BBA 66194

KINETICS OF PURIFIED CATECHOL *O*-METHYLTRANSFERASE

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(Received July 7th, 1970)

SUMMARY

An approximately 300-fold purification of catechol *O*-methyltransferase (EC 2.1.1.6) from rat liver is described. The final product shows a single band in disc gel electrophoresis. The pH function of the reaction rate exhibits two optima. The K_m-values are $4 \cdot 10^{-4}$ and $3.2 \cdot 10^{-6}$ M for epinephrine and adenosylmethionine, respectively. Since the K_m-values are independent of the concentration of the cosubstrate, a bi bi random mechanism is suggested.

INTRODUCTION

Although numerous investigations on the physiological role of catechol *O*-methyltransferase (*S*-adenosylmethionine:catechol *O*-methyltransferase, EC 2.1.1.6) have been published during the last few years[1], the kinetic data of the enzyme reported in the literature are exceedingly varied. The K_m-values for epinephrine, for instance, vary between $7 \cdot 10^{-5}$ M (ref. 2) and $0.9 \cdot 10^{-3}$ M (ref. 3), those for adenosylmethionine cover the range between $6 \cdot 10^{-6}$ M (ref. 4) and about $4 \cdot 10^{-4}$ M (ref. 5). In addition, MCCAMAN[4] found an almost complete substrate inhibition at $4 \cdot 10^{-4}$ M adenosylmethionine, whereas $v_{max}/2$ may be calculated at this substrate concentration from the data of ASSICOT AND BOHUON[5]. Similar uncertainties exist about the pH optimum[2,6,7] and the Mg^{2+} requirement[2,8,9] of catechol *O*-methyltransferase. In order to understand these discrepancies, the following points have to be considered: (1) the instability of the substrates; (2) the metal-chelating capacity of the incubation medium; (3) the instability of the enzyme itself; (4) a possible heterogenity of catechol *O*-methyltransferase[10-12] and (5) a possible influence of the concentration of one substrate on the K_m-value for the second substrate or *vice versa*. Independently SCHWABE[11] and ASSICOT AND BOHUON[7] recently found that dithiothreitol and mercaptoethanol can prevent the rapid loss of activity of purified catechol *O*-methyltransferase and, at high concentrations, may even partially reactivate the fully inactivated enzyme. By use of these reagents, highly purified catechol *O*-methyltransferase is available[7,11]. In addition, these preparations are stable enough to allow a detailed study of the kinetic behaviour of the purified enzyme.

MATERIALS AND METHODS

S-adenosyl[*Me*-^{3}H]methionine with a specific activity of 500 mC/mmole was purchased from Radiochemical Centre Amersham and purified before use, according to the method of SHAPIRO AND EHNINGER[13]. In most cases, it was diluted with (also purified) unlabeled *S*-adenosylmethionine hydrogensulfate from Boehringer (Mannheim) to a specific activity of 10 mC/mmole. L-Epinephrine-D-bitartrate was obtained from Hoechst, *N*-Tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES) from Serva (Heidelberg), Sephadex G-25 coarse, G-50 fine and DEAE-Sephadex A-50 from Pharmacia (Upsala). Hydroxyapatite was prepared according to the method of TISELIUS[14]. All other compounds were reagent grade products of Merck (Darmstadt).

Preparation of catechol O-methyltransferase from rat liver

The first two steps of the purification procedure -precipitation at pH 5 and fractionation by $(NH_4)_2SO_4$- were performed as described by AXELROD[6]. The active material was then passed through a Sephadex G-50 fine column (length 180 cm; diameter 4 cm; 0.003 M potassium phosphate buffer (pH 7.0)). The most active fractions of the eluate were applied to a hydroxyapatite column (length 8.0 cm; diameter 1.5 cm) which was equilibrated with 0.003 M potassium phosphate buffer (pH 7.0). A first peak of activity was eluted at 0.005–0.01 M, whereas a second, weaker peak was found at 0.025–0.03 M potassium phosphate. Only the first peak was purified further by DEAE-Sephadex chromatography. The elution from the DEAE-Sephadex was performed by 0.035 M potassium phosphate buffer (pH 7.0). All steps were carried out at 4° in the presence of 0.1–0.5% mercaptoethanol in order to prevent autoxidation of essential SH-groups of the enzyme.

