Biochimica et Biophysica Acta, 445 (1976) 598—609

BBA 67922

EVIDENCE FOR SULFHYDRYL GROUPS AT THE ACTIVE SITE OF CATECHOL-*O*-METHYLTRANSFERASE

RONALD T. BORCHARDT and DHIREN R. THAKKER

Department of Biochemistry, McCollum Laboratories, University of Kansas, Lawrence, Kan. 66045 (U.S.A.)

(Received March 1st, 1976)

Summary

Earlier studies using affinity labeling reagents have suggested the existence of two nucleophilic groups at the active site of catechol-*O*-methyltransferase (*S*-adenosyl-L-methionine:catechol *O*-methyltransferase, EC 2.1.1.6). Both nucleophilic residues are critical for catalytic activity. In an effort to elucidate the nature of these residues and to further characterize the relationship between the chemical structure and the catalytic function of this enzyme, inactivation studies using *N*-ethylmaleimide were undertaken. Inactivation of the enzyme by *N*-ethylmaleimide under pseudo first-order conditions exhibited a non-linear relationship between the log of the fraction of enzyme activity remaining and preincubation time. Kinetic analysis of this inactivation process suggested the modification by *N*-ethylmaleimide of two residues at the active site of the enzyme, both crucial for catalytic activity. Detailed analysis of the inactivation process including substrate protection studies, pH profiles of inactivation, and incorporation studies using *N*-ethyl[2,3-$^{14}C_2$]maleimide provided additional evidence to support this conclusion.

Introduction

Catechol-*O*-methyltransferase (*S*-adenosyl-L-methionine:catechol *O*-methyltransferase, EC 2.1.1.6) plays a major role in the extraneuronal inactivation of catecholamines and the detoxification of many xenobiotic catechols. Catechol-*O*-methyltransferase is a soluble enzyme which requires magnesium to catalyze the transfer of a methyl group from *S*-adenosylmethionine to a catechol substrate, resulting in the formation of the meta and para *O*-methylated products

Abbreviations: MalNEt, *N*-ethylmaleimide; [^{14}C]MalNEt, *N*-ethyl[2,3-$^{14}C_2$]maleimide; *N*-iodoacetyl-3,5-DMH-PEA, *N*-iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine; *N*-iodoacetyl-3,4-DMH-PEA, *N*-iodoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine; TES, *N*-tris(hydroxymethyl)-2-aminoethane sulfonic acid.

[1,2]. Earlier studies have suggested that one or more sulfhydryl groups are involved in the catalytic function of this enzyme [4—7]. These studies suggested that the *N*-haloacetyl derivatives of 3,5-dimethoxy-4-hydroxyphenylalkylamines and 3,4-dimethoxy-5-hydroxyphenylethylamines (resembling meta and para methylated products, respectively) react with two different nucleophiles at the active site of this enzyme and that both nucleophiles are crucial for the enzyme activity. Furthermore, apparent pK_a values of the nucleophiles being modified by these affinity labeling reagents suggested that one of these groups is a sulfhydryl group and the other group is either an amino group or a sulfhydryl group with a high apparent pK_a [6].

In order to provide supporting evidence for the presence of two nucleophiles at the active site of this enzyme and to gain more insight into the chemical nature of these nucleophiles, a functional group modification study using *N*-ethylmaleimide (MalNEt) was undertaken. Evidence is presented in this report, based on the kinetics of inactivation of catechol-*O*-methyltransferase by MalNEt and the incorporation of *N*-ethyl[2,3-$^{14}C_2$]maleimide ([^{14}C]MalNEt), which suggests the presence of three accessible nucleophiles on the enzyme, two of them being crucial for the enzymatic activity.

Materials and Methods

Chemicals. *S*-Adenosyl[*Me*-^{14}C]methionine (New England Nuclear, 55.0 Ci/mol) and [^{14}C]MalNEt (Amersham/Searle, 2.1 Ci/mol) were diluted to 10 μCi/ml and 9.57 μCi/ml, respectively, and stored at −29°C. *S*-Adenosylmethionine chloride (Sigma) was stored as a 0.01 M aqueous stock solution. MalNEt (Sigma) was stored as a 2.0 mM stock solution. The following compounds are available from the indicated sources: *S*-adenosylhomocysteine (Sigma); 3,4-dihydroxybenzoic acid (Aldrich); Nuclear Chicago Scintillator tissue solubilizer (Amersham/Searle). 3,4-Dihydroxyacetophenone, *N*-iodoacetyl-3,5-dimethoxy-4-hydroxyphenylethylamine (*N*-iodoacetyl-3,5-DMH-PEA) and *N*-iodoacetyl-3,4-dimethoxy-5-hydroxyphenylethylamine (*N*-iodoacetyl-3,4-DMH-PEA) were prepared using previously described procedures [5,6,8].

