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PURIFICATION AND PROPERTIES OF CATECHOL-*O*-METHYLTRANSFERASE*

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Abstract—A procedure is described for obtaining an improved purification of catechol-*O*-methyltransferase. The molecular weight of the enzyme is estimated to be 29,000 from its behavior on Sephadex G-100. The presence of multiple bands of activity after polyacrylamide gel electrophoresis is reported. It is shown that all catechols are substrates of the enzyme, but that 4-nitrocatechol can act as a non-competitive inhibitor of the *O*-methylation of another catechol.

ONE OF THE enzymes responsible for the metabolism of catecholamines is catechol-*O*-methyltransferase.[1, 2] This enzyme catalyzes the transfer of the labile methyl group of S-adenosyl-L-methionine to one of the phenolic hydroxy groups of a catechol.[1] Since the discovery of the enzyme,[1, 2] several other enzymes capable of catalyzing *O*-methylation have been discovered. These include an acetyl serotonin *O*-methyltransferase,[3] an iodophenol *O*-methyltransferase[4] and a microsomal *O*-methyltransferase, which differs from catechol *O*-methyltransferase in its pH optimum and its response to cold stress and benzypyrene.[5] Because of this diversity in biological *O*-methylation, it was decided that further attempts to purify and characterize catechol-*O*-methyltransferase were necessary.

METHODS AND MATERIALS

Preparation of the enzyme. Because rat liver was found to be one of the best sources of the enzume,[6] this organ was used as the source of the enzyme throughout the subsequent work. The purification procedure devised by Axelrod[7] was used up to and including the ammonium sulfate fractionation, with the exception that homogenization was carried out with a Waring Blendor at one-half maximum speed for 45 sec. Rat liver (65 g) was routinely processed and all operations were carried out in a cold room at 4°. Gel filtration of the ammonium sulfate precipitate was carried out on 2·5 × 45 cm columns of Bio-Gel P-60. The ammonium sulfate precipitate from 120 ml of supernatant was dissolved to a volume of 15 ml in 0·01 M phosphate buffer, pH 8·0, and applied to such a column. The column was eluted with this buffer at a flow rate of 50 ml/hr and 5-ml fractions were collected. Two such columns could be run together. The fractions of highest specific activity were pooled. The Bio-Gel P-60 columns were regenerated by passing buffer through until the eluate no longer reacted with Nessler's

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solution to give an orange precipitate. The pooled enzyme-containing fractions were applied to a 2·5 × 10 cm column of high capacity DEAE-cellulose (Cellex D, Bio-Rad Laboratories) which had been equilibrated with 0·01 M phosphate buffer, pH 8·0. Equilibration of the DEAE-cellulose with the buffer was achieved by two washes with 0·5 M acid phosphate followed by three washes with the buffer. Elution was carried out by a stepwise increase in the molarity of the phosphate buffer. Thirty ml of 0·01 M phosphate buffer, pH 8·0, was passed through the column, followed by 30 ml of 0·02 M buffer. Then the enzyme was eluted by the passage of 30 ml of 0·06 M buffer through the column.

Assay procedures. The enzymic activity was localized in the effluent from columns by an assay based on the enzymatic *O*-methylation of pyrocatecholphthalein.[8] To a mixture containing 0·2 ml of 0·1 M $MgCl_2$, 0·2 ml of $2{\cdot}5 \times 10^{-3}$ M S-adenosyl-L-methionine and 0·2 ml of 0·5 M phosphate buffer (pH 8·0), which was $5{\cdot}7 \times 10^{-4}$ M in pyrocatecholphthalein, 0·2 ml of the fraction to be tested was added. The development of a deep blue color upon the addition of 0·5 ml of 0·25 M borate buffer, pH 10·0, after 10 min of incubation at 37°, indicated the presence of the enzyme in the fraction. Fractions giving the deepest color were pooled. Quantitative determination of the activity in pooled fractions and kinetic examinations of inhibitions observed in the presence of catechols were made spectrophotofluorometrically by the method of Axelrod,[7] except that incubation times were decreased to 5 min and twice the volume of all reagents was used. All reactions were carried out in duplicate.