The protein content of the samples was determined according to the method of LOWRY *et al.*[15]. Mercaptoethanol had to be extracted with ether prior to protein determination. Since the protein tends to precipitate during this procedure, it was redissolved by addition of Triton X-100. The protein concentration was read from a calibration curve obtained with human serum albumin which was subjected to the same procedure as the sample of the enzyme preparation. The purified enzyme was run in an analytical disc electrophoresis. The apparatus according to MAURER[16], from Desaga/Heidelberg, and the gel system No. 6 (amount of cross-linker 7.5%) according to MAURER[16] was used. The gel was stained by 1% amido black 10 B in 7% acetic acid and 30% methanol[16].

Assay procedures

Determination of catechol *O*-methyltransferase activity was performed essentially according to AXELROD[17]. The samples (100–150 μl) contained the following reagents: 0.2 M potassium phosphate buffer (pH 7.3); 10 mM $MgCl_2$; 1 mM L-epinephrine-D-bitartrate and 0.25 mM *S*-adenosyl[*Me*-^{3}H]methionine with a specific activity of 10 mC/mmole. *S*-adenosyl[*Me*-^{3}H]methionine, which was purchased in sulfuric acid solution, had to be brought to pH 6 by addition of freshly prepared $BaCO_3$ immediately before use. Since *S*-adenosylmethionine is unstable under these conditions and since the degradation products of *S*-adenosyl[*Me*-^{3}H]methionine produce increased blank values in the determination of [^{3}H]metanephrine, controls were carried out if the time span of the series of experiments endangered validity. The incubation temper-

ature was 37°. The enzyme activity (U) is given in μmoles metanephrine built per min.

The kinetic experiments were performed with the purified enzyme. An incubation time was chosen during which no more than 10% of the less-concentrated substrate was consumed in order to avoid product inhibition[18] or nonlinearity of the time-reaction curves. In order to dissolve the enzyme in the incubation buffer, the samples were chromatographed at 4° on a column filled with Sephadex G-25 coarse, which was equilibrated with the desired buffer system. The detailed conditions of the respective incubations are given under RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

As is shown in Table I, catechol O-methyltransferase can be prepared by the described procedure with relatively high yield and specific activity. Only recently, ASSICOT AND BOHUON[7] achieved a preparation exhibiting about 30% higher specific

TABLE I

PURIFICATION OF PURIFIED CATECHOL O-METHYLTRANSFERASE

Fraction No.		*Units/mg protein*	*Protein (mg)*	*Purification*	*Yield (%)*
1	78 000 × *g* supernatant	0.00245	7200	1	(100)
2	Precipitation at pH 5				
3	$(NH_4)_2SO_4$ fractionation (30–50%)				
4	Chromatography on Sephadex G-50 fine	0.171	63	70	60.5
5	Apatite chromatography	0.348	15.2	142	30.0
6	DEAE-Sephadex chromatography	0.73	4.0	298	16.5

activity. However, ASSICOT AND BOHUON[7] measured the activity of their final preparation after an activation procedure with dithiothreitol, whereas the activities given in Table I are determined without previous reactivation. Thus, the specific activities of the enzyme prepared by the method described in the present paper is probably not significantly lower than the one reached by ASSICOT AND BOHUON[7].

The progress in isolating catechol O-methyltransferase, which is known to be extremely unstable in a partially purified state[2,7,10,11], is due to the use of SH-compounds during the preparation. In agreement with ASSICOT AND BOHUON[7], we found that dithiothreitol is the most suitable of the common SH-compounds to avoid oxidative inactivation of catechol O-methyltransferase. GSH even at high concentrations, GSH combined with sulfite, human albumin or liver homogenate supernatant had no significant protective effect in our experiments. Mercaptoethanol, however, can substitute for dithiothreitol in protecting as well as in partially reactivating the enzyme. A slow but steady loss of activity, however, is observed even in the presence of these SH-compounds, so that it is not likely that the specific activity of