Catechol-O-methyltransferase isolation and assay. Catechol-*O*-methyltransferase was purified from rat liver (male, Sprague-Dawley, 180—200 g) using a previously published procedure [9]. The enzyme was purified through the Sephadex G-100 stage resulting in a 910-fold purification of the enzyme as compared to the crude supernatant [9]. At this stage of purification the enzyme showed only a single major band of 23 000 molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with several minor higher molecular weight bands [9]. 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 experimental conditions used in these studies (25°C, 0—30 min), but was unstable at higher temperatures and longer incubation times. The enzyme activity was determined using *S*-adenosyl[*Me*-^{14}C]methionine and 3,4-dihydroxybenzoate as substrates according to a previously described radiochemical assay [10].

Catechol-O-methyltransferase inactivation experiments. A typical preincubation mixture consisted of the following components (in μmol): water, so that

the final volume was 0.2 ml; $MgCl_2$ (0.30); *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) buffer, pH 7.6 (10.0); MalNEt (variable) and purified enzyme preparation (2.1 μg). The preincubation step was started by addition of enzyme. After the appropriate incubation time at 25°C, the reaction mixture was assayed for residual enzyme activity by addition of 3,4-dihydroxybenzoic acid (0.5 μmol), *S*-adenosylmethionine (0.025 μmol) and *S*-adenosyl[*Me*-^{14}C]methionine (0.1 μCi) to a final volume of 0.25 ml. The assay mixture was incubated for 4 min at 37°C and the methylated products isolated as described [10]. The presence of high concentrations of both substrates (3,4-dihydroxybenzoic acid and *S*-adenosylmethionine) in the assay step, provided a means of stopping the further inactivation of the enzyme by MalNEt. The presence of both substrates in the assay mixture was shown to completely protect the enzyme from further modification by MalNEt.

Incorporation of N-ethyl[2,3-$^{14}C_2$] maleimide. A typical reaction mixture for the incorporation experiments consisted of the following components (in μmol): water so that the final volume was 0.5 ml; $MgCl_2$ (0.60); TES buffer, pH 7.3 (20.0); [^{14}C]MalNEt (0.05 μmol, 0.096 μCi) and purified enzyme (5.25 μg). The incubations were carried out at 25°C. After an appropriate incubation time, the reaction mixture was diluted with distilled water (0—4°C, 1.0 ml) to stop the reaction and then the diluted reaction mixture was immediately filtered (less than 1 min after dilution) through a Millipore filter (Type HAMK, 0.25 mm) under suction. The filter was washed with 20 ml of distilled water, dried, and the extent of incorporation was determined by placing the filter in a vial containing 10 ml of scintillation fluid (0.6% 2,5-diphenyloxazole (PPO), 1,4-bis[2(5-phenyloxazolyl)]benzene (POPOP) in toluene) and counting for radioactivity. It could be demonstrated that all of the catechol-*O*-methyltransferase was retained by the Millipore filter, since in control experiments where MalNEt was not included in the preincubation mixture, no enzyme activity was detectable in the filtrate. In addition, it appeared that the MalNEt-inactivated enzyme was also completely retained by the Millipore filter, since a good correlation was observed between the incorporation studies done by Millipore filtration and those carried out by gel electrophoresis. In the electrophoretic studies 20 μg of catechol-*O*-methyltransferase was labeled with [^{14}C]-MalNEt as described above. The excess MalNEt 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 potassium phosphate buffer (pH 7.0) which contained 0.8% sodium dodecyl sulfate. To the dissolved protein was added 5 μl 0.05% Bromophenol Blue in methanol, one drop of glycerol, and 10 μl of mercaptoethanol, and the resulting solution was then subjected to electrophoresis on polyacrylamide gels (10% acrylamide and 0.27% bisacrylamide cross-linked, pH 7.0) [9,11]. The gels were fixed and stained by incubation for 2 h at room temperature in a solution of 0.25% Coomassie Blue in methanol/acetic acid/water (45 : 9 : 46, v/v). The gels were subjected to densitometric scanning at 550 nm before slicing in order to estimate the relative amount of protein. Each gel was then cut into 3-mm slices and each slice extracted with 1.5 ml of a mixture of N.C.S. tissue solubilizer and water (9 : 1, v/v) by heating in a sealed scintillation vial at 50°C for 12 h. 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 h [6].

