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CALCIUM INHIBITION OF RAT LIVER CATECHOL-*O*-METHYLTRANSFERASE*

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Abstract—The activity of partially purified rat liver catechol-*O*-methyltransferase (COMT) was measured by an assay procedure in which 3,4-dihydroxybenzoic acid was used as a substrate for the enzyme. Optimal enzyme activity was present at a concentration of $MgCl_2$ of 10^{-3} M. The effects on COMT activity of a series of alkaline earth compounds were determined in the presence of optimal concentrations of $MgCl_2$. $CaCl_2$ and $Ca(NO_3)_2$ at concentrations of 10^{-3} M reduced COMT activity by 63 and 59 per cent respectively. $BaCl_2$ and $Sr(NO_3)_2$ (10^{-3} M) did not decrease enzyme activity, nor did additional $MgCl_2$ to a final $[Mg^{2+}]$ of 2×10^{-3} M. The inhibition of COMT by $0{\cdot}25 \times 10^{-3}$ and $0{\cdot}50 \times 10^{-3}$ M $CaCl_2$ in the presence of different concentrations of 3,4-dihydroxybenzoic acid, *S*-adenosyl-l-methionine and $MgCl_2$ was determined. In each case Lineweaver Burk plots of these data were compatible with noncompetitive or "mixed" inhibition.

Catechol-*O*-methyltransferase (EC 2.1.1.7, COMT) is the enzyme that catalyzes the *O*-methylation of norepinephrine, epinephrine, dopamine and other catechol compounds [1]. *S*-adenosyl-l-methionine functions as a methyl donor for this reaction, and magnesium ion is required for enzymatic activity [1]. Axelrod and Tomchick [1] demonstrated that other divalent cations were capable of substituting for magnesium. The most effective of these cations were Co^{2+} and Mn^{2+}. Although many studies have been carried out in which the abilities of various divalent cations to activate COMT have been examined [2, 3], the possibility that cations might inhibit the COMT reaction in the presence of optimal concentrations of magnesium has not been studied. We have examined the effects of alkaline earth cations on COMT activity in the presence of optimal concentrations of magnesium and have found that calcium inhibits COMT activity. This finding has practical implications with regard to the measurement of COMT activity in tissue homogenates and with regard to the use of COMT in assay procedures for the measurement of catecholamines. In addition, the inhibition of COMT by calcium may be involved in the physiologic regulation of the enzyme activity.

MATERIALS AND METHODS

COMT assay procedure

3,4-Dihydroxybenzoic acid (DBA) as substrate. These studies were performed with an assay procedure for the measurement of COMT activity in which 3,4-dihydroxybenzoic acid (DBA) served as a substrate for the enzyme [4]. In the presence of COMT and radioactively labeled *S*-adenosyl-l-methionine, DBA was converted to radioactively labeled 4-hydroxy-3-methoxybenzoic acid (vanillic acid). The reaction was stopped by the addition of acid to the reaction mixture, and the radioactively labeled vanillic acid was extracted into toluene prior to the determination of its radioactivity in a scintillation counter. All glassware was washed in detergent, rinsed with deionized water, soaked for 10 min in 10 mM EDTA, pH 5·5, rinsed three times with glass-distilled water and then dried in an oven.

Two hundred μl of an appropriate dilution of partially purified rat liver COMT was placed in 15-ml conical stoppered glass centrifuge tubes. The enzyme reaction was initiated by the addition of 100 μl of a mixture of the following reagents (final concentrations in 300 μl indicated): Tris (hydroxymethyl)aminomethane HCl buffer, pH 7·8, 0·08 M; $MgCl_2$, 10^{-3} M; [^{14}C]methyl-*S*-adenosyl-l-methionine (sp. act. 58·0 mCi/m-mole), $2{\cdot}8 \times 10^{-6}$ M; nonradioactive *S*-adenosyl-l-methionine HCl, $40{\cdot}4 \times 10^{-6}$ M; DBA, 10^{-3} M; and dithiothreitol, 0·043 M. The reaction mixture was incubated for 30 min at 37° in a shaker water bath, and the reaction was stopped by the addition of 100 μl of 1·0 N HCl. Five ml toluene was added to each tube. The tubes were vortexed for 10 sec, centrifuged at 700 *g* for 10 min in an International model K centrifuge, and 3·5 ml of the organic phase was added to counting vials that contained 10 ml toluene fluor [5 g 2,5-diphenyloxazole and 0·1 g 1,4-bis-2-(5-phenyloxazole)-benzene per liter]. Radioactivity was determined in a Packard 3385 liquid scintillation counter. Unless otherwise indicated, blank samples included all reagents except DBA.