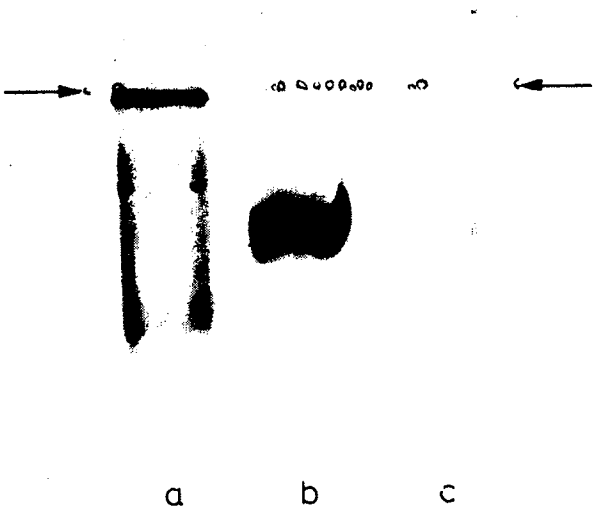

Fig. 1. Disc electrophoresis of catechol *O*-methyltransferase. The arrow indicates the beginning of the separating gel. A, Fraction No. 4 (see Table I), 120 μg protein; B, Fraction No. 5 (see Table I), 100 μg protein; C, Fraction No. 6 (see Table I), 60 μg protein.

our enzyme preparation represents the value of the native enzyme *in vivo*. Nevertheless, disc electrophoretic studies of the enzyme did not reveal any sign of impurity after DEAE-Sephadex chromatography (see Fig. 1). Only a faintly stained tail behind the single band is always observed, indicating that denaturation occurs during electrophoresis, since this phenomenon is not seen with stable proteins in the gel system used.

It should be pointed out that the electrophoretic homogeneity of our preparation implies that it is not representative of the whole catechol *O*-methyltransferase activity of the rat liver. Recently, AXELROD AND VESELL[12], and ANDERSON AND D'IORIO[10] found different fractions with catechol *O*-methyltransferase activity in starch gel and polyacrylamide electrophoretic studies, respectively, and two active fractions of catechol *O*-methyltransferase could be separated by DEAE-cellulose chromatography[7]. Thus, the occurrence of isoenzymes or at least different functional states of catechol *O*-methyltransferase in rat liver homogenate supernatant must be considered. In our preparation a heterogeneity of catechol *O*-methyltransferase was found during the chromatography on hydroxyapatite. A representative elution diagram is shown in Fig. 2. As already mentioned, only the major fraction, *i.e.* the first peak of activity, was purified further so that the kinetic data reported below are attributable only to this fraction of catechol *O*-methyltransferase. This restriction seems important because AXELROD AND VESELL[12] provided evidence that the catechol *O*-methyltransferase isoenzymes may exhibit significant kinetic differences.

Dependency of catechol-O-methyl transferase activity on pH

Since recent studies on the pH dependency of catechol *O*-methyltransferase activity revealed that the pH optimum strongly varies with the buffer system[2], we

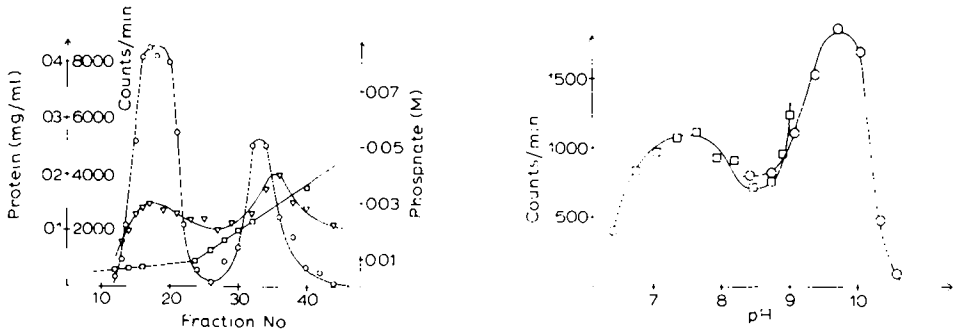

Fig. 2. Heterogeneity of rat liver catechol *O*-methyltransferase. Elution diagram of an hydroxyapatite column. ( ), catechol *O*-methyltransferase activity in counts/min (50 μl enzyme solution; incubation time, 15 min); ( ), protein content in mg/ml; ( ), phosphate concentration of the eluate (M).