Data processing. To estimate the values of the rate constants for MalNEt inactivation of catechol-*O*-methyltransferase we have used the so-called feathering technique [12,13]. In order to estimate the value of k_2 we have assumed that in Eqn. 4 (see Results) the term $e^{-(k_1+k_2)t}$ approaches zero much faster than the term $e^{-k_2 t}$ as t approaches infinity since $k_1 >> k_2$. Therefore, at large values of time (t) the terminal part of the curve in Fig. 2 can be described by Eqn. 1 and an estimate of the value of k_2 can be made from the slope of the extrapolated line. Subtracting Eqn. 1

$$A/A_0' \simeq Fe^{-k_2 t} \tag{1}$$

from Eqn. 4, one can derive Eqn. 2, from which an estimate of the value of k_1 can be made. When log A/A_0'' is plotted vs. time (Fig. 2), $k_1 + k_2$ can be

$$A/A_0 - A/A_0' = A/A_0'' \simeq (1 - F)\, e^{-(k_1+k_2)t} \tag{2}$$

calculated from the slope of the line. Therefore, having available an estimated value for k_2 one can estimate the value of k_1. An estimate of the value of F can be obtained from the y-intercept of the plot of log A/A_0' vs. time. Using the experimental data shown in Fig. 2 a curve fitting process using Eqn. 4 was attempted. Initially, approximate values of k_1, k_2 and F were required for this curve fitting procedure and best estimate were made using the feathering tech-

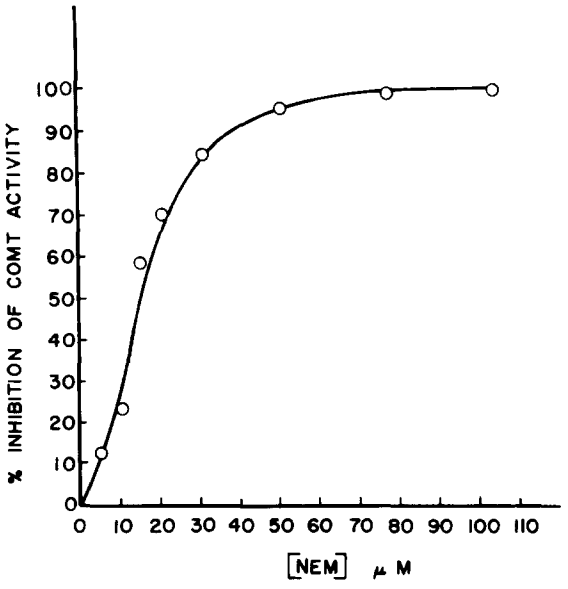


Fig. 1. The effect of MalNEt concentration on catechol-*O*-methyltransferase activity. Purified catechol-*O*-methyltransferase was preincubated with various concentrations of MalNEt (0—103 μM) for 50 min at 37°C and the enzyme activity monitored as described in Materials and Methods. Control experiments without MalNEt were incubated under the same conditions. The abbreviation NEM on the figure means *N*-ethylmaleimide.

nique described above and illustrated in Fig. 2. Accurate values of k_1, k_2 and F were subsequently calculated using a simplex computer program from the best fit to the experimental data. The resulting curve generated from Eqn. 4 using a simplex program and a Hewlett-Packard 2100 computer is shown in Fig. 2.

Results

N-Ethylmaleimide inactivation of catechol-O-methyltransferase

When catechol-*O*-methyltransferase was incubated with MalNEt, rapid and irreversible enzyme inactivation occurred indicating the presence of sulfhydryl group(s) essential for catalytic activity. The effect of MalNEt concentration on the inactivation of the enzyme is illustrated in Fig. 1. At high concentrations of MalNEt (>0.1 mM) complete inactivation of the enzyme was observed. Similar observations have been made by Axelrod and Tomchick [1] and Lutz et al. [3] using other sulfhydryl group reagents. In Fig. 2 is shown the time course of inactivation of the enzyme by MalNEt under pseudo first-order conditions. When the logarithm of the fraction of enzyme activity remaining (A/A_0) is plotted vs. preincubation time, a non-linear relationship is observed. This result is contrary