Norepinephrine as substrate. In some experiments norepinephrine was used as a substrate for the COMT reaction rather than DBA. The following changes were made in the procedure described above when norepinephrine was used as substrate: (1) norepinephrine at a final concentration of 4×10^{-3} M was substituted for DBA; (2) the reaction was terminated by the addition of 0·5 ml of 0·5 M borate buffer, pH 10; and (3) organic solvent extraction was carried out after the addition of 5 ml of water-saturated isoamyl alcohol instead of toluene. After the

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sample had been vortexed and centrifuged, 3·5 ml of the organic phase was removed and was added to a counting vial that contained 2 ml ethanol and 10 ml toluene fluor. Blanks were samples that contained the entire reaction mixture with the exception of norepinephrine.

Protein determination

Protein concentrations were determined with the method of Lowry *et al.* [5].

Thin-layer chromatography

The products of the reaction procedure were identified by thin-layer chromatography on Eastman Chromogram sheets of Silica gel, 100 μm in thickness. The solvent systems used for the chromatography were *n*-butanol saturated with 1 N HCl or isopropyl alcohol ammonium hydroxide–water (80:10:19). Chromatographic sheets were activated by drying for 15 min at 80° immediately before use. Sheets developed in *n*-butanol saturated with 1 N HCl were prerun in this solvent system and dried prior to spotting.

After development, sheets were sprayed with diazotized *p*-nitroaniline to locate spots [6]. They were then marked and cut into 1-cm-wide strips that were placed in counting vials containing 0·5 ml Soluene-100 (Packard Instrument Co., Inc). After 1 hr, 1 ml ethanol and 10 ml liquid toluene fluor were added to each counting vial, and the samples were placed in a liquid scintillation counter for the measurement of radioactivity.

Atomic absorption spectroscopy

A Jarrell–Ash single-beam atomic absorption spectrophotometer was used to measure calcium and magnesium concentrations.

Purification of rat liver COMT

Rat liver COMT was partially purified by a modification of the method of Axelrod and Tomchick [1] as described by Coyle and Henry [7]. Rat livers obtained from male Sprague–Dawley rats were homogenized in 4 vol. of 1·15% KCl in a Waring blender. The homogenate was centrifuged at 10,000 *g* for 10 min, and the precipitate was discarded. The supernatant was centrifuged at 78,000 *g* for 30 min in a Beckmann L2-65B ultracentrifuge. Once again the precipitate was discarded. Acetic acid (1 M) was slowly added to the supernatant until a pH of 5 was obtained. The suspension was centrifuged at 10,000 *g* for 10 min and the precipitate was discarded. Solid ammonium sulfate was added to this supernatant to a final concentration of 30%. After centrifugation for 10 min at 10,000 *g* the precipitate was discarded. Solid ammonium sulfate was added to this supernatant to a final concentration of 55%, and after centrifugation at 10,000 *g* for 10 min, the supernatant was discarded and the precipitate was dissolved in Tris–HCl buffer, 1 mM, pH 7·4. The dissolved precipitate was dialyzed in 20 l Tris–HCl, 1 mM, pH 7·4, for 12 hr with one buffer change. After dialysis the enzyme preparation was centrifuged at 10,000 *g* for 10 min and the precipitate formed during dialysis was discarded. The enzyme preparation was then run through a Sephadex G-200 column, and the fractions containing COMT activity were pooled and concentrated in an Amicon pressure concentrator. The final specific activity of the enzyme preparation was 550 nmoles 4-hydroxy-3-methoxybenzoic acid formed/mg of protein/hr. This preparation was purified approximately 10-fold as compared to the initial 10,000 *g* supernatant.

Statistical methods

All data were evaluated by standard statistical methods. Michaelis–Menten (K_m) values were determined by the method of Wilkinson [8] using a Fortran program written by Cleland [9]. A Control Data Corp. 3500 computer was used for these calculations.

Materials

[^{14}C]methyl-*S*-adenosyl-L-methionine (sp. act. 58 mCi/m-mole) was obtained from New England Nuclear Corp., Boston, Mass. *S*-adenosyl-L-methionine HCl, Tris (hydroxymethyl)aminomethane base, 3,4-dihydroxybenzoic acid (protocatechuic acid) and 4-hydroxy-3-methoxybenzoic acid (vanillic acid) were purchased from Sigma Chemical Co., St. Louis, Mo. Dithiothreitol (Cleland's reagent) and norepinephrine bitartrate were obtained from Calbiochem, San Diego, Calif. Chelex-100 brand of chelating resin (50–100 mesh) was purchased from Biorad Laboratories, Richmond, Calif.

