_3, CH_3

Experimental Section

Melting points were determined in open glass capillaries using a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Microanalyses were conducted on a Hewlett-Packard Model 185 B C, H, N analyzer, the University of Kansas, Lawrence, Kan.

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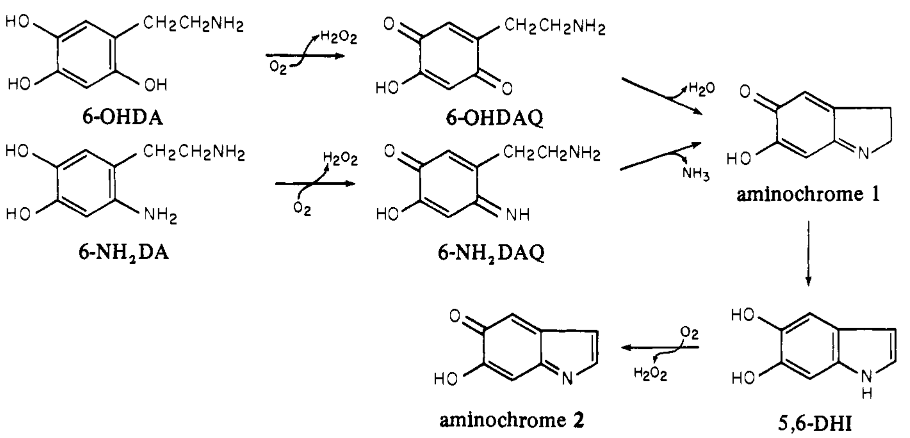
† This paper is dedicated to Professor Edward E. Smissman—esteemed teacher, scientist, and first and foremost friend.

Scheme I. Air Oxidation Pathways for 6-Aminodopamine and 6-Hydroxydopamine



Unless otherwise stated, the ir, NMR, and uv data were consistent with the assigned structures. Ir data were recorded on a Perkin-Elmer Model 727 spectrophotometer and NMR data on a Varian Associates Model T-60 spectrophotometer (Me_4Si). Scintillation counting was done on a Beckman LS-150 scintillation counter.

Materials. *S*-Adenosyl-L-methionine-*methyl*-^{14}C (SAM-$^{14}CH_3$) (New England Nuclear, 55.0 mCi/mmol) was diluted to a concentration of 10 μCi/ml and stored at -20°F. *S*-Adenosylmethionine chloride (Sigma) was stored as a 0.01 *M* aqueous stock solution. 6-Aminodopamine dihydrobromide (6-NH_2DA) was obtained from Dr. Ralph Adams and was checked for purity (melting point and NMR spectra).

1-Cyano-1-(3',4'-dimethoxyphenyl)cyclopentane (8). To 600 ml of 0.35 *M* sodium amide in liquid NH_3 was added in portions 3,4-dimethoxyphenylacetonitrile (Aldrich, 7, 17.7 g, 0.10 mol). The resulting deep red solution was stirred vigorously as 1,4-diiodobutane (31.0 g, 0.10 mol) in 100 ml of anhydrous Et_2O was added dropwise. After addition was complete (2.5 hr) the reaction mixture was allowed to reflux (-33°C) for another 2 hr, after which the NH_3 was permitted to evaporate as Et_2O (300 ml) and NH_4Cl (15 g) were added. The residue was treated with 20 ml of EtOH and then 500 ml of cold H_2O. The aqueous portion was extracted with Et_2O, the combined ethereal solutions were dried ($MgSO_4$) and filtered, and the solvent was removed under reduced pressure to yield 10.85 g of a dark oil. The oil was vacuum distilled to yield 8.70 g (38%) of the desired nitrile 8: bp 128–132° (0.10 mm). Anal. ($C_{14}H_{17}NO_2$) C, H, N.

1-(3',4'-Dimethoxyphenyl)-1-methylaminocyclopentane (9). A solution of the nitrile 8 (8.00 g, 35 mmol) in 150 ml of anhydrous Et_2O was added dropwise to a stirred solution of $LiAlH_4$ (8.3 g, 220 mmol) in 100 ml of Et_2O. The reaction mixture was stirred for 6 hr after addition of the nitrile was complete. The reaction mixture was cooled to ice-bath temperature and 10 ml of water added, followed by 30 ml of 4 *N* NaOH. The suspension was filtered and washed with Et_2O to remove occluded product. The combined ethereal fractions were dried (Na_2SO_4) and filtered, and the solvent was removed under reduced pressure to yield 8.6 g of a crude oil. Vacuum distillation afforded 6.2 g (76%) of the desired amine 9: bp 123–125° (0.1 mm). Anal. ($C_{14}H_{21}NO_2$) C, H, N.

1-(2'-Amino-4',5'-dimethoxyphenyl)-1-methylaminocyclopentane Hydrobromide (10). To a solution of amine 9 (0.7 g, 3 mmol) in a mixture of crushed ice (1 g) and H_2O (1 ml) was added, while cooling in an ice bath, 1 ml of 70% HNO_3. The reaction mixture was stirred for 5 min, after which time additional HNO_3 (7 ml) was added dropwise and the solution warmed to 10° for 2.5 hr. The yellow reaction mixture was poured into cold H_2O (150 ml) and then made strongly basic with 4 *N* NaOH maintaining the temperature below 5°. Upon standing for 18 hr at 0° yellow crystals formed which were removed by filtration. The crystalline material was unstable and was therefore immediately dissolved in EtOH (150 ml) and hydrogenated (40 lbs/in.^{2}) using PtO_2 as a catalyst. After hydrogenation was complete the reaction mixture was filtered (Celite) and to the filtrate was added 2 drops of 48% HBr. The acidic solution was concentrated in vacuo to yield a dark brown precipitate which when crystallized (*i*-PrOH–Et_2O) afforded 350 mg (28%) of the desired hygroscopic diamine 10: mp 209–212°. Anal. ($C_{14}H_{23}BrN_2O_2$) C; H: calcd, 6.43; found, 7.07. N: calcd, 8.51; found, 8.01.

1-(2'-Amino-4',5'-dihydroxyphenyl)-1-methylaminocyclopentane Dihydrobromide (4). The diamine 10 (0.35 g, 0.85 mmol) was refluxed in 48% HBr (6 ml) under argon for 1 hr. The reaction mixture was diluted with 25 ml of Et_2O and 50 ml of toluene, and the solvents were removed in vacuo at room temperature to yield 123 mg (35%) of the desired product: mp 250° dec. Anal. ($C_{12}H_{20}Br_2N_2O_2 \cdot H_2O$) C, H, N. The diamine 4 is very sensitive to air and must be stored under argon.