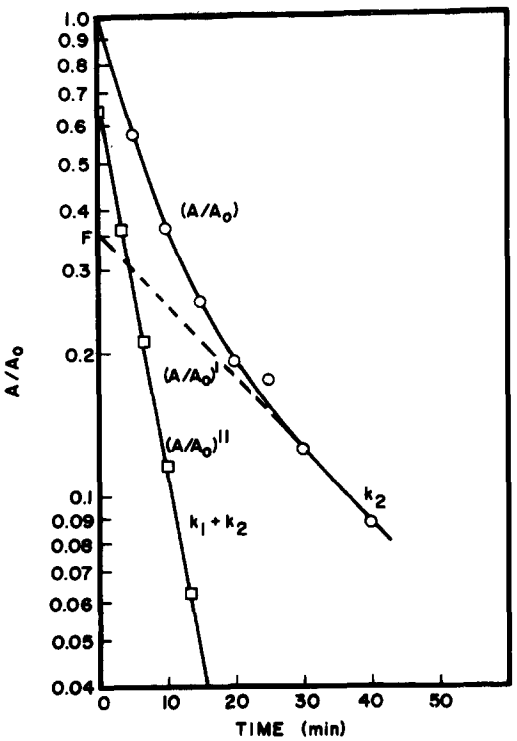


Fig. 2. Effect of MalNEt on catechol-*O*-methyltransferase activity. The enzyme purified through the Sephadex G-100 stage [9] was preincubated with MalNEt (0.1 mM) and the enzymatic activity monitored as described in Materials and Methods; A, activity at time t; A_0, activity at time zero; F, fraction of activity remaining when one group is completely modified. ○, experimental data points; ———, theoretical curve generated using Eqn. 4 as described in the Data Processing section; - - - - - -, $A/A_0' = Fe^{-k_2 t}$; □———□, $A/A_0'' = (1 - F)e^{-(k_1 + k_2)t}$. See Data Processing section for detailed analysis of this feathering technique.

to the pseudo first-order kinetics observed with affinity labeling reagents for this enzyme [4—6]. The type of data shown in Fig. 2 in which the slope of the curve decreases with time suggests that at least two residues are being modified on the protein resulting in loss of enzyme activity. The model shown in Scheme 1 to explain such kinetic behavior has been proposed by Ray and Koshland [12]. According to this model the observed activity as a function of time is given by Eqn. 3;

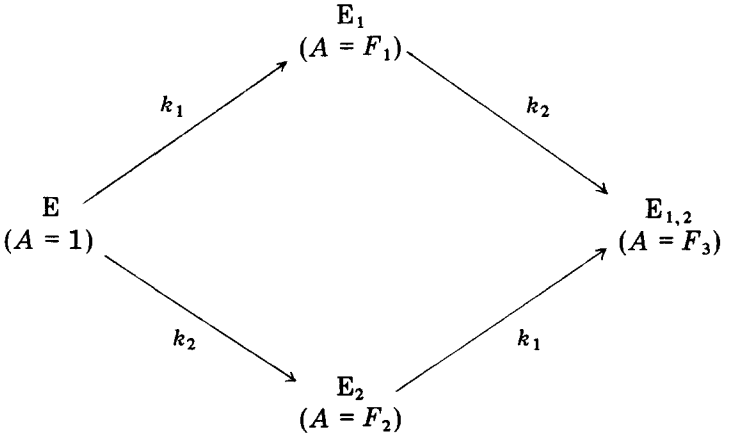
$$A/A_0 = F_3 + (1 - F_1 - F_2 + F_3)\, e^{-(k_2+k_2)t} + (F_1 - F_3)\, e^{-k_2 t}$$

$$+ (F_2 - F_3)\, e^{-k_1 t} \qquad (3)$$

where A_0 and A represent the enzyme activities at times 0 and t, respectively; F_1, F_2 and F_3 are the fractional enzyme activities after modification of group 1, group 2 and both groups on the enzyme, respectively; and k_1 and k_2 are the first-order rate constants for the modification of residues 1 and 2, respectively. Evaluation of these constants is possible if one assumes that $F_2 = F_3 = 0$, since Eqn. 3 then takes on a much simpler form under these limiting conditions as shown in Eqn. 4.

$$A/A_0 = (1 - F)\, e^{-(k_1+k_2)t} + Fe^{-k_2 t} \qquad (4)$$

The data in Fig. 2 were graphically analyzed by the feathering technique [12,13] to obtain rough estimates of the constants k_1, k_2 and F. Using these approximate values of k_1, k_2 and F, a computer-simulated curve was generated using Eqn. 4 and the experimental data points (See Materials and Methods). As can be seen in Fig. 2, a very good correspondence between the theoretical curve and experimental data was obtained indicating that the inactivation of



Scheme 1. A model to explain the kinetics of inactivation of catechol-O-methyltransferase by MalNEt. E represents the enzyme and A the enzyme activity; F_1, F_2 and F_3 are the partial enzyme activities after modification of group 1, group 2, and both residues, respectively; k_1 and k_2 are the first-order rate constants for modifications of residues 1 and 2 leading to the enzyme forms E_1 and E_2, respectively; $E_{1,2}$ is the doubly modified enzyme.