RESULTS

Characterization of assay system

Dilution of enzyme in bovine serum albumin versus water. The results of repetitive determinations of the COMT activity of partially purified rat liver enzyme diluted in ice-cold glass-distilled water or water containing Tris buffer were quite variable. Because many enzymes interact with glass when diluted with water to low protein concentrations, the COMT activity of duplicate enzyme samples diluted with water and with 0·25% bovine serum albumin (BSA) was compared. These experiments were carried out both in glass tubes and in polycarbonate plastic tubes. The results in the two types of tubes were the same when enzyme samples were diluted with 0·25% BSA. Enzyme diluted with water gave only 40 per cent as much activity as did an identical sample diluted with 0·25% BSA when assayed in glass tubes, but the activity of water-diluted enzyme assayed in plastic tubes was higher. These samples yielded 56 per cent of the activity of identical samples diluted with BSA. The results of the experiment were the same whether or not Tris buffer was present in the water or BSA prior to dilution. Therefore, an interaction with glass is at least partially responsible for the lower and more variable COMT activity of samples diluted with water, but the use of plastic tubes is not sufficient to eliminate the problem. Since many of the experiments described below involve divalent cations that are capable of binding to BSA, most experiments were performed with both water-diluted and BSA-diluted enzyme.

Time course. The time course for the assay procedure was linear for at least 60 min when the enzyme was diluted in either BSA or water. An incubation time of 30 min was used for all experiments.

Effect of increasing concentrations of enzyme. Activity increased in a linear fashion with increasing

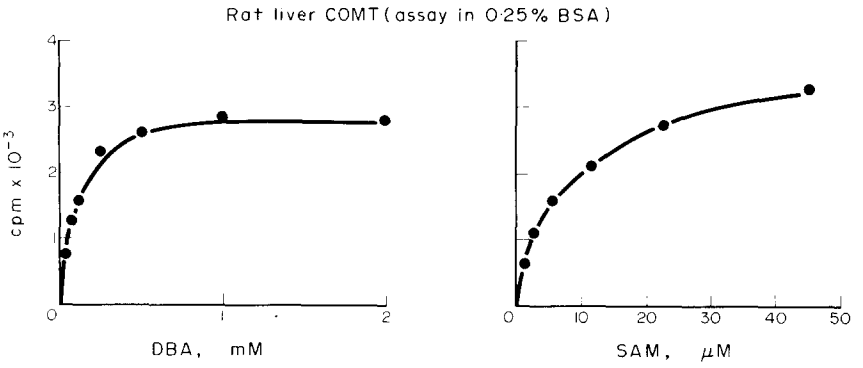

Fig. 1. Effect of substrate concentrations on COMT activity. COMT activity was determined in the presence of increasing concentrations of 3,4-dihydroxybenzoic acid (DBA) and *S*-adenosyl-1-methionine (SAM). The enzyme was diluted in 0·25% bovine serum albumin (BSA). Each point represents the mean of three determinations.

quantities of enzyme over a range from at least 0·1 to 0·5 μl enzyme preparation per assay (1·4 to 7·0 μg protein). This was true whether dilution was performed with water or with 0·25% BSA. The usual quantity of enzyme used in most of these studies was 0·25 μl/assay.

Relationship of enzyme activity to substrate concentration. The activity of rat liver COMT was measured in the presence of varying concentrations of 3,4-dihydroxybenzoic acid (DBA) and *S*-adenosyl-1-methionine (SAM), the two co-substrates of the reaction. The results of experiments in which the enzyme was diluted with 0·25% BSA are shown in Fig. 1. These data and data from similar experiments in which the enzyme was diluted with water were used to calculate Michaelis–Menten constants (K_m) for DBA and SAM at 37° under the conditions of assay described above. Calculated K_m values for DBA and SAM were 10^{-4} and 7×10^{-6} M, respectively, when the enzyme was diluted with BSA and 7×10^{-5} and $2{\cdot}6 \times 10^{-6}$ M with dilution in water. These values agree with those determined by McCaman [10] using DBA as a substrate for COMT in experiments in which homogenates of rat brain served as the source of the enzyme and with results that we have reported using DBA as a substrate for the determination of COMT activity in human red blood cells [4]. The apparent K_m value for SAM was slightly lower when the enzyme was diluted in water than when 0·25% BSA was present. This difference may be due to binding of SAM to BSA.

