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Catechol *O*-Methyltransferase. 1. Kinetics of Tropolone Inhibition

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In an attempt to clarify the mechanism by which tropolones inhibit catechol *O*-methyltransferase (COMT), kinetic studies have been performed on COMT purified from rat liver. Rate studies indicate that tropolone is a linear noncompetitive inhibitor with respect to 3,4-dihydroxybenzoic acid (K_{ii} = 247 μM, K_{is} = 22 μM) and an uncompetitive inhibitor with respect to *S*-adenosyl-L-methionine; a complex relationship exists with respect to magnesium. Similar results were observed with *l*-norepinephrine and 3,4-dihydroxyacetophenone as substrates, as well as with 4-methyltropolone and β-thujaplicin as inhibitors. The patterns of inhibition and the kinetic parameters for tropolone inhibition remained constant through various stages of enzyme purification. The effect of pH on the substrate kinetic parameters (K_m and V_{max}) and tropolone inhibition parameters (K_{is} and K_{ii}) was also investigated. The ability of tropolone to inhibit COMT was found to decrease with increasing pH which can be attributed to an increase in K_{is}. This probably reflects the ionization of tropolone rather than dissociation of a group on the enzyme. The K_{ii} for tropolone was observed to decrease with increasing pH. The significance of this data relative to the mechanism of O-methylation by COMT is discussed.

The inactivation of catecholamines and the detoxification of many zenobiotic catechols is dependent upon the enzyme catechol *O*-methyltransferase (COMT) (E.C. 2.1.1.6).† COMT is a soluble, magnesium-requiring enzyme which transfers a methyl group from *S*-adenosylmethionine (SAM) to a catechol substrate resulting in the formation of the meta and para O-methylated derivatives.[1,2] Because of the importance of COMT in the extraneuronal inactivation of norepinephrine, inhibition of this route of metabolism has generated considerable research interest.[3-10]

Several classes of synthetic inhibitors of COMT have been identified: (a) substrates such as catechols,[3] desmethylpapaverine,[4] *o*-dihydroxyphenylacetamides,[5] and pyrogallol;[6] (b) product type inhibitors such as 3-hydroxy-4-methoxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, 3,5-dihydroxy-4-methoxybenzoic acid,[7] and *S*-adenosylhomocysteine;[8] and (c) dead-end inhibitors such as tropolones[9] and pyridoxal 5′-PO_4.[10]

Since dead-end inhibitors have proven useful in the elucidation of certain enzyme mechanisms,[11] and since the kinetic mechanism of COMT appears to be the subject of considerable controversy,[12-14,‡] it was felt that an extensive investigation of the kinetics of tropolone inhibition on COMT could provide information relative to the mechanism of methyl transfer. The present paper reports the results of such an investigation and provides information about the kinetic mechanism for COMT.

Results and Discussion

In Vitro Inhibition Patterns. The inhibition patterns toward COMT for three tropolone derivatives were determined using plots of reciprocal velocities against reciprocals of the substrate concentrations. As shown in Figure 1, a noncompetitive type of inhibition was observed when DHB was the variable substrate and tropolone was the inhibitor. Furthermore, this inhibition pattern can be classified as linear noncompetitve since replots of the slopes and intercepts *vs.* inhibitor concentrations are linear (Figure 2).[15] This type of linear relationship with a dead-end inhibitor provides evidence against the possibility that two or more molecules of the inhibitor add simultaneously to the same form of the enzyme.[15] This, however, does not rule out the possibility that tropolone may be combining with two different forms of the enzyme producing the noncompetitive

†Abbreviations used are: SAM, *S*-adenosyl-L-methionine; DHB, 3,4-dihydroxybenzoic acid; *l*-NE, *l*-norepinephrine; DHA, 3,4-dihydroxyacetophenone; COMT, catechol *O*-methyltransferase (E.C. 2.1.1.6); K_{is}, inhibition constant for the slope; K_{ii}, inhibition constant for the intercept.

‡C. Creveling, personal communication.

