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INHIBITION OF RAT LIVER CATECHOL-*O*-METHYLTRANSFERASE BY LANTHANUM, NEODYMIUM AND EUROPIUM*

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Abstract—Partially purified rat liver catechol-*O*-methyltransferase (COMT) is inhibited by lanthanum, and by the lanthanides, neodymium and europium. The 50 per cent inhibitory concentrations are 3×10^{-6}, 1.2×10^{-6}, and 8.5×10^{-7} M for La^{3+}, Nd^{3+} and Eu^{3+} respectively. The inhibition of COMT by these ions is reversible. Lineweaver–Burk plots of the results of experiments in which the enzyme activity was measured in the presence of varying concentrations of magnesium, an activator of COMT, and different concentrations of La^{3+}, Nd^{3+} and Eu^{3+} were compatible with non-competitive or "mixed" inhibition. Double reciprocal plots of the results of experiments in which COMT activity was determined in the presence of different concentrations of La^{3+} and varying quantities of the two co-substrates for the reaction, *S*-adenosyl-l-methionine and 3,4-dihydroxybenzoic acid, were also compatible with non-competitive or "mixed inhibition". The characteristics of the inhibition of rat liver COMT by La^{3+}, Nd^{3+} and Eu^{3+} are similar to those of the inhibition of COMT by Ca^{2+}. However, lanthanum, neodymium and europium are two to three orders of magnitude more potent inhibitors of COMT than is calcium.

Catechol-*O*-methyltransferase, (EC 2.1.1.7, COMT), catalyzes the *O*-methylation of catechol compounds such as norepinephrine, epinephrine and dopamine [1]. *S*-adenosyl-l-methionine functions as a methyl donor for the reaction, and magnesium is required for COMT activity [1]. It has been reported recently that calcium inhibits COMT in the presence of optimal concentrations of magnesium [2]. The calcium inhibition of COMT is reversible and is non-competitive with respect to magnesium and both of the co-substrates for the reaction, *S*-adenosyl-l-methionine and the catechol compound [2]. Lanthanum and the lanthanides can mimic the effects of calcium and other alkaline earths in many biochemical systems [3]. It is thought that the lanthanides replace calcium in these systems [4–6]. The lanthanide rare earths have spectral and magnetic properties that make them valuable chemical probes for the study of alkaline earth interactions with enzymes and other proteins [7]. The effects on rat liver COMT activity of compounds of lanthanum and of the lanthanides, neodymium and europium, were studied to determine whether they might inhibit COMT. All three ions were reversible inhibitors of COMT. The kinetic characteristics of the inhibition were similar in type to those of the inhibition of COMT by calcium. Lanthanum and the lanthanides might be useful in future studies of the nature of the interaction of calcium with COMT.

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METHODS

COMT assay procedure

COMT activity was measured by the procedure of Raymond and Weinshilboum [2, 8] as modified to measure the activity of partially purified rat liver enzyme. 3,4-Dihydroxybenzoic acid (DBA) was used as a substrate for the reaction, and [^{14}C]S-adenosyl-l-methionine (SAM) was the methyl donor. The final pH of the reaction mixture was 7.8 in the presence of 0.08 M Tris–HCl buffer. DBA was converted to radioactively labeled 4-hydroxy-3-methoxybenzoic acid (vanillic acid) by COMT, and the product was separated by organic solvent extraction prior to the measurement of radioactivity in a liquid scintillation counter. The assay procedure has been described in detail elsewhere [2, 8]. Specifically, 5–10 μl of partially purified rat liver COMT was added to 10 ml of glass-distilled water. A suspension of Chelex-100 chelating resin in water was added to the diluted enzyme preparation in a proportion of 1 vol. of Chelex-100 suspension to 9 vol. of diluted enzyme. This suspension was rotated gently at 12 rev/min for 1 hr and was then centrifuged at 10,000 *g* for 10 min. The supernatant was removed, and 200-μl aliquots of the supernatant were added to reaction tubes for the determination of COMT activity. Each assay tube contained from 0.1 to 0.2 μl of partially purified enzyme (1.4 to 2.8 μg protein)/200 μl final volume. This quantity is in the range in which activity is related in a linear fashion to the quantity of enzyme present [2]. All incubations were carried out at 37° for 45 min. Blanks were samples to which no DBA was added. Chelex-100 was prepared as previously described [8].