Relationship of enzyme activity to magnesium concentration. The relationship of enzyme activity to increasing concentrations of magnesium ion is shown in Fig. 2. The optimal concentration of $MgCl_2$ was approximately 1 mM. When these data were used to calculate apparent K_m values for magnesium, values of $2{\cdot}5 \times 10^{-4}$ and $1{\cdot}8 \times 10^{-4}$ M were obtained for enzyme diluted with BSA and with water respectively. The small quantities of BSA that were used in these experiments to obtain more reproducible enzyme activity did not significantly alter the concentration of magnesium needed to obtain maximal COMT activity.

Effect of pH on COMT activity. The effect of pH on COMT activity is shown in Fig. 3. These assays were performed with enzyme diluted with BSA. All pH values were measured at 20° in the presence of the entire reaction mixture including 0·08 M Tris–HCl buffer. A pH optimum of 7·8 to 8·0 was found with either DBA or norepinephrine as substrate. Other workers have described a bimodal pH curve with one peak at pH 7·5 to 8 and another at pH 9 or above [11]. Although the total counts per

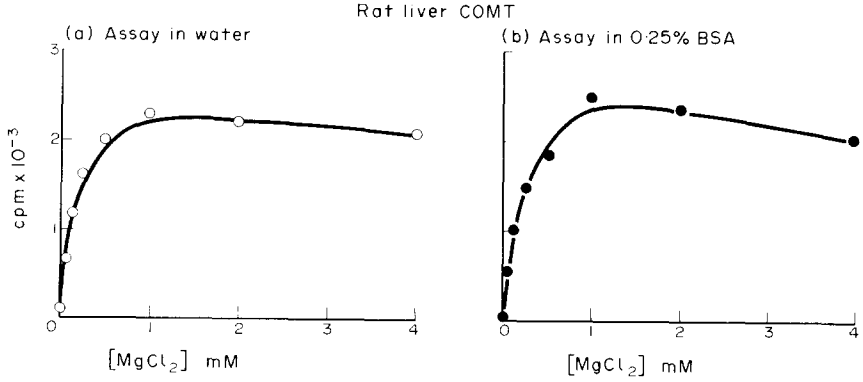

Fig. 2. Effect of magnesium concentration on COMT activity. COMT activity was determined in the presence of increasing concentrations of $MgCl_2$. The enzyme was diluted either with water (○——○) or with 0·25% bovine serum albumin (●——●). Twice as much enzyme protein was added to samples diluted with water as was present in samples diluted with 0·25% BSA. Each point represents the mean of three determinations.

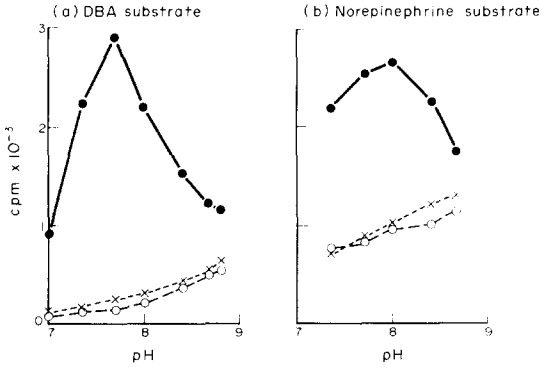

Fig. 3. Effect of pH on COMT activity. COMT activity was determined at various pH values with either 3,4-dihydroxybenzoic acid (DBA) or norepinephrine as substrate. Blank samples were either heated to 95° for 5 min (× ×) or did not include substrate (○ ○). The total activity after subtraction of the no substrate blanks is shown (● ●). Each point represents the average of two determinations.

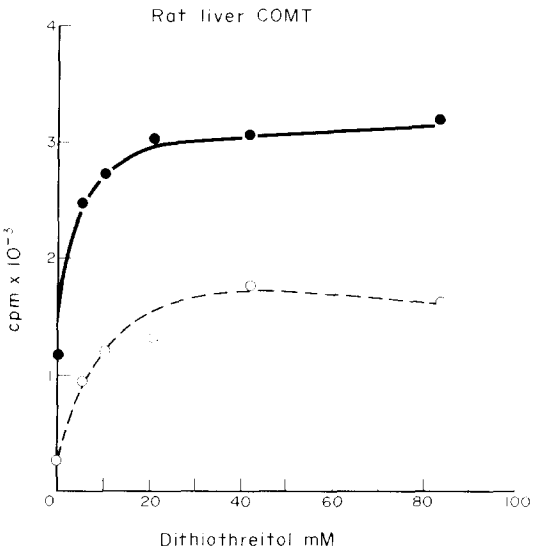

Fig. 4. Effect of dithiothreitol on COMT activity. COMT activity was measured in the presence of increasing concentrations of dithiothreitol. The enzyme was diluted either in water (○ ○) or in 0·25% bovine serum albumin (● ●). Each point represents the mean of three determinations.

min (cpm) of extractable radioactivity with both substrates began to increase as the pH of the incubation mixture increased, this was due entirely to increases in blank values. There was no significant difference in the behavior of blank samples in which substrate was omitted or in which the enzyme was heated to 95° for 5 min. A possible explanation for the increase in organic solvent-extractable cpm with an increase in the pH of the reaction mixture might be that the radioactive *S*-adenosylmethionine breaks down more rapidly at high pH values through a nonenzymatic process.