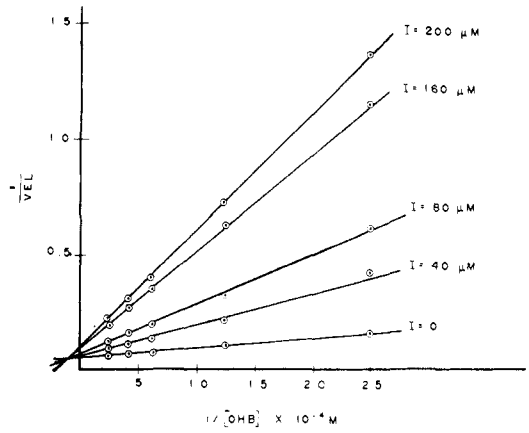


Figure 1. Reciprocal plots with DHB as the variable substrate and tropolone as the inhibitor. Assay conditions are the same as in Table I. Vel = nmol of product/mg of N Kjeldahl/min.

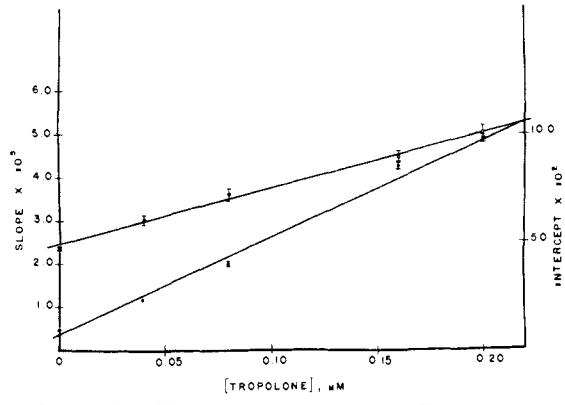


Figure 2. Replot of intercepts and slopes from Figure 1 against tropolone concentration. Equation 1 was used to calculate intercepts and slopes. Where significant ±S.E.M. are also shown. ●, intercept; ○, slope.

pattern. The noncompetitive pattern observed could in fact result from tropolone binding with one form of the enzyme producing competitive kinetics and one form producing uncompetitive kinetics. Using eq 3 for linear noncompetitive kinetics, a K_{is} of 22 (±1.0) μM and a K_{ii} of 247 (±16) μM for tropolone were calculated. (In linear inhibitions, K_{is} and K_{ii} represent the concentration of the inhibitor that doubles the slope or intercept term, respectively.) As shown in Table I similar results were observed in the cases of 4-methyltropolone and β-thujaplicin supporting the general noncompetitive pattern for this class of inhibitors.

These results contradict earlier work by Belleau and Burba[9b] and Mavrides, *et al.*,[9c] in which their preliminary data supported a competitive mechanism for tropolone inhibition. Earlier, however, Belleau and Burba[9a] had proposed a noncompetitive mechanism for this inhibition. To provide further evidence for a noncompetitive inhibitory mechanism, several other substrates of COMT were studied and the results of tropolone inhibition of their O-methylation are summarized in Table II. In each case noncompetitive inhibition was observed. Particularly interesting is the case of *l*-NE where K_{ii} and K_{is} for tropolone were of similar magnitude.

To eliminate the possible effect of saturation on the enzyme with the nonvaried substrates (SAM and Mg^{2+}) the inhibitory patterns varying DHB at half-maximal concentrations of SAM or Mg^{2+} were determined and found to be

Table I. Inhibition Constants for Various Substituted Tropolones toward COMT[a]

Inhibitor	Inhibition constants, μM[b] K_{ii} ± S.E.M.	K_{is} ± S.E.M.
Tropolone	247 ± 16	22 ± 1.0
4-Methyltropolone	143 ± 21	8.9 ± 1.7
β-Thujaplicin	283 ± 44	10.1 ± 2.2

[a]COMT was purified through the calcium phosphate gel step (ref 7) resulting in a preparation which contained 3.53 mg of protein per milliliter with a specific activity of 60.47 nmol/mg of N Kjeldahl/min (DHB as substrate). Assay conditions: DHB concentrations 40–400 μM; SAM concentration 1.0 mM; TES buffer pH 7.60 and inhibitor concentrations ranging from 16 to 200 μM. [b]Data fitted to eq 3.

identical with results obtained under saturating conditions (noncompetitive). Further evidence in support of this noncompetitive inhibition comes from data on a series of 8-hydroxyquinoline derivatives which have been shown *in vitro* to be potent noncompetitive inhibitors of COMT. They appear to act at a similar site and by a similar mechanism to that of the tropolones.[16]

During extensive purification of COMT, the inhibitory constants for tropolone were determined and as shown in Table III did not vary significantly. These data as well as data from the meta and para O-methylation studies of Creveling, *et al.*,[17,18] and gel electrophoresis studies of this purified enzyme in our laboratory would provide strong evidence that only one enzyme is involved. §