TABLE I

SUBSTRATE PROTECTION OF CATECHOL-*O*-METHYLTRANSFERASE FROM INACTIVATION BY *N*-ETHYLMALEIMIDE

Inactivation experiments were carried out as described in the Materials and Methods. A standard preincubation mixture consisted of the following components (in μmol): water, so that the final volume was 0.2 ml; MgCl (0.30); TES buffer, pH 7.6 (10.0); MalNEt (0.02); purified enzyme (2.1 μg) and the indicated additions. Incubations were carried out at 25°C for 0 or 30 min, after which time the samples were assayed for residual activity.

Reaction mixture	Additions (mM)		Percent residual activity after 30 min, 25°C *
	3,4-Dipydroxy-acetophenone	*S*-Adenosyl-homocysteine	
1	—	—	12
2	0.1	—	34
3	1.0	—	59
4	5.0	—	78
5	10.0	—	83
6	—	0.01	20
7	—	0.025	29
8	—	0.075	43
9	10.0	0.10	98

* Residual activity after 30 min was calcualted relative to the activity of the control samples preincubated for 0 min.

catechol-*O*-methyltransferase by MalNEt can be satisfactorily described by Eqn. 4. The values of k_1, k_2 and F calculated from the data shown in Fig. 1 using the simplex fit are 0.139 min^{-1}, 0.035 min^{-1} and 0.357, respectively. The fact that the experimental data could be fit to Eqn. 4 doesn't prove, but only suggests, that the enzyme has two nucleophiles at its active site which behave in a manner consistent with the model shown in Scheme 1.

In order to gain more insight into the inactivation process, substrate protection studies were undertaken. MalNEt inactivation studies were carried out in

TABLE II

EFFECT OF pH ON INACTIVATION OF CATECHOL-*O*-METHYLTRANSFERASE BY *N*-ETHYLMALEIMIDE

Inactivation experiments were carried out as described in Table I. The final concentration of MalNEt was 0.02 mM. Preincubations were carried out at 25°C for 20 min, after which time samples were assayed for residual activity.

pH preincubation mixture	Percent residual activity after 20 min, 25°C *
6.5	88
7.0	69
7.5	56
8.0	51
8.25	50
8.5	14
9.0	10

* Residual activity after 20 min was calculated relative to the activity of the control samples at the same pH, preincubated for 0 min.

the presence of various concentrations of the catechol substrate 3,4-dihydroxyacetophenone or *S*-adenosylhomocysteine, a product inhibitor of catechol-*O*-methyltransferase. The data shown in Table I indicate that inclusion of either 3,4-dihydroxyacetophenone or *S*-adenosylhomocysteine in the preincubation mixture with MalNEt results in partial protection of the enzyme from inactivation. However, if both 3,4-dihydroxyacetophenone and *S*-adenosylhomocysteine are included in the preincubation mixture together, complete protection of the enzyme from inactivation is observed.

In order to ascertain the chemical nature of the groups being modified by MalNEt and to determine if these functional groups behave similar to those groups modified by the affinity labeling agents for the enzyme (*N*-iodoacetyl-3,5-DMH-PEA and *N*-iodoacetyl-3,4-DMH-PEA [4—6]), inactivation of the enzyme by MalNEt was studied as a function of pH. The results shown in Table II indicate that the extent of inactivation of the enzyme by MalNEt increases as pH increases. This would be the type of pH dependency expected for modification of the sulfhydryl groups and is consistent with data observed earlier for affinity labeling agents [4,6].