Fig. 3. Reaction rate of catechol *O*-methyltransferase as a function of pH. The conditions of incubation were as follows: catechol *O*-methyl transferase activity, $2 \cdot 10^{-4}$ U/sample; specific activity, 0.73 U/mg protein; epinephrine, 2 mM; *S*-adenosyl[*Me*-^{3}H]methionine, 0.2 mM, 10 mC/mmole; Mg^{2+}, 2 mM; ( ), TES/NaOH buffer, 0.025 M; ( ), sarkosine/NaOH buffer, 0.025 M. Mean values of each two determinations are given. The standard deviation amounts to about 47 counts/min.

reinvestigated the influence of the pH on the reaction rate of the enzyme. As can be seen from Table II, we could reproduce the pH optimum of 7.8 in phosphate buffer. A nearly identical pH optimum (7.6, see Fig. 3) is seen with TES buffer (pK 7.14 at 37° (ref. 19)) which has no magnesium-complexing activity[19]. However, the reaction rate of catechol *O*-methyltransferase, having passed a minimum at about pH 8.5, rises again. In sarkosine buffer (pK 9.93 at 37°) at about pH 8.5, the reaction rate of catechol *O*-methyltransferase approximates the values found in TES buffer and shows an optimum near pH 9.7. The steep decline of the activity at higher pH values is due to the inactivation of the enzyme under these conditions, as could be shown by preincubation of a sample at pH 10.6 and successive determination of activity at pH 9.7. The pH dependency found by NIKODIJEVIC *et al.*[2] in Tris buffer seems not to be entirely different from the present results in that it exhibits a shoulder at about pH 7.5–8.0, where a flat optimum is seen in phosphate[2,6,7,9] or TES buffer systems.

TABLE II

PH OPTIMA OF CATECHOL *O*-METHYLTRANSFERASE

pH optimum	*Buffer system*	*Ref.*
7.5–8.2	Phosphate (0.05 M)	6
7.9	Phosphate (0.01 M)	2
9.1	Tris (0.01 M)	2
10.0	Glycine (0.01 M)	2
7.8	Phosphate (0.1 M)	9
8.2–8.5 (7.6–8.9)	Phosphate (0.07 M)	7
7.8	Phosphate (0.2 M)	This investigation
7.6	TES (0.02 M)	This investigation
9.7 or above	Sarkosine (0.02 M)	This investigation

Thus, the pH function of catechol *O*-methyltransferase apparently shows two peaks. For cited reasons we do not think that this phenomenon can be explained by an heterogeneity of the investigated sample of enzyme. It may be tentatively assumed that the first optimum is attributable to the intrinsic properties of the enzyme, whereas the second rise in reaction rate may be due to an increased supply of more reactive anionic species of substrate to the active site of the enzyme. In the pH range 7–11 a complex mixture of dissociation products of epinephrine are present[20], and it is difficult to predict which species is the actual substrate for catechol *O*-methyltransferase. In addition, it has been discussed that not epinephrine itself but its magnesium complex, whose physical properties are not exactly known as yet, is bound to the enzyme[1,8,9]. This lack of information and the mentioned instability of catechol *O*-methyltransferase at high pH renders it difficult to give a conclusive interpretation of the pH function.

Kinetic constants of catechol-O-methyl transferase

As already mentioned, quite a series of reasons may be responsible for the deviations of the kinetic data of catechol *O*-methyltransferase reported in the literature[2-9]. We are mainly interested in the question of whether the contradictory results are conditioned by the properties of the enzyme itself, *e.g.* by an ordered reaction mechanism[21]. Therefore, the determination of the K_m-values was performed at different concentrations of cosubstrate. The results, however, indicate (Figs. 4 and 5) that the K_m-values for both substrates are independent of the concentration of the respective cosubstrate. That means that the K_m-values equal the dissociation constants of the enzyme–substrate complexes and that the dissociation constants are not influenced by the binding of cosubstrate to the enzyme. Using the nomenclature