When SAM was the variable substrate and tropolone the inhibitor, an uncompetitive pattern of inhibition was observed (Figure 3). Identical uncompetitive patterns were observed when *l*-NE or DHA were used as substrates, as well as if 4-methyltropolone or β-thujaplicin were used as inhibitors. Utilization of half-maximal concentrations of catechol substrates or Mg^{2+} rather than saturation conditions of these substrates did not change the inhibition pattern. This uncompetitive pattern of inhibition with varying SAM was found to be independent of pH. This type of uncompetitive dead-end inhibition is characteristically produced by combination of the inhibitor with an enzyme form that occurs after combination of the variable substrate and before release of all the products.[11a,b] From a mechanistic standpoint the uncompetitive pattern observed here would indicate that tropolone was adding to a form of the enzyme after the addition of SAM.

A complex relationship exists between Mg^{2+} concentration, inhibitor concentration, and enzymatic activity. The buffer used in these studies was TES which has been shown to have no magnesium-complexing activity.[19] As shown in Figure 4 the double reciprocal plot of enzyme activity *vs.* Mg^{2+} at various concentrations of tropolone showed essentially parallel curves. In the absence of inhibitor at very low concentrations of Mg^{2+} little effect on enzyme activity was noted. However, an increased Mg^{2+} concentration produced an activation of reaction rate followed by a progressive inhibition at concentrations of Mg^{2+} greater than 1 mM. These results are consistent with similar observations by earlier investigators.[7,20] A paralleling effect was observed in the presence of increasing concentrations of tropolone. Even though the interrelationship between Mg^{2+} and inhibitor is not clear, the presence of inhibition at Mg^{2+} concentrations greater than that of tropolone and the apparent inability to reverse the inhibition with excess magnesium argues against chelation of

§ R. T. Borchardt, unpublished data.

Table II. Tropolone Inhibition of COMT[a]

Substrate	Substrate constants[b]		Inhibition constants, μM[c]	
	K_m ± S.E.M.[d]	V_{max} ± S.E.M.[e]	K_{ii} ± S.E.M.	K_{is} ± S.E.M.
3,4-Dihydroxybenzoic acid	90.0 ± 4.0	20.7 ± 0.28	247 ± 16	22 ± 1
l-Norepinephrine[f]	393 ± 43	7.67 ± 0.34	30.5 ± 4.4	44.3 ± 1.4
3,4-Dihydroxyacetophenone	8.04 ± 0.37	10.0 ± 0.88	270 ± 20	50.6 ± 4.4

[a]Kinetic assay conditions are the same as those outlined in Table I except *l*-NE concentrations of 160–1600 μM, DHA concentrations 16–80 μM, and tropolone concentrations 40–200 μM. [b]Data fitted to eq 1. [c]Data fitted to eq 3. [d]μM. [e]μmol of product /mg of N Kjeldahl/min. [f]Bitartrate salt.

Table III. Effect of Enzyme Purity on Tropolone Inhibition

Enzyme fraction	Purification,[a] -fold	Inhibition constants, μM[b,c]	
		K_{ii} ± S.E.M.	K_{is} ± S.E.M.
100,000*g* supernatant	8	245 ± 25	19.1 ± 3.2
30–50% $(NH_4)_2SO_4$	92	237 ± 14	21.4 ± 2.3
Sephadex G-25	192	271 ± 22	18.9 ± 2.7
Calcium phosphate gel	280	247 ± 16	22.0 ± 1.1

[a]COMT was purified from rat liver as described earlier (ref 7). [b]Assay conditions (the assay procedures were as described in the Experimental Section): 3,4-dihydroxybenzoic acid was utilized as substrate in concentrations ranging from 40 to 400 μM; SAM concentration, 1.0 mM; TES buffer pH 7.60; tropolone concentrations ranging from 40 to 200 μM. [c]Data fitted to eq 3.