Incorporation of N-ethyl[2,3-$^{14}C_2$] maleimide

To obtain further evidence to substantiate the presence of two nucleophilic groups at the active site of catechol-*O*-methyltransferase, incorporation of [^{14}C]MalNEt into the enzyme was investigated. In Fig. 3 the rate of enzyme inactivation is compared with the amount of [^{14}C]MalNEt incorporated into

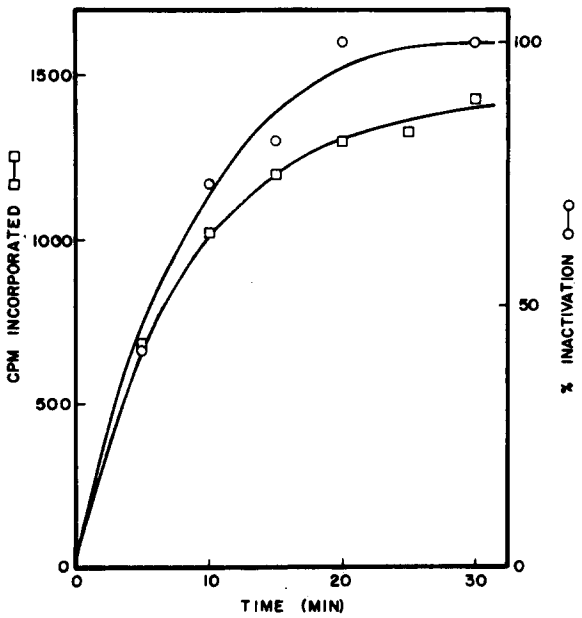


Fig. 3. Extent of catechol-*O*-methyltransferase inactivation produced by [^{14}C]MalNEt as compared to cpm of radioactivity incorporated. Preincubations with the enzyme were carried out using [^{14}C]MalNEt (100 μM) at 25° C. The enzyme activity remaining and the radioactivity incorporated were determined as described in the text.

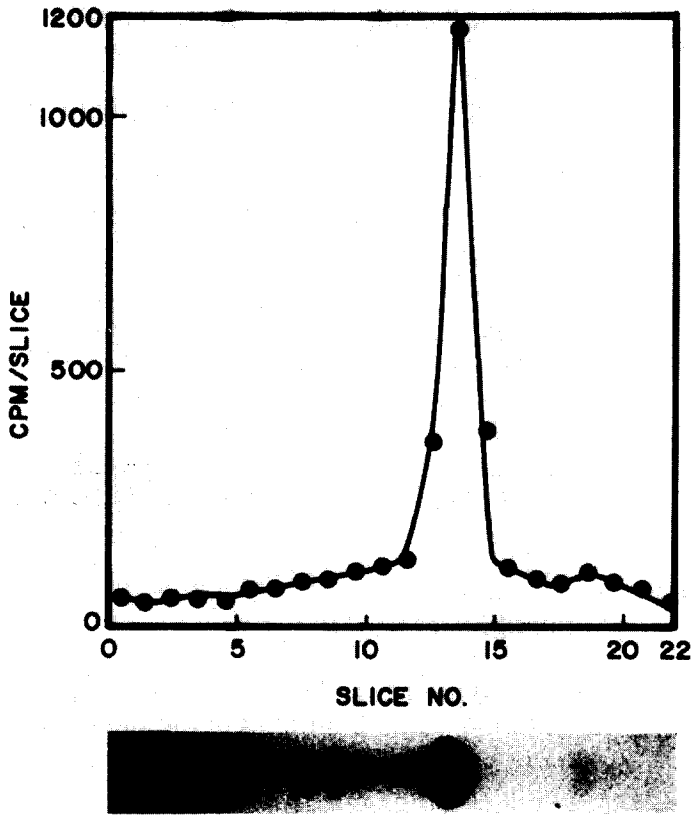


Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic pattern of catechol-*O*-methyltransferase inactivation by [^{14}C]MalNEt and the corresponding plot of cpm of ^{14}C radioactivity per slice. Enzyme purified through the Sephadex G-100 chromatographic step was used for these studies. Gels stained with Coomassie Blue were cut into 3-mm slices and each slice extracted with N.C.S. tissue solubilizer and the radioactivity determined as described in Materials and Methods.

the protein. It is apparent from these data that the incorporation of radioactivity parallels the loss in enzyme activity. The extent of incorporation of [^{14}C]MalNEt, when the enzyme was completely inactivated, was calculated to be approximately 2.5 molecules of MalNEt per molecule of the enzyme using 23 000 daltons as the molecular weight of catechol-*O*-methyltransferase [9,14]. Since the enzyme preparation used in this experiment was slightly contaminated with 2—3 minor proteins, it was necessary to estimate the amount of [^{14}C]MalNEt incorporated specifically into the 23 000 molecular weight band. Hence, the enzyme preparation was treated with [^{14}C]MalNEt, then subjected to sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis and the radioactivity in the catechol-*O*-methyltransferase band determined. Fig. 4 shows the stained polyacrylamide gel and a plot of cpm of radioactivity per slice vs. slice number. It is apparent from Fig. 4 that most of the radioactivity is incorporated into the catechol-*O*-methyltransferase band (M_r 23 000). Calculations showed that 92% of the total radioactivity was incorporated into this band so that in subsequent experiments, when the stoichiometry of the

TABLE III

PROTECTION OF CATECHOL-*O*-METHYLTRANSFERASE FROM INCORPORATION OF *N*-ETHYL-[2,3-$^{14}C_2$]MALEIMIDE

The incorporation experiments were carried out exactly as described in Materials and Methods except the indicated additions were made in the preincubation mixtures.