3-(2'-Amino-4',5'-dimethoxyphenyl)-3-methyl-2-aminobutane Dihydrobromide (11b). The 3-(3',4'-dimethoxyphenyl)-3-methyl-2-butanone used in this synthetic procedure was prepared from 2-(3',4'-dimethoxyphenyl)-2-methylpropionitrile according to the procedure of Finkelstein et al.[8] A mixture of 3-(3',4'-dimethoxyphenyl)-3-methyl-2-butanone (4.70 g, 21.7 mmol) and ammonium formate (20.0 g) was heated on an oil bath at 180° for 3 hr. The reflux condenser was replaced with a distillation head and the H_2O removed over a period of 2 hr. The oily residue was poured into 150 ml of cold H_2O to which 10 ml of 4 *N* NaOH had been added. The aqueous solution was extracted four times with a mixture of Et_2O–C_6H_6 (1:1; total 250 ml). The organic solvent was removed under reduced pressure and the resulting residue dissolved in 50 ml of 6 *N* HCl and heated at reflux for 2 hr. The cooled acidic solution was extracted with Et_2O (75 ml), then made strongly basic with 4 *N* NaOH, and again extracted with Et_2O (four times, total 250 ml). The ethereal solution was dried ($CaSO_4$) and the Et_2O removed in vacuo to yield 4.34 g (93%) of the desired 3-(3',4'-dimethoxyphenyl)-3-methyl-2-aminobutane (11a). The amine was used without further purification, since its spectral properties corresponded to those previously reported.[8]

The 3-(3',4'-dimethoxyphenyl)-3-methyl-2-aminobutane (11a, 4.34 g, 20 mmol) was dissolved in glacial acetic acid (35 ml) and cooled to 15°, after which time 7 ml of 70% HNO_3 was added dropwise. After the addition the reaction mixture was allowed to return to ambient temperature and stirred for 2 hr. The reaction mixture was poured into 600 g of ice and neutralized with 4 *N* NaOH. The solution was concentrated under reduced pressure to approximately 100 ml and then continuously extracted with Et_2O for 72 hr. The ethereal extract was concentrated under reduced pressure to yield a viscous oil. The oil was dissolved in 200 ml of EtOH and hydrogenated (37 lbs/in.^{2}) using PtO_2 as a catalyst for 4 hr. The ethanolic solution was filtered (Celite) and 5 ml of 48% HBr added. The ethanol was removed under reduced pressure and the residue crystallized (*i*-PrOH–Et_2O) to yield 2.17 g (27%) of the desired diamine (**11b**): mp 200° dec. Anal. ($C_{13}H_{12}Br_2N_2O_2$) C, H, N.

3-(2'-Amino-4',5'-dihydroxyphenyl)-3-methyl-2-aminobutane Dihydrobromide (3). The diamine 11b (0.25 g, 0.63 mmol) was hydrolyzed under argon using 48% HBr (10 ml) as described above for the preparation of 4. The reaction mixture was diluted with EtOH (50 ml) and C_6H_6 (100 ml), after which the solvent was removed under reduced pressure. The residue was triturated with Et_2O–*i*-PrOH (20 ml, 9:1) and the resulting solid material rapidly suction filtered to yield 102 mg (48%) of the desired product **3**: mp 250° dec. Anal. ($C_{11}H_{20}Br_2N_2O_2$) C, H, N.

***N,N*-Dimethyl-2-(2′-nitro-4′,5′-dimethoxyphenyl)ethylamine Hydrochloride (13).** The 2-(2′-nitro-4′,5′-dimethoxyphenyl)ethylamine (**12**) used in this synthetic route was prepared according to the procedure of Harley-Mason.[9] The amine **12** (11.0 g, 54 mmol) was converted to the corresponding *N,N*-dimethylamine **13** using formaldehyde and formic acid as previously described.[10] The *N,N*-dimethylamine **13** was isolated as the hydrochloride salt: 5.18 g (41%); mp 223–225° dec (lit.[10] mp 225–226°).

***N,N,N*-Trimethyl-2-(2′-nitro-4′,5′-dimethoxyphenyl)ethylammonium Iodide (14).** To a solution of the amine **13** (1.0 g, 3.4 mmol) in 75 ml of anhydrous Et_2O was added CH_3I (2.00 g). The reaction mixture was refluxed for 2 min on a steam bath and then allowed to stand at ambient temperature for 18 hr. The solid material which formed was removed by filtration to yield 1.55 g (90%) of **14**: mp 214–216° dec. Anal. ($C_{13}H_{21}IN_2O_4$) C, H, N.

***N,N*-Dimethyl-2-(2′-amino-4′,5′-dihydroxyphenyl)ethylamine Dihydrobromide (5).** The diamine **5** was prepared from **13** by a modification of the procedure reported previously by Nerland and Smissman.[10] Amine **13** (5.10 g, 18 mmol) was reduced using PtO_2 as a catalyst to yield 3.13 g (60%) of *N,N*-dimethyl-2-(2′-amino-4′,5′-dimethoxyphenyl)ethylamine dihydrobromide: mp 240–244° (lit.[10] mp 244–246°).

N,N-Dimethyl-2-(2′-amino-4′,5′-dimethoxyphenyl)ethylamine dihydrobromide (1.0 g, 3.3 mmol) was hydrolyzed to **5** using 48% HBr (10 ml) under an argon atmosphere. The reaction mixture was refluxed for 45 min after which it was diluted with 20 ml of cold H_2O and 10 ml of EtOH. The solvents were removed in vacuo at 100° and the residue was dissolved in 30 ml of *i*-PrOH. The *i*-PrOH solution was decanted from the insoluble material and the solution diluted with 200 ml of hot Et_2O. The resulting white solid which formed was removed by filtration and dried (P_2O_5) at ambient temperature in vacuo to yield 1.12 g (83%): mp 163–167° dec. Anal. ($C_{10}H_{18}Br_2N_2O_2$) C, H, N.