Effect of dithiothreitol on enzyme activity. The activity of COMT has been reported to decrease during the purification of the enzyme [12]. It is thought that this phenomenon is due to inactivation of the enzyme. Other workers have found that this loss of activity can be prevented by the addition of reducing agents such as 6-mercaptoethanol or dithiothreitol to the enzyme [12]. Dithiothreitol increased the COMT activity measured in our enzyme preparation maximally when present in concentrations of 0·04 M (Fig. 4). This was true whether the enzyme was diluted in BSA or water. Identical quantities of enzyme were assayed after dilution with water or BSA to obtain the data shown in Fig. 4. Note that in the presence of 0·04 M dithiothreitol, the water-diluted enzyme gave only about half of the activity present after dilution in BSA.

Identification of reaction product by thin-layer chromatography. Thin-layer chromatography of a dried toluene extract from an assay of rat liver COMT in which DBA was used as a substrate was carried out. The R_f for the radioactive product was the same as that of the authentic vanillic acid in both solvent systems used. Of the total radioactivity applied to the chromatography plates, 92 and 93 per cent migrated with authentic vanillic acid in the *n*-butanol saturated with HCl and isopropyl alcohol ammonium hydroxide water solvent systems respectively.

Table 1. Rat liver COMT activity in the presence of alkaline earth compounds.*

Compound added	Relative COMT activity Initial [$MgCl_2$]	
	Zero	1 mM
$MgCl_2$, 1 mM	100	104†
$CaCl_2$, 1 mM	4	37
$BaCl_2$, 1 mM	0	106
NaCl, 2 mM	0	109
$Be(NO_3)_2$, 1 mM	0	72
$Mg(NO_3)_2$, 1 mM	102	113†
$Sr(NO_3)_2$, 1 mM	0	101
$Ca(NO_3)_2$, 1 mM	3	41
$NaNO_3$, 2 mM	2	99

* All results are expressed as percentages of COMT activity in the presence of 1 mM $MgCl_2$ alone. Each figure represents the average of three determinations.

† The final concentration of Mg^{2+} in these samples was 2 mM.

Effects of alkaline earth compounds on COMT activity

The effects of alkaline earth compounds on COMT activity were determined (Table 1). These compounds were added to reaction mixtures that initially contained either no $MgCl_2$ or $MgCl_2$ at the optimal concentration of 1 mM. With the exception of $MgCl_2$ itself, the different alkaline earth compounds tested were added to final concentrations of 1 mM. Since all the compounds studied were either chlorides or nitrates, NaCl and $NaNO_3$ in a 2 mM concentration were also tested to determine whether the anions altered COMT activity. As can be seen in Table 1, only in the presence of $CaCl_2$ and $Ca(NO_3)_2$ was there COMT activity in the absence of magnesium, and this activity was less than 5 per cent of that found with 1 mM $MgCl_2$. In the presence of optimal $MgCl_2$

the addition of further $MgCl_2$, of $BaCl_2$ or of $Sr(NO_3)_2$ did not result in a decrease in COMT activity. The addition of 1 mM $CaCl_2$ and $Ca(NO_3)_2$, on the other hand, resulted in 63 and 59 per cent decreases in enzyme activity in the presence of 1 mM $MgCl_2$. $Be(NO_3)_2$ demonstrated a less striking 28 per cent inhibition of COMT activity in the presence of 1 mM $MgCl_2$. The addition of $CaCl_2$ to a final concentration of 1 mM in a sample that contained 1 mM $MgCl_2$ after the termination of the COMT reaction with HCl but before organic solvent extraction did not change the extractable radioactivity. Thus, the inhibition of COMT by calcium is not an artifact related to an effect of calcium on the extraction step of the assay procedure.

An experiment similar to that described above was carried out with norepinephrine as the substrate for COMT rather than DBA. With this "natural" substrate, 1 mM $CaCl_2$ gave 6 per cent as much COMT activity as did 1 mM $MgCl_2$ alone, and there was a 55 per cent inhibition of COMT by 1 mM $CaCl_2$ in the presence of 1 mM $MgCl_2$.