Additions (mM)				nmol of [^{14}C]MalNEt per nmol of enzyme, 30 min, 25°C *
N-Iodoacetyl-3,5-DMH-PEA	*N*-Iodoacetyl-3,4-DMH-PEA	3,4-Dihydroxy-acetophenone	*S*-Adenosyl-homocysteine	
—	—	—	—	2.52
—	—	10	—	1.63
—	—	—	0.1	1.66
—	—	10	0.1	0.80
1.0	—	—	—	1.55
—	1.0	—	—	1.80

* nmol of [^{14}C]MalNEt incorporated were calculated by correcting for incorporation at 0 min and for the non-specific incorporation into other proteins. These were the average of at least three determinations.

reaction between MalNEt and the enzyme was studied, we corrected for the non-specific incorporation.

In Table III are listed the results of a study in which the extent of incorporation of [^{14}C]MalNEt was determined in the absence and presence of substrates or inhibitors of catechol-*O*-methyltransferase. Using [^{14}C]MalNEt alone, 2.52 mol of [^{14}C]MalNEt were incorporated per mol of enzyme, indicating the presence of approximately three accessible nucleophiles on the enzyme. Inporation studies were carried out under conditions similar to those used in the kinetic experiments (30 min, 25°C). Under the conditions used complete inactivation of the enzyme was observed. Incorporation of 2.52 mole of [^{14}C]-MalNEt per mol of enzyme rather than 3.0 mol suggests that one residue may not have been modfied in all the enzyme molecules present in the incubation mixture under the experimental conditions. When 3,4-dihydroxyacetophenone or *S*-adenosylhomocysteine were included in the incubation mixture, approximately one less molecule of [^{14}C]MalNEt was incorporated per molecule of the enzyme (the ratio of [^{14}C]MalNEt to enzyme decreases from 2.52 to 1.33 and 1.36, respectively) suggesting that one functional group is protected by both 3,4-dihydroxyacetophenone and *S*-adenosylhomocysteine. The ratios obtained in these protection experiments again suggest that the residue that is partially modified must be located outside the active site of the enzyme. When both 3,4-dihydroxyacetophenone and *S*-adenosylhomocysteine were present together in the incubation mixture, 0.80 mol of [^{14}C]MalNEt was incorporated per mol of enzyme, which amounted to protection of two residues from modification by MalNEt per molecule of enzyme. Under these conditions total retention of enzyme activity was observed (Table I) indicating that the residue that is being partially modified is outside the active site of the enzyme and not crucial for enzyme activity. This additive nature of the protection provided by 3,4-dihydroxyacetophenone and *S*-adenosylhomocysteine, both against inactivation of the enzyme and incorporation of [^{14}C]MalNEt, provides considerable support to the idea that the functional groups being protected by the ligands are

two different nucleophiles present at the active site of this enzyme. The affinity labeling agents *N*-iodoacetyl-3,5-DMH-PEA or *N*-iodoacetyl-3,4-DMH-PEA, when included in the incubation mixture, protected one functional group each from modification by [^{14}C]MalNEt (Table III). These observations further substantiate our earlier conclusions that two of the three nucleophiles on the enzyme being modified by MalNEt are at the active site and that these are the same functional groups which are being modified by the two classes of affinity labeling agents described in earlier publications from our laboratory [4—6]. The third residue which is not protected by either substrates or inhibitors of catechol-*O*-methyltransferase must be situated outside the active site of the enzyme and is not crucial for catalytic activity.