***N,N,N*-Trimethyl-2-(2′-amino-4′,5′-dihydroxyphenyl)ethylammonium Bromide Hydrobromide (6).** A suspension of amine **14** (1.40 g, 3.5 mmol) in 100 ml of absolute EtOH was hydrogenated (47 lbs/in.^{2}) using PtO_2 (150 mg) as a catalyst for 4 hr. The pale yellow solution was filtered and 6 ml of 48% HBr added. The EtOH–H_2O was removed in vacuo to yield 1.42 g (100%) of the desired *N,N,N*-trimethyl-2-(2′-amino-4′,5′-dimethoxyphenyl)ethylammonium bromide hydrobromide: mp 254–256°. This material was used without further purification.

N,N,N-Trimethyl-2-(2′-amino-4′,5′-dimethoxyphenyl)ethylammonium bromide hydrobromide (1.5 g, 3.5 mmol) was dissolved in 50 ml of 48% HBr under argon and the solution refluxed for 2 hr with stirring. The reaction mixture was diluted with 100 ml of cold H_2O and the solvent removed in vacuo at ambient temperature. The residue was recrystallized (*i*-PrOH–EtOH) to yield 750 mg (57%): mp 263–265° dec. Anal. ($C_{11}H_{20}Br_2N_2O_2$) C, H, N.

COMT Isolation and Assay. COMT was purified from rat liver (male, Sprague–Dawley, 180–200 g) according to the methods previously described.[11-13] Purification was carried through the calcium phosphate stage resulting in approximately a 50-fold purification of the COMT activity.[13] The enzyme activity was determined using *S*-adenosylmethionine-*methyl*-^{14}C and 3,4-dihydroxybenzoate or 3,4-dihydroxyacetophenone (DHA) as substrates according to a previously described radiochemical assay.[11,12]

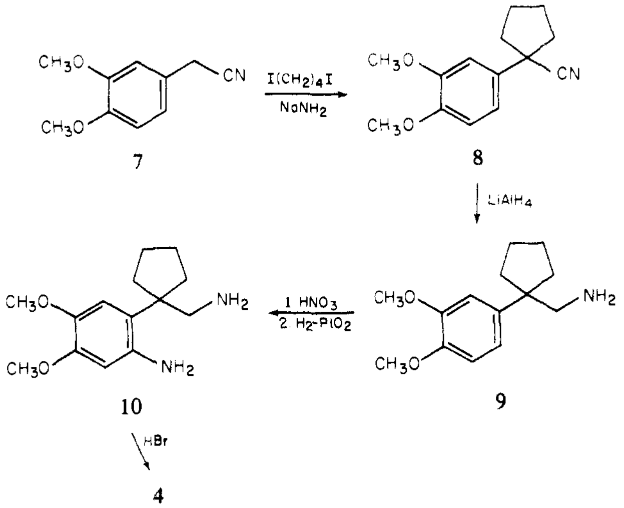
COMT Inactivation Experiments. The COMT inactivation experiments were carried out using procedures similar to those described earlier from our laboratory.[6,14] A typical preincubation mixture consisted of the following components: water, so that the final volume was 3.20 ml; magnesium chloride (4.80); phosphate buffer, pH 7.60 (400); inhibitor (variable); and purified enzyme preparation. The preincubation step was started by the addition of enzyme, and incubation was carried out at 37°. After the appropriate preincubation time an aliquot (0.20 ml) of the preincubation mixture was removed and assayed by addition of 0.05 μCi of *S*-adenosylmethionine-*methyl*-^{14}C, *S*-adenosylmethionine (0.25 μmol), and 3,4-dihydroxybenzoate or DHA (0.50 μmol) to a final volume of 0.25 ml. The assay mixtures were incubated for 5 min at 37° and the reaction was stopped by addition of 0.10 ml of 1.0 *N* HCl. The methylated products were extracted using 10 ml of toluene–isoamyl alcohol (7:3) as previously described.[6,14] In the anaerobic experiments (Table I) the preincubation mixtures and inhibitor solutions were prepared in a similar way to those described above, except the anaerobic experiments were done in sealed ampules under nitrogen and samples were removed using a syringe. Enzyme assays in these anaerobic experiments were also carried out in ampules under nitrogen.

Table I. Effects of Antioxidants, Catalase, and Anaerobic Conditions on the Inactivation of COMT by 6-NH_2DA

Rxn mix-ture	Preincub conditions[a]	Additions	% residual act. after 40 min, 37°[d]
1	Aerobic		2
2	Anaerobic		103
3	Aerobic	Sodium metabisulfite[b]	77
4	Aerobic	Catalase[c]	1

[a] The standard preincubation mixture consisted of 6-NH_2DA (189 μ*M*), magnesium chloride (1.38 m*M*), phosphate buffer, pH 7.60, enzyme preparation, and water to a final volume of 1.32 ml. Anaerobic samples were handled as described in the Experimental Section. [b] Sodium metabisulfite concentration = 0.15 *M*. [c] 250 μg of catalase (activity = 11000 units/mg) was added to the preincubation mixture. [d] Residual activity was calculated from controls which were not first incubated but were directly assayed after addition of the inhibitor. Values are the averages of duplicate determinations.

Scheme II



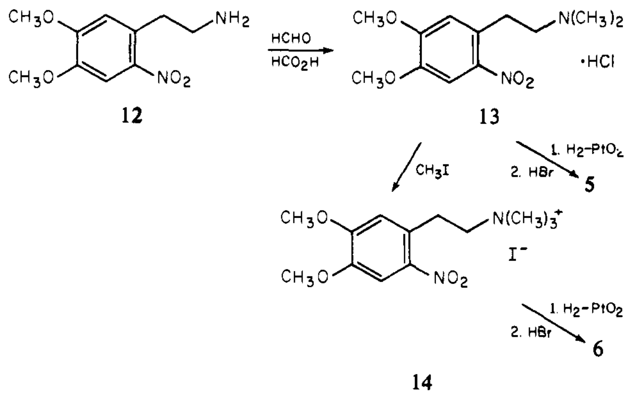
Because of stability problems, stock solutions of 6-NH_2DA and compounds **3–6** were prepared fresh for each inactivation experiment. Stock solutions of 5 μmol/ml in H_2O were prepared in sealed ampules under nitrogen and aliquots were removed as needed for inactivation experiments. In general no evidence of oxidation was observed in these stock solutions for up to 4 hr.