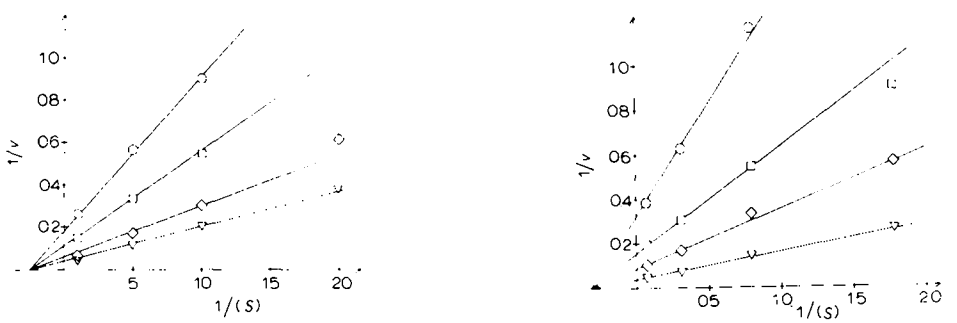

Fig. 4. Determination of the K_m-value of catechol *O*-methyltransferase for epinephrine. The experiments were performed in 0.04 M TES buffer (pH 7.3) in the presence of $2 \cdot 10^{-3}$ M Mg^{2+} at 37°. Mean values of each 3 determinations are given. ○—○, concn. S-adenosylmethionine hydrogensulphate: $0.56 \cdot 10^{-6}$ M; □—□, concn. of S-adenosylmethionine hydrogensulfate: $1.25 \cdot 10^{-6}$ M; ◇—◇, concn. of S-adenosylmethionine hydrogensulfate: $3.18 \cdot 10^{-6}$ M; ▽—▽, concn. of S-adenosylmethionine hydrogensulfate: $13.4 \cdot 10^{-6}$ M. [S] is given in mM epinephrine, v in 10^{-12} moles metanephrine per min.

Fig. 5. Determination of the K_m-value of catechol *O*-methyltransferase for adenosylmethionine. The values are calculated from the experiments shown in Fig. 4. ○—○, concn. of epinephrine: $0.5 \cdot 10^{-4}$ M; □—□, concn. of epinephrine: $1.0 \cdot 10^{-4}$ M; ◇—◇, concn. of epinephrine: $2.0 \cdot 10^{-4}$ M; ▽—▽, concn. of epinephrine: $10.0 \cdot 10^{-4}$ M. [S] is given in μM *S*-adenosylmethionine hydrogensulfate. v in 10^{-12} moles metanephrine per min.

of CLELAND[21], the type of reaction of catechol *O*-methyltransferase may be classified as a bi bi random mechanism. In this connection it may be mentioned that recently similar kinetic properties have been demonstrated for phenylethanolamine-*N*-methyl transferase by CONNET AND KIRSHNER[22].

The K_m-value for epinephrine of $4 \cdot 10^{-4}$ M is in reasonable agreement with the results of NIKODIJEVIC *et al.*[2] ($2.6 \cdot 10^{-4}$ M at pH 7.8) and AXELROD AND VESELL[12] ($2 \cdot 10^{-4}$ M at pH 7.9 for one of the two isozymes of rat liver). The comparable low deviation of our value may be due to the low pH at which the determinations were performed, since NIKODIJEVIC[2] reported an even smaller K_m-value ($7.2 \cdot 10^{-5}$ M) in Tris buffer (pH 9.1). Considering the present and cited[2,12] results, it may be suggested that the pH of the incubation medium has an influence on the formation of the enzyme–magnesium–epinephrine complex. This question will be the subject of a further investigation.

The dependency of the reaction rate on the *S*-adenosylmethionine concentration shown in Fig. 5 strongly differs from the values presented by ASSICOT AND BOHUON[5] and MCCAMAN[4]. From the data shown in Fig. 5 a K_m of *S*-adenosylmethionine of $3.2 \cdot 10^{-6}$ M may be calculated, which is appreciably lower than the values reported so far[4,5]. In addition, a significant substrate inhibition by *S*-adenosylmethionine hydrogensulfate could not be seen up to a concentration of $4 \cdot 10^{-3}$ M.

As to the biological aspects of these data we may state that rat liver catechol *O*-methyltransferase always operates at substrate saturation with regard to *S*-adenosylmethionine (about 26 μg/g of liver tissue[23]). However, epinephrine will be metabolized at a pseudo-first-order reaction rate, since substrate saturation of the enzyme by epinephrine will not be reached, even under extreme conditions *in vivo* (mean epinephrine concentration of rat liver tissue: $22 \cdot 10^{-9}$ g/g (ref. 24)). Due to its kinetic properties, catechol *O*-methyltransferase, therefore, seems well suited to regulate the level of epinephrine under physiological conditions.

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