Lithium salts are widely used in the treatment of manic-depressive disease. It has been speculated that abnormal metabolism of catecholamines may be related to the pathogenesis of affective disease [13]. Therefore, even though lithium is not an alkaline earth, it was of interest to determine whether lithium alters COMT activity. LiCl in final concentrations of 1, 2 and 4 mM neither activated COMT in the absence of $MgCl_2$ nor inhibited the enzyme in the presence of 1 mM $MgCl_2$.

Effect of various concentrations of calcium

The results shown in Table 1 were obtained with only a single concentration of the alkaline earth compounds, 1 mM. This concentration was chosen because 1 mM $MgCl_2$ is optimal for the determination of COMT activity. To test the effect of different concentrations of alkaline earth cations on COMT, the enzyme activity was measured in the presence of 1 mM $MgCl_2$ with the addition of increasing quantities of $CaCl_2$, $BaCl_2$ and $MgCl_2$. Additional $BaCl_2$ and $MgCl_2$ up to concentrations of 4 mM (final concentration of $MgCl_2$, 5 mM) did not result in dramatic changes in COMT activity (Fig. 5). However, increasing concentrations of $CaCl_2$ resulted in a large decrease in COMT activity. There was approximately a 50 per cent inhibition of COMT in the presence of 0·50 mM $CaCl_2$ added to a reaction mixture that contained 1·0 mM $MgCl_2$. The results were the same whether the enzyme was diluted with water or with BSA.

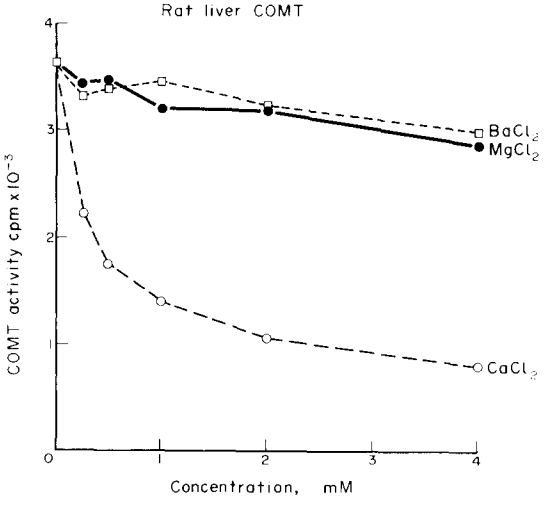

Fig. 5. Effect of alkaline earth compounds on COMT activity. COMT activity was measured in the presence of increasing concentrations of alkaline earth compounds. Each assay included 1 mM $MgCl_2$, and the concentrations indicated are in addition to the initial $MgCl_2$. Each point represents the mean of three determinations.

An experiment was carried out to insure that the inhibition of COMT by calcium was not due to the formation of a precipitate that might result in the removal of magnesium from solution. The highest concentration of $CaCl_2$ used in the previous experiment, 4 mM, was added to the entire reaction mixture including enzyme diluted with either water or 0·25% BSA. After incubation at 37° for 30 min, samples were divided into two aliquots, and half of the sample was subjected to centrifugation at 100,000 *g* for 1 hr. No visible precipitate was present in the bottom of the tubes after centrifugation, and the calcium and magnesium concentrations measured by atomic absorption spectroscopy of samples removed carefully from the tops of centrifuged tubes were identical with those found in uncentrifuged aliquots. This is evidence that magnesium was not removed from solution by the formation of a precipitate in the presence of $CaCl_2$.

An additional experiment was carried out to determine whether the inhibition of COMT by calcium is reversible. COMT was preincubated for 2 hr at 4° with 1·0 mM $CaCl_2$. Beads of Chelex-100 chelating resin were then added to some samples. Duplicate samples to which no calcium had been added served as controls. After 1 hr of gentle mixing with the Chelex-100 beads, the resin was removed by centrifugation, and COMT activity was measured. Enzyme activity was also determined in samples that had been in contact with $CaCl_2$ for 3 hr at 4° and in samples that had not been exposed to $CaCl_2$ but were exposed to Chelex-100. As can be seen in Table 2, a sample preincubated with $CaCl_2$ for 3 hr at 4° yielded only 43 per cent as much activity as one which had not been exposed to calcium. However, treatment with the chelating resin resulted in an increase in COMT activity to 95 per cent of control levels. These results indicate that the inhibition of COMT by calcium is reversible.