When the inhibitors were pre-preincubated to permit air oxidation of the compound (Figure 4), this was accomplished by preparing 5 μmol/ml solutions of the inhibitors in 0.1 *M* PO_4, pH 7.6. The samples were exposed to the air and incubated at 37° for the indicated times.

Results and Discussion

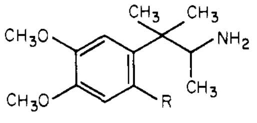
Chemistry. In Scheme II is outlined the steps involved in the preparation of the cyclopentane derivative **4**. The 1-cyano-1-(3′,4′-dimethoxyphenyl)cyclopentane (**8**) was prepared by reaction of the nitrile **7** with 1,4-diiodobutane and sodium amide in liquid NH_3. The nitrile **8** was reduced to the amine **9** using $LiAlH_4$. Amine **9** was converted to the desired 1-(2′-amino-4′,5′-dimethoxyphenyl)-1-methylaminocyclopentane (**10**) by nitration with

Scheme III



70% HNO$_3$ followed by catalytic reduction. Hydrolysis of **10** using 48% HBr afforded the desired cyclopentane derivative **4**.

The analog **3** was prepared by a pathway similar to that outlined above for the preparation of **4**. The intermediate 3-(3',4'-dimethoxyphenyl)-3-methyl-2-aminobutane (**11a**) was prepared according to the procedure of Finkelstein et al.[8] Nitration of **11a** followed by catalytic reduction afforded the desired diamine **11b**. Hydrolysis of **11b** with 48% HBr afforded the desired **3**.



11a, R = H
b, R = NH$_2$

The analogs **5** and **6** were prepared by the steps outlined in Scheme III. The intermediate *N*,*N*-dimethyl-2-(2'-nitro-4',5'-dimethoxyphenyl)ethylamine (**13**) was prepared by an Eschweiler–Clark methylation of **12** according to the procedure of Smissman and Nerland.[10] The *N*,*N*-dimethylamine **13** was reduced catalytically and then hydrolyzed with 48% HBr to yield the *N*,*N*-dimethyl-6-NH$_2$DA derivative **5**. Alternately, methylation of **13** with methyl iodide to afford the intermediate *N*,*N*,*N*-trimethylamine **14**, followed by catalytic hydrogenation and hydrolysis with 48% HBr, gave the other desired derivative, *N*,*N*,*N*-trimethyl-6-NH$_2$DA (**6**). All of the intermediates and final compounds in this series were characterized by their ir, NMR, and uv spectra, their chromatographic properties, and elemental analyses.

COMT Inactivation Studies. Similar to our earlier observations with 6-OHDA,[6] we have found that 6-NH$_2$DA rapidly and irreversibly inactivates COMT (Table I). This inactivation of COMT by 6-NH$_2$DA is completely irreversible, since the enzyme activity cannot be recovered after removal of the excess inhibitor by dialysis or gel filtration. Air oxidation of 6-NH$_2$DA appears to be a crucial step in the mechanism of inactivation since inclusion of an antioxidant (sodium metabisulfite) in the preincubation mixture or carrying out the preincubation under anaerobic conditions results in nearly complete protection of the enzyme from inactivation (Table I). Addition of sodium metabisulfite after treatment of the enzyme with 6-NH$_2$DA did not reverse the inactivation. These observations strongly support the premise that the products resulting from air oxidation of 6-NH$_2$DA are the toxic species toward COMT. The possibility that hydrogen peroxide, which would be generated upon air oxidation of 6-NH$_2$DA, is the toxic species was ruled out by the fact that inclusion of catalase (Table I) in the preincubation mixture did not afford protection of COMT from inactivation. These preliminary data, therefore, would suggest that 6-NH$_2$DA inactivation of COMT occurs by a mechanism similar to that of 6-OHDA,[6] where the reactive species are probably the quinoid oxidation products.

Table II. Substrate Protection of COMT from Inactivation by 6-NH$_2$DA

Rxn mixture	Additions,[a] m*M*			% residual act. after 40 min, 37°[b]
	DHA	SAM	SAH	
1				2
2	2.5			10
3	10			17
4		0.76		26
5			0.76	52
6	2.5	0.4		63
7	10	0.4		85
8	10		0.4	94

[a] The standard preincubation mixture described in Table I was used except the indicated concentrations of DHA, SAM, or SAH were included. [b] Residual activity after 40 min was calculated relative to the activity of the control samples preincubated for 0 min.

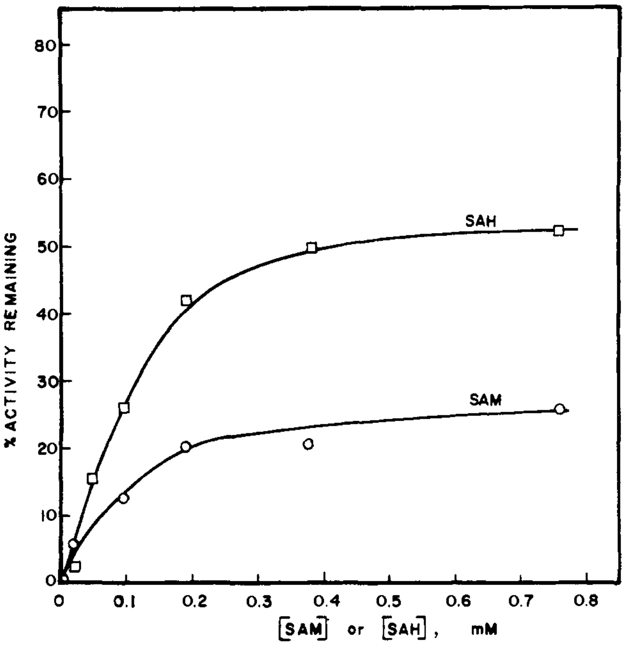


Figure 1. *S*-Adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) protection of COMT from inactivation by 6-aminodopamine (6-NH$_2$DA). Preliminary incubation mixtures prepared as described in the Experimental Section, containing purified enzyme, 6-NH$_2$DA (0.19 m*M*), and varying concentrations of SAM (○–○) or SAH (□–□), were incubated at 37° and aliquots were removed at zero time (controls) and 40 min. These samples were checked for enzyme activity and each point is the average of duplicate determinations. The percentage of activity remaining after 40 min of preliminary incubation is compared to controls.