Discussion

Earlier studies of catechol-*O*-methyltransferase using functional group reagents have suggested the existence of one or more sulfhydryl groups at the active site of this enzyme, which are crucial for enzyme catalysis [1—3]. Recently, using affinity labeling reagents, we concluded that the active site of this enzyme consists of two different nucleophilic residues [4—6]. In the present study kinetic evidence obtained from the inactivation of the enzyme by MalNEt confirmed our earlier observations and expanded our knowledge concerning the properties of these nucleophilic residues. Detailed kinetic analysis showed that the inactivation of catechol-*O*-methyltransferase by MalNEt could be satisfactorily described by a model system involving the modification of two protein nucleophiles, both crucial for enzyme activity (Scheme 1). When the nucleophilic group, which reacts with a rate constant k_1 (group 1), is completely modified the enzyme is only partially inactivated, whereas when the group reacting with a rate constant k_2 (group 2) is completely modified, the enzyme loses all its catalytic activity. The model system outlined in Scheme 1, as well as the experimental data obtained for MalNEt inactivation of the enzyme, can be described by Eqn. 4 under the limiting conditions that $F_2 = F_3 = 0$. Substrate protection studies showed that the groups being modified by MalNEt were present at the active site of the enzyme. The presence of either 3,4-dihydroxyacetophenone or *S*-adenosylhomocysteine affords partial protection of the enzyme from inactivation by MalNEt. The presence of both a catechol substrate and *S*-adenosylhomocysteine results in complete protection of the enzyme from inactivation.

The incorporation studies substantiated the above conclusions and in addition suggested the presence of a third accessible nucleophilic group on the enzyme which is not essential for catalytic activity and which is situated outside of the active site of the enzyme. The experimental evidence presented here supports our earlier conclusion from affinity labeling studies [6] that one of the nucleophilic groups is probably a sulfhydryl group of a cysteine residue. In addition, our earlier data [6] had suggested that the other nucleophilic group was either an amino group or a sulfhydryl group. From the results reported here using MalNEt, we would have to conclude that both nucleophilic groups at the active site of catechol-*O*-methyltransferase are probably sulfhydryl groups. Whether these nucleophilic residues at that active site of the enzyme

are involved in the binding of the substrates or in actual enzymatic catalysis has not been established. Coward et al. [15] have suggested that a nucleophilic residue at the active site of catechol-*O*-methyltransferase may be involved as a general base in catalysis of this transmethylation reaction. However, no experimental evidence exists to support this conclusion, therefore the function of these nucleophilic residues in the transmethylation catalyzed by catechol-*O*-methyltransferase is at this point unclear. However, from the present studies and our earlier work [4—6], it is apparent that these nucleophilic residues are crucial for the transmethylation catalyzed by this enzyme. Studies to determine the exact nature of the nucleophilic residues present at the active site of this enzyme and their role in enzyme catalysis are presently being carried out in our laboratory.

Acknowledgements

The authors gratefully acknowledge the excellent technical assistance of Patricia Davis. This work was supported by a Research Grant from the National Institutes of Neurological Diseases and Stroke (NS-10918) and a Grant-in-Aid from the American Heart Association. R.T.B. is an Established Investigator of the American Heart Association.

References

1 Axelrod, J. and Tomchick, R. (1958) J. Biol. Chem. 233, 702—705
2 Guldberg, H.C. and Marsden, C.A. (1975) Pharmacol. Rev. 27, 135—206
3 Lutz, W.B., Creveling, C.R., Daly, J.W., Witkop, B. and Goldberg, L.I. (1972) J. Med. Chem. 15, 795—802
4 Borchardt, R.T. and Thakker, D.R. (1973) Biochem. Biophys. Res. Commun. 54, 1233—1239
5 Borchardt, R.T. and Thakker, D.R. (1975) J. Med. Chem. 18, 152—158
6 Borchardt, R.T. and Thakker, D.R. (1975) Biochemistry 14, 4543—4551
7 Borchardt, R.T. (1975) Mol. Pharmacol. 11, 436—449
8 Smissman, E.E. and Borchardt, R.T. (1971) J. Med. Chem. 14, 702—707
9 Borchardt, R.T., Cheng, C.F. and Thakker, D.R. (1975) Biochem. Biophys. Res. Commun. 63, 69—77
10 Borchardt, R.T. (1973) J. Med. Chem. 16, 377—382
11 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
12 Ray, Jr., W.J. and Koshland, D.E. (1961) J. Biol. Chem. 236, 1973—1979
13 Notari, R.E. (1971) Biopharmaceutics and Pharmacokinetics, p. 129, Marcel Dekker, New York
14 Buhuon, C. and Assicot, M. (1973) Frontiers in Catecholamine Research (Usdin, E. and Snyder, S., eds.), p. 107, Pergamon Press, New York
15 Coward, J.K., Slisz, E.P. and Wu, F. Y-H. (1973) Biochemistry 12, 2291—2297