Table 2. Reversibility of COMT inhibition by calcium*

Additions		COMT activity	
$CaCl_2$	Chelex-100	cpm	% Control
0	0	1548 ± 19	100
0	100 µl	1622 ± 45	105
1 mM	0	660 ± 4	43
1 mM	100 µl	1470 ± 3	95

* Samples of COMT diluted with 0·25% BSA (1 ml final volume) were pre-incubated with $CaCl_2$ and were then exposed to Chelex-100 chelating resin for 1 hr (100 µl of a suspension of the resin/1 ml diluted enzyme) prior to assay. All results are the mean ± S. E. M. of three determinations.

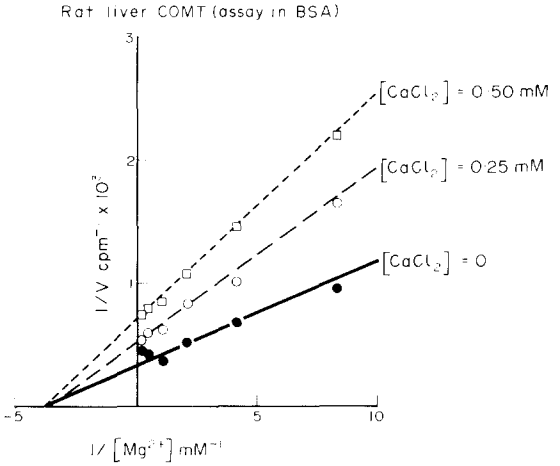

Fig. 6. Relationship between reaction velocity and magnesium concentration. A Lineweaver Burk plot is shown of the effect of two different concentrations of $CaCl_2$ on the velocity of the COMT reaction in the presence of various concentrations of $MgCl_2$. Each point represents the mean of three determinations.

Kinetic studies

Magnesium. Because it seemed likely that the inhibition of COMT by calcium might involve a competitive interaction with magnesium, COMT activity was measured in the presence of varying quantities of $MgCl_2$ either with or without added $CaCl_2$. Lineweaver–Burk plots of the results of these experiments are shown in Fig. 6. When the enzyme was diluted with BSA, the apparent K_m for magnesium in the absence of calcium was $2{\cdot}5 \times 10^{-4}$ M, while that in the presence of 0·25 mM $CaCl_2$ was $2{\cdot}7 \times 10^{-4}$ M and that in the presence of 0·5 mM $CaCl_2$ was $2{\cdot}5 \times 10^{-4}$ M. When the assay was carried out with the enzyme diluted with water rather than 0·25% BSA, the results were very similar with apparent K_m values of $1{\cdot}8 \times 10^{-4}$, $2{\cdot}3 \times 10^{-4}$ and $2{\cdot}4 \times 10^{-4}$ M in the presence of no calcium, 0·25 mM $CaCl_2$ and 0·5 mM $CaCl_2$ respectively. The Lineweaver–Burk plots of both of these sets of data were compatible

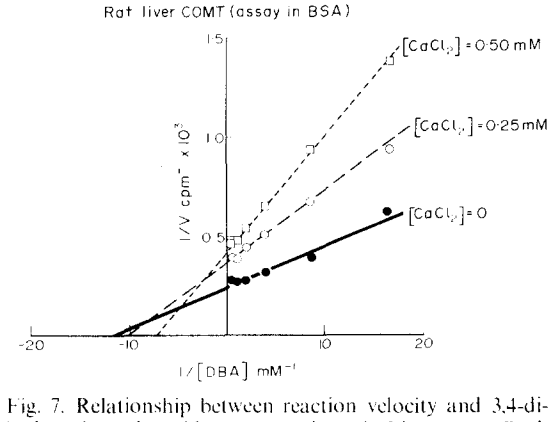

Fig. 7. Relationship between reaction velocity and 3,4-dihydroxybenzoic acid concentration. A Lineweaver–Burk plot is shown of the effect of two different concentrations of $CaCl_2$ on the velocity of the COMT reaction in the presence of various concentrations of 3,4-dihydroxybenzoic acid (DBA). Each point represents the mean of three determinations.