Compounds were screened as potential substrates for the enzyme by their ability to inhibit the enzymatic *O*-methylation of pyrocatecholphthalein. A 0·1 ml amount of potential substrates dissolved in alcohol was added to incubation mixtures containing 0·2 ml of 0·1 $MgCl_2$, 0·4 ml of $2{\cdot}5 \times 10^{-3}$ M S-adenosyl-L-methionine, 0·4 ml of 0·5 M phosphate buffer (pH 8·0), 0·1 ml of pyrocatecholphthalein dissolved in alcohol, 0·6 ml of water and 0·2 ml of enzyme (P-60 eluate). A final pyrocatecholphthalein concentration of $1{\cdot}5 \times 10^{-4}$ M and a final potential substrate concentration of $1{\cdot}5 \times 10^{-4}$ M were used. Blanks were prepared by the addition of water instead of methyl donor, and controls by the omission of the potential substrates, 0·1 ml alcohol being added instead. Enzyme activity was determined by the quantitative assay for catechol-*O*-methyltransferase that is possible when pyrocatecholphthalein is used as substrate.[8] All reactions were done in triplicate.

The ability of the enzyme to catalyse the *O*-methylation of various catechols was examined by the use of S-adenosyl-L-methionine-methyl-^{14}C with a sp. act. of 40 mc/m-mole as the methyl donor. The contents of vials containing 10 μc of the labeled methyl donor were diluted to 10 ml with water and 13·1 mg of nonradioactive S-adenosyl-L-methionine was added. Incubation mixtures containing 0·2 ml of 0·5 M phosphate buffer (pH 8·0), 0·2 ml of 0·1 M $MgCl_2$ and 0·4 ml of the radioactive S-adenosyl-L-methionine solution were prepared. Potential substrates were added in 0·2 ml of alcohol to a final concentration of 1×10^{-3} M. Reactions were initiated by the addition of 0·5 ml enzyme (P-60 eluate) and incubations were carried out for 10 min at 37°. Reactions were stopped by the addition of 1 ml of 10% sulfosalicylic acid. Blanks were prepared by the addition of radioactive methyldonor after the addition of sulfosalicylic acid. All reactions were done in triplicate. The acidified incubation mixtures were filtered and the filtrate was extracted 3 times with 5 ml ethylacetate. Aliquots (0·5 ml) of the pooled ethylacetate extracts containing the *O*-methylated

substrate were pipetted into scintillation vials containing 9·5 ml of 0·4% diphenyloxazole dissolved in 50% ethanol–50% toluene and the radioactivity was measured in a liquid scintillation counter.

Enzyme activity on polyacrylamide gels after electrophoresis was located by incubating the gels in 3-ml test tubes containing 1·5 ml of $5{\cdot}7 \times 10^{-4}$ M pyrocatecholphthalein in 0·5 M phosphate buffer (pH 8·0), 0·2 ml of 2×10^{-2} M S-adenosyl-L-methionine and 0·3 ml of 0·1 M $MgCl_2$ at 37° for 15 min. The gels were then placed in 3-ml test tubes containing 2 ml of 0·25 M borate buffer, pH 10·0.

Molecular weight determination. The molecular weight of the enzyme was estimated from elution volume data by the method of Squire.[9] A column of Sephadex G-100 was used and the column constants were calculated graphically with the aid of two proteins of known molecular weight. The proteins used were twice crystallized pepsin (mol. wt. 35,500) and thrice crystallized α-chymotrypsin (mol. wt. 22,500). The Sephadex G-100 (particle size 40–120 μ) was allowed to swell in an excess of 0·01 M acetate buffer (pH 6·0) containing 0·01 M NaCl for 3 days. A column 1·5 by 70 cm was then poured in the cold room. At a final operating pressure of 15 cm, a constant flow rate of 3 ml/hr was obtained. One ml of 0·5% protein solutions, which contained 0·1% in Blue Dextran 2000, was applied to the column and 2-ml fractions were collected. The void volume of the column was determined from the elution volume of Blue Dextran 2000. The elution volumes of proteins were determined by measuring the absorbance of the eluate from columns at 280 mμ. In the case of catechol-*O*-methyltransferase, the elution volume was also determined by assaying for enzyme activity in the eluate. The enzyme preparation used for the determination of the molecular weight of the enzyme was lyophilized P-60 eluate.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out by using the discontinuous anionic system of Ornstein[10] and Davis.[11] Apparatus and reagents were obtained from Canal Industrial Corp., Bethesda, Md. A technique in which the sample gel was omitted was adopted. Samples dissolved in 10% sucrose were pipetted on tope of the stacking gel immediately prior to starting the electrophoresis. Samples (100 μl) were applied containing 200 μg protein for gels which were to be assayed for enzyme activity. Electrophoresis was carried out in a cold room at 4° at a current of 3 mA per tube until the tracking dye had migrated 5 cm into the separating gel.