All glassware was washed in detergent, rinsed with deionized water, soaked for 10 min in 10 mM EDTA,

pH 5.5, and was rinsed three times with glass-distilled water before use. Stock solutions of $LaCl_3$, $NdCl_3$, $EuCl_3$, $MgCl_2$ and $CaCl_2$ were prepared and stored in polystyrene tubes.

Purification of COMT

Rat liver COMT was purified by a modification of the method of Axelrod and Tomchick [1] as described elsewhere [2]. The procedure involved homogenization of rat liver in 1.15% KCl followed by centrifugation, acid precipitation, ammonium sulfate precipitation, and passage through a Sephadex G-200 column. The specific activity of the final enzyme preparation was 550 nmoles of 4-hydroxy-3-methoxybenzoic acid formed/mg of protein/hr.

Data analysis

Michaelis–Menten (K_m) constants were determined by the method of Wilkinson [9] using a Fortran program written by Cleland [10]. 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. Tris–hydroxymethylaminomethane base, 3,4-dihydroxybenzoic acid and *S*-adenosyl-l-methionine hydrochloride were purchased from Sigma Chemical Co., St. Louis, Mo. Dithiothreitol (Cleland's reagent) was obtained from CalBiochem, La Jolla, Calif. Chelex-100 chelating resin, 50–100 mesh, was purchased from BioRad Laboratories, Richmond, Calif. $LaCl_3$ was obtained from Fisher Scientific Co., Fair Lawn, N.J., and $NdCl_3$ and $EuCl_3$ were purchased from Alfa Products, Beverly, Mass.

RESULTS

Characteristics of assay procedure

The characteristics of the procedure used to measure COMT activity have been described elsewhere [2]. The effects of time of incubation, quantity of enzyme, pH, magnesium concentration, co-substrate concentrations, and concentrations of dithiothreitol have all been studied, and the reaction product has been identified as authentic vanillic acid by thin-layer chromatography. Unless otherwise stated, all assays were carried out under optimal conditions.

Although it has been reported that magnesium or a related divalent cation is needed for COMT activity [1], preliminary experiments revealed that some enzyme activity was present in our partially purified rat liver enzyme preparation in the absence of exogenously added $MgCl_2$. Exposure of the enzyme to the solid chelating resin, Chelex-100, eliminated this residual activity. These results suggested that magnesium was present in the enzyme preparation. Therefore, enzyme diluted with glass-distilled water was always exposed to Chelex-100 prior to its use in the studies described below (see Methods for details).

Inhibition of COMT by lanthanum, neodymium and europium

A preliminary attempt to determine whether $LaCl_3$, $NdCl_3$ and $EuCl_3$ either inhibit or activate COMT was made. Enzyme activity was measured in the presence of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M concentrations of rare earth compounds either with or without the addition of an optimal concentration of $MgCl_2$, 10^{-3} M. COMT was dramatically inhibited by $LaCl_3$ at concentrations of 10^{-3} to 10^{-5} M, and by $NdCl_3$ and $EuCl_3$ at all concentrations studied. None of these compounds activated COMT in the absence of magnesium at any of the concentrations tested. This initial "screening" experiment was followed by a study in which the exact concentrations of the rare earth compounds needed to inhibit the enzyme were determined (Fig. 1). $MgCl_2$, 1 mM, was present in all reaction tubes. The concentrations at which a 50 per cent inhibition of COMT occurred were found to be: $LaCl_3$, 3×10^{-6} M; $NdCl_3$, 1.2×10^{-6} M; and $EuCl_3$, 8.5×10^{-7} M. The inhibition of COMT by $CaCl_2$ is shown in the figure for comparison. The 50% inhibitory concentration of COMT by calcium is 4.5×10^{-4} M. Lanthanum and the lanthanides, neodymium and europium, are two to three orders of magnitude more potent as inhibitors of COMT than is calcium.