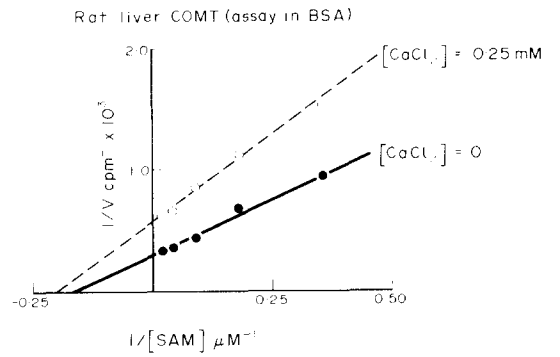

Fig. 8. Relationship between reaction velocity and *S*-adenosyl-1-methionine (SAM) concentration. A Lineweaver–Burk plot is shown of the effect of $CaCl_2$ on the velocity of the COMT reaction in the presence of various concentrations of SAM. Each point represents the mean of three determinations.

with noncompetitive or "mixed" inhibition of COMT by calcium in the presence of varying concentrations of magnesium. Since the apparent activity of the enzyme decreased in the presence of concentrations of $MgCl_2$ over 1 mM (Fig. 2), the two data points obtained at the highest concentrations of magnesium in the absence of calcium were not used in the calculation of the K_m values.

3,4-Dihydroxybenzoic acid. COMT activity of partially purified rat liver enzyme was also determined in the presence of varying concentrations of DBA either with or without added $CaCl_2$. All assays were carried out in the presence of 1 mM $MgCl_2$. A Lineweaver–Burk plot of the results is shown in Fig. 7. The two points representing results of assays in the absence of $CaCl_2$ at the highest DBA concentrations were neglected in the calculations of the apparent K_m values because the enzyme activity decreased slightly at these concentrations of substrate. The apparent K_m for DBA in the absence of $CaCl_2$ was 9×10^{-5} M, while the K_m in the presence of 0·25 mM $CaCl_2$ was 10^{-4} M and that in the presence of 0·5 mM $CaCl_2$ was $1{\cdot}3 \times 10^{-4}$ M. The Lineweaver–Burk plots of these data were compatible with noncompetitive or "mixed" inhibition.

S-adenosyl-1-methionine. The assay procedure was also carried out in the presence of increasing concentrations of *S*-adenosyl-1-methionine, either with or without 0·25 mM $CaCl_2$. These assays were performed with enzyme diluted with 0·25% BSA. As can be seen in Fig. 8, these data were also compatible with noncompetitive or "mixed" inhibition. The apparent K_m values without and with $CaCl_2$ present were $5{\cdot}8 \times 10^{-6}$ and 5×10^{-6} M respectively.

DISCUSSION

The enzyme catechol-*O*-methyltransferase requires the presence of a divalent cation such as magnesium [1]. We have demonstrated that calcium is capable of inhibiting COMT activity in the presence of optimal concentrations of magnesium. This inhibition is reversible. Kinetic analysis of the inhibition of COMT by calcium shows that the kinetics of inhibition are noncompetitive or "mixed" with respect to both of the co-substrates for the reaction, 3,4-dihydroxyben-

zoic acid and *S*-adenosyl-1-methionine, as well as with respect to magnesium. The finding of inhibition of COMT by calcium has implications of importance with regard to the measurement of the enzyme activity and to the possible physiologic regulation of the enzyme.

First, the determination of COMT activity in crude tissue preparations must be corrected for inhibition of the enzyme by calcium. For example, COMT activity has been measured in lysates of erythrocytes. Although there is little calcium in the red blood cell itself, the calcium concentration in serum is high. Some assays for COMT activity in blood have used lysates of whole blood [14]. In these assays, and in assays carried out in other tissues such as liver where contamination with serum occurs, it is necessary to remove calcium from homogenates prior to the determination of COMT activity. We have recently described a procedure in which Chelex-100 chelating resin beads are mixed with a lysate of whole blood to remove calcium prior to the determination of COMT activity [4]. This approach has greatly increased both the sensitivity and accuracy of the COMT assay in this tissue. Similar considerations apply to situations in which COMT has been used to measure the concentrations of catecholamines in various body fluids—especially plasma [15]. Sensitivity is of great importance in these catecholamine assays, and we have found that the sensitivity of these methods for the determination of catecholamines in plasma can be greatly enhanced by the addition of EDTA to the reaction mixture (R. Weinshilboum and F. Raymond, unpublished observation). The increase in sensitivity is almost certainly due to the chelation of calcium in plasma by EDTA.

Finally, the possibility must be considered that the inhibition of COMT by calcium might represent a physiologic mechanism for the regulation of the enzyme activity. This might occur in tissues such as cardiac muscle and vascular smooth muscle in which rapid fluctuations in intracellular calcium concentrations occur. Depending upon the relationship of the free magnesium concentration to the free calcium concentration at any given time, it is conceivable that the activity of catechol-*O*-methyltransferase, an important catecholamine degradative enzyme, might be rapidly increased or decreased. Further studies will be necessary to test this hypothesis.

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