Protein estimation. Proteins were estimated in solutions by u.v. absorption method;[12] on polyacrylamide gels they were detected by Amido-Schwarz stain.[11]

The results of a typical purification of the enzyme are shown in Table 1. Approximately 200-fold purification is obtained with about a 10 per cent yield in activity. The purest enzyme preparation proved to be quite unstable. Approximately one-half of the activity was lost within 2 hr. The addition of sulfhydryl compounds did not prevent this loss of activity. Because of the lability of the purest enzyme preparation, the enzyme was routinely used after passage through Bio-Gel P-60. At this stage the enzyme was very stable and all the activity could be preserved for at least 2 months if the preparations were lyophilized. The entire purification could be carried out in about 10 hr.

The molecular weight of the enzyme was calculated to be 29,000. Symmetrical enzyme activity and protein peaks were located in the eluate from the Sephadex G-100 column. The calculated column constants ($g = 0{\cdot}95$ and $C_{\frac{1}{3}} = 40{\cdot}0$) were of

the same order of magnitude as those given by Squire[9] for Sephadex G-100. Three determinations of the molecular weight led to identical results.

Polyacrylamide gel electrophoresis of the P-60 eluate showed that at least 13 protein bands were present. Further purification of the enzyme by passage through DEAE-cellulose reduced the number of visible protein bands to eight. The assay of

TABLE 1. THE PURIFICATION OF CATECHOL-*O*-METHYLTRANSFERASE*

Procedure	Volume (ml)	Activity (units/ml)	Total units	Protein (mg/ml)	Sp. act. (units/mg protein)	Yield (%)	Purification
Supernatant of 40,000 *g* centrifugation	200	46·9	9380	37·0	1·26	100	1
30–50% Ammonium sulfate fractionation of pH 5 supernatant, followed by passagethroughBio-GelP-60	37·5	72·4	2715	1·5	48·3	29	38
DEAE eluate	21	46·8	983	0·20	234	10	186

* One unit is defined as 1 μg metanephrine formed per 5 min of incubation.

the gels for enzyme activity resulted in the formation of three blue bands of activity when the P-60 eluate was subjected to electrophoresis. Only two bands were located after further purification of the enzyme by passage through DEAE-cellulose. The assay procedure colored the entire gels blue. When borate was added, the gels were decolorized except in certain regions. These colored bands reached a maximum intensity about 15 min after the addition of borate. After this time they became diffuse and within 4 hr they had disappeared. The bands were located between 2·5 and 3·0 cm into the separating gel when the tracking dye was allowed to migrate 5·0 cm into this gel. Blank gels in which either no methyl donor was added to the incubation mixture or noncatalytic protein was applied to the gel developed no activity bands. An attempt was made to fractionate the activity bands by elution from large-scale disc electrophoresis. The instability noted after DEAE-cellulose chromatography was also apparently present in this kind of treatment and no activity could be detected in the eluates.

The screen of potential substrates is summarized in Table 2. Of the compounds tested, only catechols proved to be capable of inhibiting the enzymatic *O*-methylation

TABLE 2. ABILITY OF VARIOUS COMPOUNDS TO INHIBIT THE ENZYMATIC *O*-METHYLATION OF PYROCATECHOLPHTHALEIN*

Compound	% Inhibition after 10 min of incubation	Compound	t % Inhibition after 10 min of incubation
O-nitrophenol	0	4-Methylcatechol	40
O-aminophenol	0	3-Methylcatechol	36
O-chlorophenol	0	4-Isopropylcatechol	35
3,4-Dimercaptotoluene	0	3-Isopropylcatechol	40
4-Nitrocatechol	86	4-*tert*-Octylcatechol	55
4-Chlorocatechol	42	Purpurogallin	82
4-Hydroxycatechol	49	4-Methyldaphnetine	82

* Pyrocatecholphthalein and inhibitor concentrations of $1{\cdot}5 \times 10^{-4}$ M were used.

of pyrocatecholphthalein. The ability of catechols to inhibit the enzyme appears to be dependent on the nature, size and position of the substituent on the catechols. However, none of these factors could prevent the catechols from inhibiting the enzyme.