Chloride was the anion in all of the rare earth compounds studied. Therefore, the effects of sodium chloride on COMT activity in concentrations of 1, 3, 6 and 9×10^{-6} M were determined. At none of these concentrations did sodium chloride activate COMT in the absence of magnesium chloride, nor was there any inhibition of COMT in the presence of 1 mM $MgCl_2$. The effects of lanthanum, neodymium and europium on COMT cannot be explained on the basis of an effect of the anion in the compounds studied.

Because of the possibility that lanthanum, neodymium and europium might interact with the product of the COMT reaction or might interfere with the organic solvent extraction step of the assay, an experiment was performed in which $LaCl_3$, $NdCl_3$ and $EuCl_3$ were added to the reaction mixture both before and after incubation. The results of this experiment are shown in Table 1. Magnesium chloride, 1 mM, was present during the enzyme reaction in all cases except for the one experiment in which it was added only after the incubation. No inhibition of COMT occurred when the rare earth compounds were added

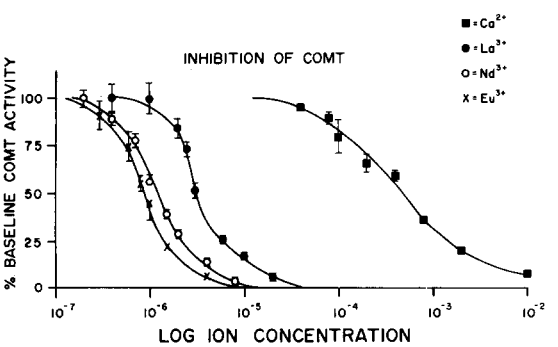

Fig. 1. Effect of varying concentrations of lanthanum, neodymium, europium and calcium on COMT activity. Each point represents the mean ± S. E. M. of five determinations for La^{3+} (●), Nd^{3+} (○), Eu^{3+} (X), and three determinations for Ca^{2+} (■). The abcissa shows the negative logarithm of the molar concentrations of these ions.

Table 1. Effect of ion added before or after reaction on rat liver COMT activity*

Compound	Concn (M)	Added before (cpm)	(% Control)	Added after (cpm)	(% Control)
$MgCl_2$	1×10^{-3}	1567 ± 91	100	22 ± 7	1.4
		$MgCl_2$ (1 mM) present during reaction			
$LaCl_3$	2×10^{-5}	23 ± 5	1.5	1632 ± 32	104
$NdCl_3$	8×10^{-6}	45 ± 19	3	1613 ± 72	103
$EuCl_3$	4×10^{-6}	63 ± 22	4	1438 ± 42	92

*Control represents the enzyme activity in the presence of 1 mM $MgCl_2$ added prior to incubation. Each value represents the mean ± S. E. M. of three determinations; cpm represents counts/min.

after the enzyme reaction but prior to the organic solvent extraction. Therefore, the inhibition of COMT by lanthanum, neodymium and europium is not due to an artifact involving interference with the organic solvent extraction step of the assay procedure.