To provide evidence that the mechanism by which 6-NH$_2$DA causes inactivation of COMT involves a selective alteration of an amino acid at the active site of the enzyme, various substrate protection studies were carried out and the results are shown in Table II. If the preincubation of COMT with 6-NH$_2$DA is performed in the presence of *S*-adenosylmethionine (SAM) or *S*-adenosylhomocysteine (SAH), the inactivation of the enzyme is greatly reduced. (To demonstrate the specificity of this interaction, methionine and methionine-*S*-methylsulfonium iodide at concentrations up to 2 m*M* were found not to protect the enzyme from inactivation.) The protection of COMT by SAM (or SAH) is of particular interest, since this appears to be a saturable process as shown in Figure 1. Concentrations of SAM (or SAH) above ~0.3 m*M* did not provide additional protection of COMT from inactivation by 6-NH2DA. This type of saturable protection by SAM or SAH is similar to that observed for the inactivation of COMT by 6-OHDA[6] and *N*-iodoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine.[15] These data suggest that when SAM (or SAH) binds to COMT, the enzyme perhaps undergoes a conformational change which decreases either the accessability or the nucleophilicity of the active site amino acid moiety which is being modified, thereby affording partial protection from inactivation.

Since the oxidation products of 6-NH2DA (e.g., 6-NH2DAQ, aminochromes **1** and **2**) are structurally related to inhibitors which bind at the catechol binding site[16,17] (α-hydroxycarbonyl compounds), it would be expected that catechol substrates should protect the enzyme from inactivation by 6-NH2DA. When 3,4-dihydroxyacetophenone (DHA) was included in the preincubation mixture containing 6-NH2DA only slight protection was observed as is shown in Table II. However, if both SAM (or SAH) and DHA were included in the preincubation mixture substantial protection was observed. The protection observed when both substrates were present in the preincubation mixture is much greater than the protection produced by SAM (or SAH) alone or than that expected from a simple combination of the two substrates. We believe this enhanced protection results because SAM (or SAH) facilitates the binding of the catechol substrate. The SAM–COMT complex (or SAH–COMT complex) must have a higher affinity for DHA than does the enzyme alone, thereby allowing the catechol substrate to compete more effectively with the inhibitor for binding to the enzyme. Similar results have been observed in our laboratory for protection of COMT from inactivation by 6-OHDA,[6] adrenochrome,[6] and *N*-iodoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine.[15]

To further explore the mechanism of the interaction between COMT and the oxidation products of 6-NH2DA, the time course of COMT inactivation by 6-NH2DA was studied and the results are shown in Figure 2. At each concentration of 6-NH2DA studied a linear relationship was observed when the logarithm of the percentage of activity remaining was plotted vs. preincubation time suggesting the inactivation follows pseudo-first-order kinetics. 6-NH2DA is known to be extremely unstable at neutral and alkaline pH and that under such conditions it is rapidly oxidized to 6-NH2DAQ.[7] Under the preincubation conditions used in this study (pH 7.60, 37°), it would be expected that complete oxidation of 6-NH2DA to 6-NH2DAQ would occur in the first few minutes of the preincubation, so that the time course for inactivation of COMT shown in Figure 1 really represents that of 6-NH2DAQ and/or its further rearrangement and oxidation products (Scheme I) generated in situ. The linearity of the plots shown in Figure 2 are of particular interest, since similar plots for 6-OHDA inactivation of COMT[6] exhibited a nonlinear relationship with an apparent lag time before rapid inactivation of COMT occurred. The lag time for 6-OHDA inactivation of COMT was shown to result because of the relatively slow cyclization of 6-OHDAQ to aminochromes **1** and **2**, which then inactivated the enzyme at a faster rate than 6-OHDAQ itself.[6] The linearity of the plots shown in Figure 2 suggests no similar lag time in the formation of the toxic species from 6-NH2DA. It is known that the rate of cyclization of 6-NH2DAQ to aminochrome **1** ($t_{1/2} \simeq$ 30 sec, 37°) is much faster than the similar cyclization of 6-OHDAQ ($t_{1/2}$ = 36 min, 37°).[18]

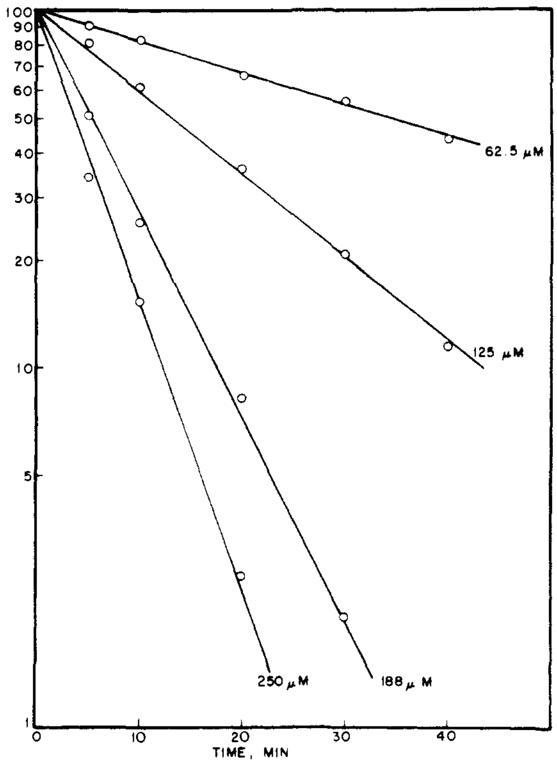
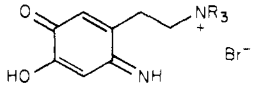


Figure 2. Effect of 6-aminodopamine (6-NH$_2$DA) on COMT activity. Purified COMT was first incubated with 6-NH$_2$DA and the residual enzyme activity was monitored as a function of time as described in the Experimental Section. Inhibitor concentrations indicated were those present in the preincubation mixtures.

Therefore, the linearity observed in Figure 2 could result because of the rapid production of the aminochrome **1** and **2** from 6-NH2DAQ and their subsequent interaction with COMT, or it could result from 6-NH2DAQ being as reactive or more reactive toward the enzyme than the aminochromes. The latter possibility is of particular interest, since in our earlier studies[6] we concluded that the 6-OHDAQ was much less reactive toward COMT than the aminochrome products.