The values of various compounds as substrates for the enzyme relative to the value of 4-methylcatechol taken as 1·0 are given in Table 3. It is apparent that all catechols

TABLE 3. RELATIVE *O*-METHYLATION WITH VARIOUS SUBSTRATES*

Compound	Substrate value relative to 4-methylcatechol	Compound	Substrate value relative to 4-methylcatechol
4-Chlorocatechol	1·7	Purpurogallin	0·4
4-Nitrocatechol	0·5	4-Methyldaphnetine	1·5
4-Hydroxycatechol	0·2	β-Thujaplicin	0·0
Catechol	2·8	3,5-Diiodo-4-hydroxybenzoic acid	0·0
4-Isopropylcatechol	1·1	3,4-Dihydroxybenzoic acid	2·5
3-Isopropylcatechol	0·2	3,4-Dihydroxyphenylacetic acid	2·5
4-*tert*-Octylcatechol	0·5	3,4-Dihydroxycinnamic acid	2·7
3,5-Diisopropylcatechol	0·1	3,4-Dihydroxymandelic acid	1·9

* The radioactivity observed with 4-methylcatechol as substrate was utilized as a basis of comparison and designated as 1. Enzymatic *O*-methylation was carried out in the presence of S-adenosyl-L-methionine-methyl-^{14}C.

are substrates for the enzyme. However, the ability of a compound to be a substrate varies with the nature, size and position of substituents. Of the monosubstituted catechols, 4-nitrocatechol proved to be the best inhibitor of the *O*-methylation of pyrocatecholphthalein. The best substrate of the compounds tested was found to be catechol. The enzyme preparation was also tested on physiological substrates such as adrenaline, which was found to have a relative substrate value of 1·6. This finding agrees with those previously reported,[1] indicating that catechol was methylated by the enzyme approximately 1·6 times faster than the physiological substrates adrenaline, noradrenaline and dopamine.

A kinetic examination of the inhibition of the *O*-methylation of adrenaline caused by catechol and 4-nitrocatechol is shown in Fig. 1. It can be seen that, whereas catechol inhibits the enzyme competitively, the inhibition caused by 4-nitrocatechol is noncompetitive. The inhibitor constants (K_i values) for catechol and 4-nitrocatechol were calculated to be $2{\cdot}3 \times 10^{-4}$ and $3{\cdot}0 \times 10^{-6}$ M respectively.

DISCUSSION

The maximum previously reported purification of catechol *O*-methyltransferase from rat liver is 30-fold.[1] By applying the techniques of gel filtration and ion-exchange chromatography to the purification of the enzyme, it has been possible to obtain a 200-fold purification. The lability of this preparation prevented further attempts at purification. The reason for this lability is unknown.

The value of the molecular weight of 29,000, determined by gel filtration, is consistent with the behavior of the enzyme on Bio-Gel P-60. The enzyme is significantly retarded on this gel, which has an exclusion limit of 60,000. Molecular weight values obtained by physicochemical methods are not available to make comparisons.

However, since anomalous behavior on Sephadex has usually been encountered only with enzymes capable of binding carbohydrates,[9] and it is unlikely that catechol *O*-methyltransferase is such a protein, it is highly probable that the molecular weight determined on Sephadex is representative of the true molecular weight of the enzyme.

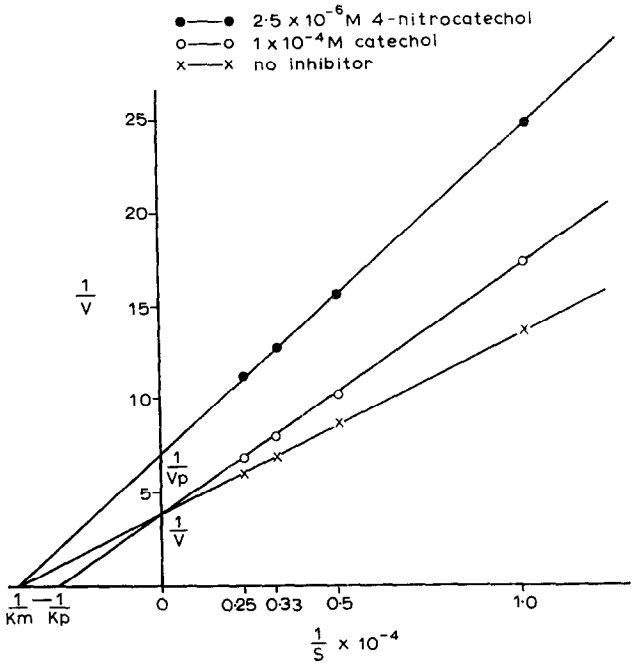

FIG. 1. Lineweaver–Burk plots of the inhibition of the *O*-methylation of adrenaline produced by the presence of 1×10^{-4} M catechol (○———○) and $2{\cdot}5 \times 10^{-6}$ M 4-nitrocatechol (●———●) and in the absence of inhibitor (×———×).