Reversibility of inhibition

Since the inhibition of COMT by calcium is reversible, an experiment was carried out to determine whether the inhibition of this enzyme by lanthanum, neodymium and europium is also reversible. Rat liver COMT diluted with glass-distilled water to the final enzyme concentrations used in the assay was incubated with $LaCl_3$, $NdCl_3$ and $EuCl_3$ at 4° for 1 hr. Control samples were incubated at 4° in the absence of inhibitory compounds. A suspension of Chelex-100, 100 μl of suspension/ml of diluted enzyme, was then added to test tubes that contained COMT and inhibitors as well as to control samples without rare earth compounds. The tubes were then mixed by gentle rotation at 12 rev/min for 1 hr at 4° to determine whether exposure to Chelex could reverse the inhibition by chelation of the lanthanides. Controls which contained enzyme either with or without the inhibitory compounds were also rotated for 1 hr in the absence of Chelex-100 but after the addition of 100 μl water/ml of enzyme. After centrifugation to remove the beads of chelating resin, 200-μl samples of supernatant from each tube were added to reaction tubes, and $MgCl_2$ was added prior to the determination of enzyme activity. The results of these experiments are shown in Table 2. The inhibition of COMT by lanthanum, neodymium and europium is reversible after exposure to a solid chelating resin. In addition, it was noted that the enzyme activity was lower in samples tumbled in glass tubes in the absence of Chelex than in samples in which Chelex was present. Although this unexpected finding has not been adequately explained, it may be the result of interaction of the enzyme with glass in the absence of Chelex.

Kinetic studies

Magnesium. Because magnesium or other divalent cations are necessary for COMT activity [1], it seemed possible that lanthanum, neodymium and europium might inhibit COMT by competing with magnesium. Therefore, kinetic studies were carried out in which COMT activity was measured in the presence of either no rare earth or of two different inhibitory concentrations of a lanthanide compound. Varying concentrations of $MgCl_2$ were added to the reaction mixtures, and Lineweaver–Burk plots of the results of these experiments are shown in Figs. 2–4. Apparent K_m constants, values of maximum velocity (V_{max}), and

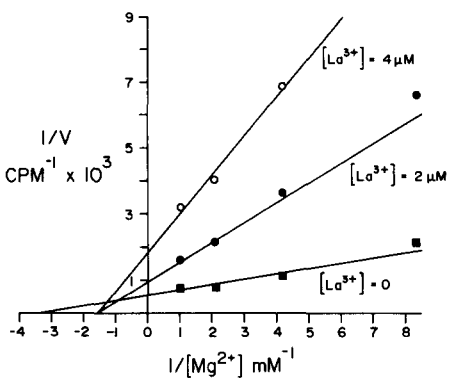

Fig. 2. Lineweaver–Burk plot of the effect of two different concentrations of La^{3+} on the velocity of the COMT reaction in the presence of various concentrations of $MgCl_2$. Each point represents the mean of three determinations. A fourth point ($1/[Mg^{2+}] = 8.3$, $1/V = 14.6$) in the presence of 4 μM La^{3+} was used in the kinetic analysis but is not shown in the figure because it was off scale.

Table 2. Reversibility of inhibition of rat liver COMT activity*

Compound	Concn (M)	Chelex (cpm)	(% Control)	No Chelex (cpm)	(% Control)
		1113 ± 31	100	844 ± 76	76
$LaCl_3$	2×10^{-5}	914 ± 32	82	11 ± 3	1
$NdCl_3$	8×10^{-6}	1135 ± 50	102	5 ± 3	0.5
$EuCl_3$	4×10^{-6}	1118 ± 36	100	55 ± 10	5

*Each value represents the mean ± S. E. M. of three determinations. The "control" was a sample of rat liver COMT treated with Chelex-100 in the absence of inhibitory ions.