To evaluate the reactivity of the transient intermediate 6-NH2DAQ, analogs **5** and **6** were synthesized and tested for their ability to inactivate COMT. The compounds **5** and **6** provided a way of generating in situ derivatives of 6-NH2DAQ, since Adams et al.[18] recently showed by electrochemical studies that these compounds rapidly oxidize to the intermediate quinoid species **15** and **16**; but because of their structures, these quinoid intermediates do not undergo intramolecular cyclization to the corresponding aminochromes. We have observed from spectrophotometric experiments, however, that the quinoids **15** and **16** under aerobic conditions are unstable and appear to rapidly polymerize.



15, R = CH_3, CH_3, H
16, R = CH_3, CH_3, CH_3

When analogs **5** and **6** were incubated with purified COMT rapid and irreversible inactivation of the enzyme was observed. Similar to our observations for 6-NH2DA, the inactivation of COMT by **5** and **6** could be prevented by including sodium metabisulfite in the preincubation mixtures or by excluding oxygen; however, catalase did not protect the enzyme from inactivation. The time course for inactivation of COMT by several concentrations of

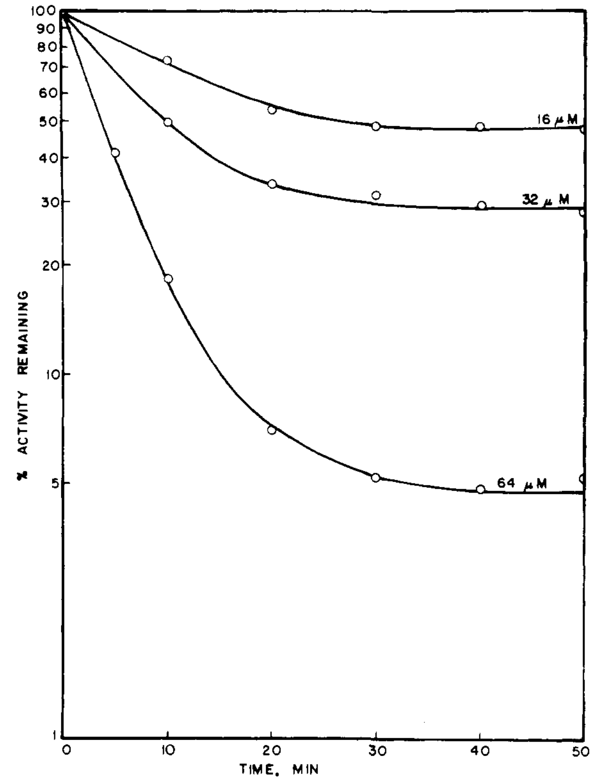


Figure 3. Effect of *N*,*N*,*N*-trimethyl-2-(2'-amino-4',5'-dihydroxyphenyl)ethylammonium bromide (6) on COMT activity. Purified COMT was first incubated with compound 6 and the residual enzyme activity was monitored as a function of time as described in the Experimental Section. Inhibitor concentrations indicated were those present in the preincubation mixtures.

compound **6** is shown in Figure 3 (plots of the same type have been observed for compound **5**). Of particular interest is the potency of these compounds in inactivating COMT. The quinoid **16** generated in situ from oxidation of **6** appears to be a very reactive species with the enzyme. However, the plots shown in Figure 3 suggest that with prolonged preincubation the rate of inactivation decreases. This appears to be the result of the unstable nature of the quinoid **16**. The transient nature of the species toxic to COMT is demonstrated by the data shown in Figure 4. In this experiment analog **6** was incubated alone in pH 7.60 buffer (37°) for various times (pre-preincubation time), after which aliquots were removed and tested for their ability to inactivate COMT. As can be seen in Figure 4, prolonged pre-preincubation of the analog **6** results in a decrease in its ability to inactivate COMT. This decrease in potency apparently relates to the chemical instability of the intermediate quinoid **16** under the preincubation conditions, an instability which is supported by spectrophotometric studies.

Of particular interest was the observation that quinoids **15** and **16** (therefore possibly 6-NH_2DAQ) are very reactive with COMT. This is in contrast to the activity of 6-OHDAQ and 2-hydroxy-5-methyl-1,4-benzoquinone which were previously shown to be quite unreactive toward COMT.[6] This increased ability of **15** and **16** for inactivation of COMT may result from the increased reactivity of these imine-containing quinoid systems (6-NH_2DAQ, **15** and **16**) to attack by nucleophiles, as compared to the corresponding *p*-quinones (6-OHDAQ, 2-hydroxy-5-methyl-1,4-benzoquinone). Evidence to support this higher chemical reactivity to nucleophilic attack of 6-NH_2DAQ as compared to 6-OHDAQ can be seen from their relative rates of intramolecular cyclization to aminochrome **1**.[18]

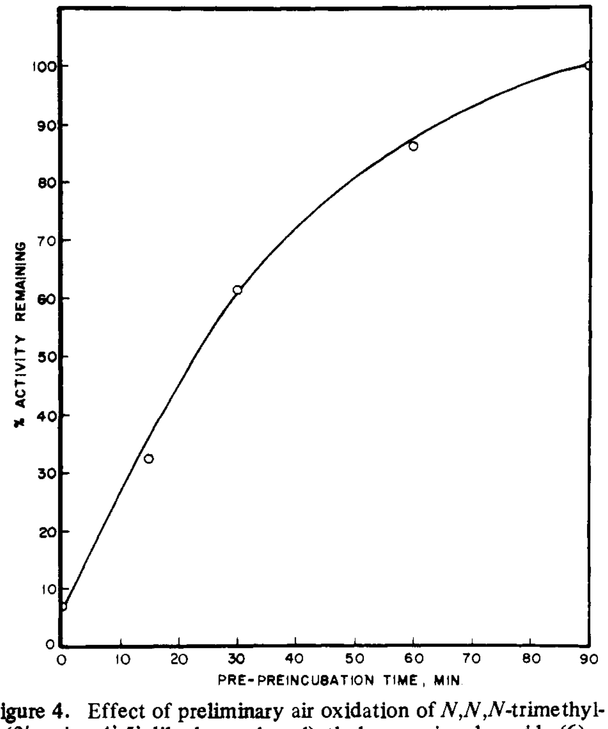
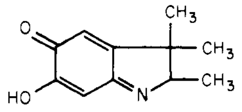


Figure 4. Effect of preliminary air oxidation of *N*,*N*,*N*-trimethyl-2-(2'-amino-4',5'-dihydroxyphenyl)ethylammonium bromide (6) on inactivation of COMT. A solution of compound 6 (5.0 m*M*) in phosphate buffer, pH 7.60, was pre-preincubated at 37° for the indicated times to allow for air oxidation. Aliquots of this pre-preincubation solution were removed at the indicated times and added to preliminary incubation mixtures containing purified enzyme (final concentration of 6 in the preliminary incubation mixture = 64 μ*M*) and incubation was carried out at 37° for 20 min, after which the samples were assayed (see Experimental Section) to determine the residual enzyme activity. Points represent the averages of duplicate determinations.