Polyacrylamide gel electrophoresis by the method of Ornstein[10] and Davis[11] revealed that contaminating proteins were present in the purest enzyme preparation. The reason for the multiple bands of activity that were observed after electrophoresis is unknown. Possibly the application of preparative discontinuous electrophoresis[13] to the study of the enzyme may furnish an explanation.

The studies of the specificity of the enzyme show that only catechols are capable of being substrates for the enzyme. However, indications that the enzyme is capable of binding some catechols at a site other than the substrate binding site were obtained. In a case where one enzyme is acting on two substrates simultaneously, it is to be expected that the second substrate will act as a competitive inhibitor of the first substrate.[14] However, it was found that 4-nitrocatechol inhibited the enzyme non-competitively and that it was a poor substrate for the enzyme. Noncompetitive inhibition can be interpreted as being due to the binding of the inhibitor at a site other than the substrate binding site.[15, 16] It can then affect the reactivity without affecting the binding of substrate. Since 4-nitrocatechol was found to be a substrate, it must be bound to the substrate binding site. But the noncompetitive inhibition observed in its presence indicates that it may also be bound at another site. This binding at two

sites may be due to the increase of the acid dissociation constant (p*Ka*) of the phenolic hydroxyls caused by the presence of the electron-withdrawing nitro group. This effect would result in the formation of a greater number of phenoxide ions in a solution of 4-nitrocatechol at pH 8·0 than would be present in an equimolar catechol solution at the same pH. Since catechol with a p*Ka* of $3{\cdot}3 \times 10^{-10}$ is an excellent substrate at pH 8·0,[17] it is reasonable to assume that the enzyme requires the hydroxyl form for binding to the substrate site. Thus, the non-ionic form of 4-nitrocatechol may be bound to the substrate site. However, in the presence of excess substrate (adrenaline) this competition for the substrate site may be of no importance. The ionic form of 4-nitrocatechol, on the other hand, may be bound at another site on the enzyme, and noncompetitive inhibition may result.

REFERENCES

1. J. Axelrod and R. Tomchick, *J. biol. Chem.* **233**, 702 (1958).
2. J. Pellerin and A. D'Iorio, *Can. J. Biochem. Physiol.* **36**, 491 (1958).
3. J. Axelrod and H. Weissbach, *J. biol. Chem.* **236**, 211 (1961).
4. K. Tomita, M. C. Chung-Ja and H. A. Lardy, *J. biol. Chem.* **239**, 1202 (1964).
5. J. K. Inscoe, J. Daly and J. Axelrod, *Biochem. Pharmac.* **14**, 1257 (1965).
6. J. Axelrod, *Physiol. Rev.* **39**, 751 (1959).
7. J. Axelrod, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), vol. 5, p. 748. Academic Press, New York (1962).
8. P. J. Anderson and A. D'Iorio, *Can. J. Biochem. Physiol.* **44**, 397 (1966).
9. P. G. Squire, *Archs Biochem. Biophys.* **107**, 471 (1964).
10. L. Ornstein, *Ann. N.Y. Acad. Sci.* **121**, 321 (1964).
11. B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
12. E. Layne, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), vol. 3, p. 454. Academic Press, New York (1957).
13. T. Jovin, A. Chramback and M. Naughton, *Analyt Biochem.* **9**, 35 (1964).
14. M. Dixon and E. C. Webb, *Enzymes*, 2nd edn., p. 84. Longmans Green, London (1964).
15. H. Gutfreund, in *An Introduction to the Study of Enzymes*, p. 100. Blackwell, Oxford (1965).
16. K. J. Laidler, *The Chemical Kinetics of Enzyme Action*, p. 79. Oxford University Press, Oxford (1958).
17. J. D. Roberts and M. C. Caserio, *Basic Principles of Organic Chemistry*, p. 902. W. A. Benjamin, New York (1964).