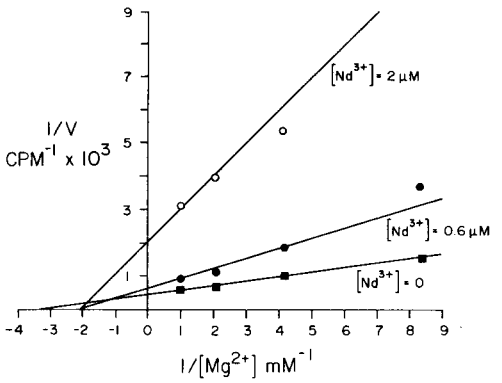

Fig. 3. Lineweaver–Burk plot of the effect of two different concentrations of Nd^{3+} on the velocity of the COMT reaction in the presence of various concentrations of $MgCl_2$. Each point represents the mean of three determinations. A fourth point ($1/[Mg^{2+}] = 8.3$, $1/V = 20.6$) in the presence of 2 μM Nd^{3+} was used in the kinetic analysis but is not shown in the figure because it was off scale.

of the $1/V$ values at the intercepts on the ordinates of these double reciprocal plots are shown in Table 3. The apparent K_m for magnesium in the absence of lanthanides was 3×10^{-4} M, a figure that compares favorably with that reported previously [2]. The double reciprocal plots of data obtained in the presence of $LaCl_3$, $NdCl_3$ and $EuCl_3$ were all compatible with non-competitive or "mixed" inhibition. Although the term "mixed" inhibition is widely used to describe double reciprocal plots in which inhibitors alter V_{max} but in which the lines do not cross on the abscissa, as Cleland [11] points out, the term "mixed" inhibition really conveys no additional kinetic information over that conveyed by the term non-competitive. Therefore, in the subsequent discussion the term non-competitive inhibition will be used to describe all Lineweaver–Burk plots in which both the slopes and intercepts are increased in the presence of the inhibitor. The inhibition of COMT by calcium is also non-competitive with respect to magnesium [2].

3,4-Dihydroxybenzoic acid. COMT activity was measured in the presence of varying concentrations of DBA either with or without added $LaCl_3$ at concentrations of 2 and 4×10^{-6} M. Figure 5 shows a Lineweaver–Burk plot of the results of these experiments. The apparent K_m for DBA in the absence of lanthanum was 10^{-4} M. Values for V_{max} and K_m, and the $1/V$ values at the intercepts on the ordinate are shown in Table 4. The double reciprocal plot of the

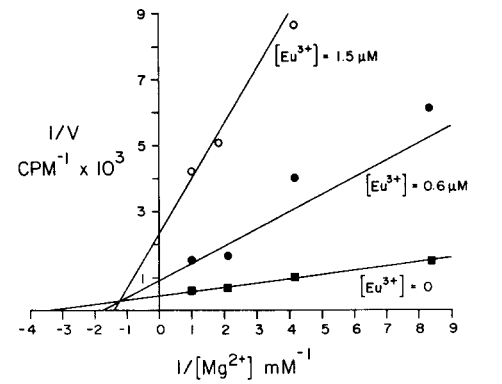

Fig. 4. Lineweaver–Burk plot of the effect of two different concentrations of Eu^{3+} on the velocity of the COMT reaction in the presence of various concentrations of $MgCl_2$. Each point represents the mean of three determinations. A fourth point ($1/[Mg^{2+}] = 8.3$, $1/V = 44.0$) in the presence of 1.5 μM Eu^{3+} was used in the kinetic analysis but is not shown in the figure because it was off scale.

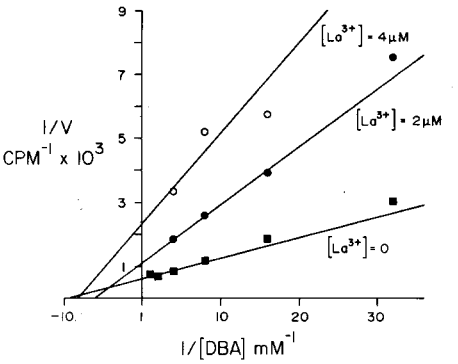

Fig. 5. Lineweaver–Burk plot of the effect of two different concentrations of La^{3+} on the velocity of the COMT reaction in the presence of various concentrations of 3,4-dihydroxybenzoic acid (DBA). Each point represents the mean of three determinations. A fourth point ($1/[DBA] = 32$, $1/V = 13.1$) in the presence of 4 μM La^{3+} was used in the kinetic analysis but is not shown in the figure because it was off scale.