In earlier studies from our laboratory[6] we had evaluated the reactivity toward COMT of adrenochrome and aminochrome **2**, which was generated in situ by air oxidation of 5,6-DHI. From these studies we concluded that aminochrome **2** and possibly aminochrome **1** were the intermediates in the oxidation of 6-OHDA responsible for inactivation of COMT. Our data suggested that these aminochromes (**1** and **2**) were more reactive toward COMT than 6-OHDAQ. To evaluate further the reactivity of compounds structurally related to aminochrome **1**, we prepared compounds **3** and **4**, which we expected would rapidly oxidize to *p*-quinoids and then cyclize to aminochromes **17** and **18**. Since the position adjacent to the aromatic ring in **17** and **18** is blocked with alkyl substituents, rearrangement to the corresponding dihydroxyindoles should be prohibited. In support of the above prediction recent electrochemical data[18] indicate that compounds **17** and **18** are rapidly formed upon oxidation of **3** and **4** but that these aminochromes do not rearrange to the corresponding 5,6-DHI's. In addition, it was observed[18] that the rates of cyclization to form aminochromes **17** and **18** were extremely fast ($t_{1/2}$ = <0.01 sec, 37°), so that no significant buildup of the intermediate *p*-quinoids would be expected. Compounds **3** and **4** provided a convenient method for generating in situ relatively stable analogs of aminochrome **1**.



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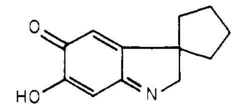

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When analogs **3** or **4** were incubated with purified

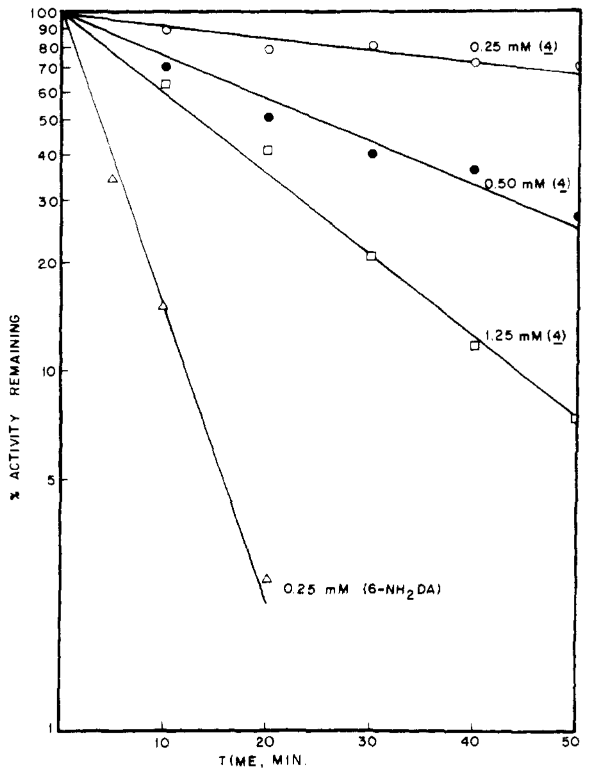

Figure 5. Effect of 1-(2'-amino-4',5'-dihydroxyphenyl)-1-methylaminocyclopentane (4) on COMT activity. Samples containing compound 4 at the indicated concentrations and purified COMT were preincubated and the residual COMT activity was monitored as a function of time as described in the Experimental Section. For comparison the inactivation of COMT by 6-NH$_2$DA (0.25 m*M*) (△—△) is also shown in this figure. Points represent the averages of duplicate determinations.

COMT, inactivation of the enzymes resulted. The inactivation of COMT produced by 3 or 4 could be prevented by antioxidants or anaerobic conditions, similar to that observed for 6-NH$_2$DA. Shown in Figure 5 are the time course for inactivation of COMT by various concentrations of analog 4 (similar data were obtained for analog 3). Somewhat surprising was the relatively weak inhibitory activities of 3 and 4 as compared to 6-NH$_2$DA (Figure 5). The COMT inactivating abilities of 3 and 4, however, are analogous to those results previously seen with adrenochrome or aminochrome 2.[6] The relatively weak inhibitory activity of 3 and 4 may result in part from poor enzymatic binding of the aminochromes 17 and 18 because of the bulky alkyl substituents on the indoline ring.

The data observed with these 6-NH$_2$DA analogs would suggest that the COMT inactivation produced by 6-NH$_2$DA probably results from the interaction of the enzyme with aminochromes 1 and 2 and 6-NH$_2$DAQ. The most active species toward COMT in the oxidation pathway of 6-NH$_2$DA would appear to be 6-NH$_2$DAQ; however, aminochromes 1 and 2 would also contribute significantly to the inactivation of this enzyme.

Conclusion

In the present study we have attempted to elucidate the mechanism by which 6-NH$_2$DA produces inactivation of COMT in vitro. By investigating what effects antioxidants, anaerobic conditions, and catalase had on this inactivation process, it was concluded that the toxic species toward COMT were the quinoid type products generated upon air oxidation of 6-NH$_2$DA. In addition, the results from substrate protection studies and kinetic studies of inactivation suggested that the inactivation process involved a specific modification of an amino acid at the active site of COMT. These observations appeared to be consistent with our earlier findings on the mechanism of 6-OHDA inactivation of this enzyme.[6]

We have also explored in more detail the reactivity toward COMT of the specific intermediates in the oxidation pathway of 6-NH$_2$DA. The results of these studies suggested that in the oxidation pathway of 6-NH$_2$DA the most reactive intermediate with COMT appears to be 6-NH$_2$DAQ. The high reactivity of 6-NH$_2$DAQ toward COMT as compared to 6-OHDAQ appears to be the major difference in the mechanisms by which 6-OHDA and 6-NH$_2$DA produce inactivation of this enzyme. The difference in reactivity of 6-OHDAQ and 6-NH$_2$DAQ toward COMT, we believe, is associated with the greater electrophilic nature of 6-NH$_2$DAQ as compared to 6-OHDAQ. Since this inactivation of COMT appears to result from alkylation of a nucleophile at the active site of COMT,[6] an order of reactivity of 6-NH$_2$DAQ >> 6-OHDAQ is reasonable. Work is continuing in our laboratory in an effort to identify the specific amino acid residue on COMT which is being modified by these reagents.

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References and Notes

(1) (a) Established Investigator of the American Heart Association. (b) Deceased July 14, 1974.
(2) (a) Abbreviations used are COMT, catechol *O*-methyltransferase; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosylhomocysteine; DHA, 3,4-dihydroxyacetophenone; 6-NH$_2$DA, 6-aminodopamine; 6-NH$_2$DAQ, 6-aminodopamine-*p*-quinone; 6-OHDA, 6-hydroxydopamine; 6-OHDAQ, 6-hydroxydopamine-*p*-quinone; 5,6-DHI, 5,6-dihydroxyindole. (b) V. G. Longo, *Behav. Biol.*, **9**, 397 (1973); (c) G. Jonsson and C. Sachs, *J. Neurochem.*, **21**, 117 (1973); (d) A. Oke, R. Freeman, and R. N. Adams, *Eur. J. Pharmacol.*, **26**, 125 (1974).
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Potential Oxidative Pathways of Brain Catecholamines†

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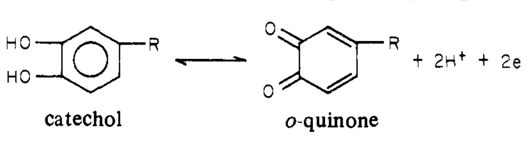
The possibility that catecholamines can be oxidized via aberrant pathways in vivo is open to question, but in vitro oxidation via aerobic manipulations is established. Assuming oxidation does occur, we have examined quantitatively the fast chemical reactions of the initial oxidation products, the *o*-quinones. The nature and rates of these reactions were studied under conditions simulating closely those which presumably exist in mammalian brain. The results are in close accord with existing literature and especially support oxidation pathways recently reported in [^{3}H]-norepinephrine binding to particulate cell fractions.

The possibility of aberrant oxidations of catecholamines (CA) has remained an active issue in the development of biochemical theories of mental illness, particularly schizophrenia. The original adrenochrome hypothesis seems relegated to history. However, the possibility of CA oxidations continues to surface in various reports of disturbances in oxidative metabolism, circulating rheomelanins,[1] and formation of the powerful neurotoxin, 6-hydroxydopamine.[2,3] Very recently Maguire et al.[4] have postulated that oxidative reactions of norepinephrine (NE) are involved in binding of [^{3}H]-NE with particulate fractions. They suggest that these findings may invalidate previous binding studies of adrenergic receptors.

Whether or not such aberrant oxidations can occur in vivo remains an unanswered question. That problem is not the object of the present study. Instead, we assume such reactions might occur under certain conditions in CNS. If so, the oxidized CA's produced even at picogram levels or less could have serious functional significance. The studies herein provide quantitative data about what would happen to the oxidized intermediates under conditions which exist in vivo in CNS. In addition, the results are highly pertinent to the particulate binding studies mentioned above.

The CA oxidations referred to are, of course, not the usual monoamine oxidase degradations, but conversion of the catechol moiety to the corresponding *o*-quinone as

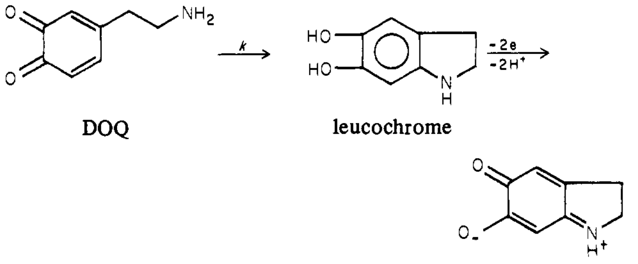


catechol *o*-quinone

where R = $CH_2CH_2NH_2$ = dopamine (DA)
= $CH(OH)CH_2NH_2$ = norepinephrine (NE)
= CH_3 = 4-methylcatechol model analog

Other CA metabolites where the side chain is an alcohol or an acid function may be considered similarly. Dopamine is of primary interest in the present study and most of the reactions employed it or 4-methylcatechol as a model compound.

The major distinguishing feature of the primary oxidation product (the *o*-quinone) is that it is an electron-deficient species and therefore highly reactive with respect to nucleophiles. The most readily available nucleophile is the side-chain amine group of the *o*-quinone itself; hence, an intracyclization can take place near neutral pH as illustrated for dopamine-*o*-quinone (DOQ).

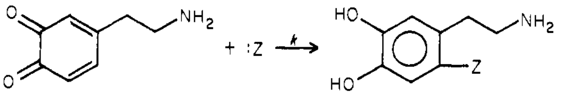


DOQ leucochrome

"dopamine-chrome"

The initial cyclized product, the leucochrome, is, of course, much more easily oxidized than the parent catechol; hence, oxidation proceeds further to the well-known aminochrome, in this case, dopamine-chrome. The complete electrochemical elucidation of this reaction has been reported previously[5] and Harrison et al.[6] have shown that chemical oxidations proceed similarly.

This intramolecular cyclization is just a special case of general nucleophilic reactions of the *o*-quinone. Despite the direct availability of the ethylamine side chain, the present studies show that more "aggressive" external nucleophiles can easily compete with the intracyclization as



where :Z represents a typical nucleophile species. Depending on the nature of Z, these addition products can also easily undergo further oxidation.

Hence, three reaction pathways of the original *o*-quinone must be considered as potentially important: (1) intracyclization yielding aminochromes, (2) addition of external nucleophiles known to be present in high concentration in brain, and (3) rereduction to the original catechol by endogenous reductants before either reaction 1 or 2 has time to occur.

The relative rates of these chemical reactions (all of which are possible in the CNS milieu) dictate the fate of any oxidized CA. From the rates one can predict what

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† This paper is dedicated to the memory of Professor Edward E. Smissman, a dear friend and colleague whose enthusiastic support and interest in our studies is sorely missed.