Table 3. Results of kinetic studies of effects of lanthanum, neodymium and europium on COMT activity in the presence of varying concentrations of magnesium

Compound	Concn	$1/V \pm$ S. E. M.	V_{max}	$K_m \pm$ S. E. M.
$LaCl_3$	0	0.5 ± 0.1	1914	$3.1 \pm 1.1 \times 10^{-4}$ M
$LaCl_3$	2 μM	1.0 ± 0.1	1021	$6.2 \pm 1.1 \times 10^{-4}$ M
$LaCl_3$	4 μM	1.9 ± 0.3	515	$6.0 \pm 2.0 \times 10^{-4}$ M
$NdCl_3$	0	0.5 ± 0.0†	2212	$3.0 \pm 0.6 \times 10^{-4}$ M
$NdCl_3$	0.6 μM	0.6 ± 0.1	1648	$4.8 \pm 1.5 \times 10^{-4}$ M
$NdCl_3$	2.0 μM	2.0 ± 0.6	498	$5.1 \pm 3.2 \times 10^{-4}$ M
$EuCl_3$	0	0.5 ± 0.0†	2212	$3.0 \pm 0.6 \times 10^{-4}$ M
$EuCl_3$	0.6 μM	0.9 ± 0.3	1106	$5.9 \pm 4.5 \times 10^{-4}$ M
$EuCl_3$	1.5 μM	2.4 ± 1.0	422	$7.0 \pm 5.6 \times 10^{-4}$ M

*COMT activity was measured in the presence of different concentrations of $MgCl_2$ either with no rare earth present or in the presence of two concentrations of the inhibitor. All results represent the mean ± S. E. M. of three determinations.

†S. E. M. < 0.05.

Table 4. Results of kinetic studies of effects of lanthanum on COMT activity in the presence of varying concentrations of 3,4-dihydroxybenzoic acid (DBA) and S-adenosyl-l-methionine (SAM)*

$[La^{3+}]$	$1/V \pm$ S. E. M.	V_{max}	$K_m \pm$ S. E. M.
Varying concentrations of DBA			
0	0.6 ± 0.1	2073	$1.7 \pm 0.1 \times 10^{-4}$ M
2 μM	1.1 ± 0.1	901	$1.6 \pm 0.2 \times 10^{-4}$ M
4 μM	2.4 ± 0.1	425	$1.2 \pm 0.1 \times 10^{-4}$ M
Varying concentrations of SAM			
0	0.5 ± 0.0†	1992	$6.2 \pm 0.4 \times 10^{-6}$ M
2 μM	0.9 ± 0.1	1160	$8.5 \pm 0.4 \times 10^{-6}$ M
4 μM	1.4 ± 0.1	137	$9.7 \pm 1.0 \times 10^{-6}$ M

* COMT activity was measured in the presence of different concentrations of DBA and SAM either with no $LaCl_3$ present or with two concentrations of the inhibitor present. All results represent mean ± S. E. M. of three determinations.

†S. E. M. < 0.05.

results of these experiments was compatible with non-competitive inhibition. Lanthanum, like calcium, demonstrates a pattern of non-competitive inhibition with respect to DBA [2].

S-adenosyl-l-methionine. COMT activity was also determined in the presence of various concentrations of SAM either with or without added $LaCl_3$ (2 or 4×10^{-6} M). A double reciprocal plot of the results was made (Fig. 6) and kinetic data obtained from this plot are shown in Table 4. The apparent K_m value for SAM in the absence of lanthanum is similar to that reported previously [2]. This Lineweaver–Burk plot is also compatible with non-competitive inhibition, the same type of inhibition that calcium shows with respect to SAM [2].

Relationship between inhibition and ionic radius of lanthanides

The potency of lanthanum, neodymium and europium as inhibitors of COMT is inversely related to their ionic radii. The ionic radii of these species are

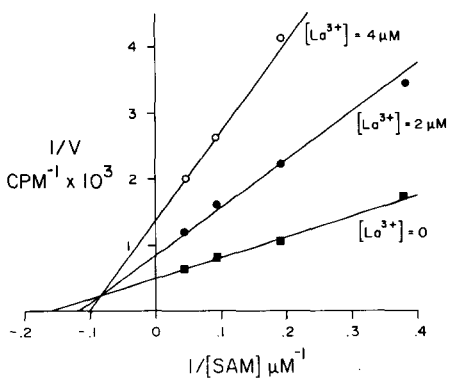

Fig. 6. Lineweaver–Burk plot of the effect of two different concentrations of La^{3+} on the velocity of the COMT reaction in the presence of various concentrations of S-adenosyl-l-methionine (SAM). Each point represents the mean of three determinations. A fourth point ($1/[SAM] = 0.38$, $1/V = 6.3$) in the presence of 4 μM La^{3+} was used in the kinetic analysis but is not shown in the figure because it was off scale.

La^{3+}, 1.061 Å; Nd^{3+}, 0.995 Å; and Eu^{3+}, 0.950 Å [12]. The ionic radius of Ca^{2+} is 0.99 Å [12]. Although the ionic radius of Nd^{3+} is closer to that of Ca^{2+} than is that of Eu^{3+}, europium is a slightly more potent COMT inhibitor than is neodymium. Although this observation is of interest with respect to the hypothesis that the lanthanides may interact with the COMT at the same site or sites as does calcium, Gomez *et al.* [6] caution that the relationship of the biochemical effects of the lanthanides to their ionic radii is often complex and cannot be easily related to the ionic radii.

DISCUSSION

Calcium is an inhibitor of COMT [2], and the present study has shown that lanthanum and the lanthanides, neodymium and europium, also inhibit this enzyme. These rare earths are two orders of magnitude more potent as inhibitors of COMT than is calcium. The inhibition of COMT by lanthanides is reversible, and kinetic analysis shows that this inhibition is non-competitive with respect to magnesium and with respect to the two co-substrates for the reaction, S-adenosyl-l-methionine and 3,4-dihydroxybenzoic acid. All of these characteristics are similar to those shown by calcium as an inhibitor of COMT [2], and these similarities raise the possibility that the lanthanides may interact with the same sites in COMT as does calcium.

Lanthanum and the lanthanides have proven to be useful chemical probes of the interactions of alkaline earth ions with other biological and biochemical systems [13]. These rare earths have spectral and magnetic properties that make it possible to carry out absorption, fluorescent, and nuclear magnetic resonance studies that cannot be performed with calcium or magnesium [7]. Lanthanum, neodymium and europium may be useful in future studies of the interaction of calcium with COMT if they act as isomorphic replacements for calcium in this enzyme.

Finally, the lanthanides may represent a new class of COMT inhibitor. Competitive inhibitors of COMT such as pyrogallol and S-adenosyl-l-homocystine have been described [14, 15]. Some compounds such as tropolone inhibit the enzyme by complex mechanisms [16], while sulfur analogs of catechol irreversibility inhibit the enzyme—presumably by the formation of disulfide bonds to the protein [17]. Many other types of COMT inhibitors have been described [18, 19]. Although inhibitors of COMT have been tested in clinical medicine in the treatment of psychiatric and neurologic diseases [20, 21], it is unlikely that the lanthanides will prove of value as COMT inhibitors *in vivo*. Lanthanide compounds are toxic, and lanthanum probably does not gain access to the interior of the cell where most of the COMT resides [3]. However, these limitations will not prevent the use of the lanthanides as chemical probes to study the molecular basis of the activity of COMT, an important catecholamine metabolic enzyme.

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