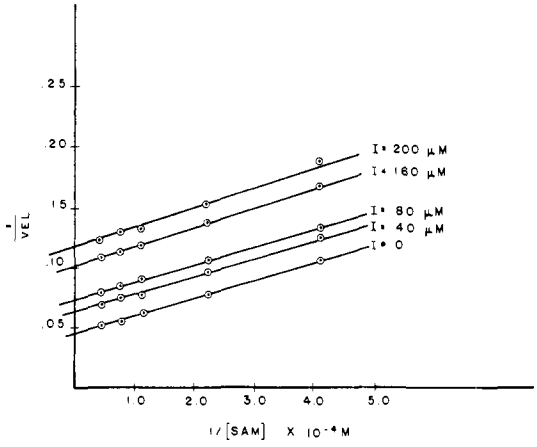


Figure 3. Reciprocal plots with SAM as the variable substrate and tropolone as the inhibitor. Assay conditions are the same as in Table I except SAM concentrations, 24–210 μM. DHB concentration, 2 mM. Vel = nmol of product/mg of N Kjeldahl/min.

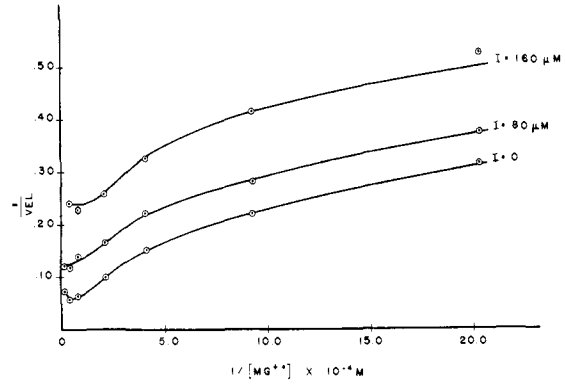


Figure 4. Reciprocal plots with Mg^{2+} as the variable component and tropolone as the inhibitor. Assay conditions are the same as in Table I except SAM concentration, 1.0 mM. DHB concentration, 2 mM. Vel = nmol of product /mg of N Kjeldahl/min.

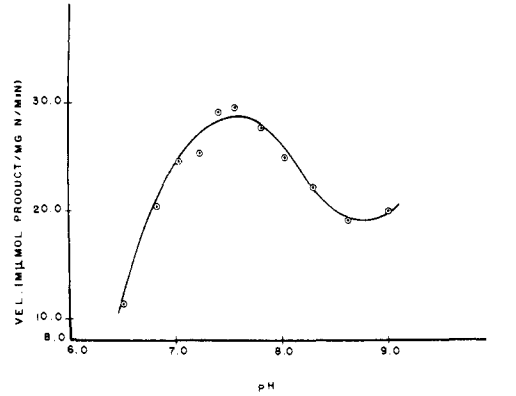


Figure 5. Reaction rate of COMT as a function of pH. Assay conditions are the same as in Table I except SAM concentration, 1.0 mM. DHB concentration, 2.0 mM. Mg^{2+} concentration, 1.2 mM. TES buffer concentration, 40 mM.

the free metal as a mechanism of tropolone inhibition. This does not, however, rule out the possibility that tropolone is bound by an enzyme-Mg^{2+} complex.

Effect of pH on Kinetic Constants. Considerable information about the functional groups present at or near the absorption pocket on an enzyme can be obtained by studying the variation of the kinetic parameters with pH. According to Dixon,[21] changes in the slope of plots of pK_m *vs.* pH reflect dissociation of ionizing groups that occur in the substrate, the free enzyme, or the enzyme-substrate complex. If the curvature of the pH profile for a particular substrate results from dissociation of groups present at or near the binding site on the enzyme, then other substrates or inhibitors which bind to the same site should show similar inflection points. Since at least part of the inhibition produced by tropolone on COMT can be classified as competitive with respect to the catechol substrate (indicating that tropolone is competing for the same form of the enzyme as the substrate), the effect of pH on the kinetic constants for substrate and tropolone should provide information relative to similarities or differences in their specific binding sites.

The effect of pH on the rate of O-methylation of DHB by purified COMT is shown in Figure 5. A pH optimum of 7.6 was observed. Previous studies using other substrates have shown a similar pH optimum,[12,22] as well as a second optimum at pH 9.7. The first optimum is attributable to the intrinsic properties of the enzyme, whereas the second probably results from an increased concentration of the anionic species of the catechol substrate. Since our interests were strictly in the groups associated with the binding sites on the enzyme, the pH range for our studies was restricted to pH 6.5–9.0.

Figure 6 shows the variation of the pK_m of DHB with pH. A single inflection point in this pH range was observed indicating dissociation of a group on the enzyme with a pK_a = 7.4–7.6. Figure 7 shows the variation of log V_{max} with pH showing an optimum V_{max} at pH 7.5 with a steady decrease in V_{max} with increasing pH.

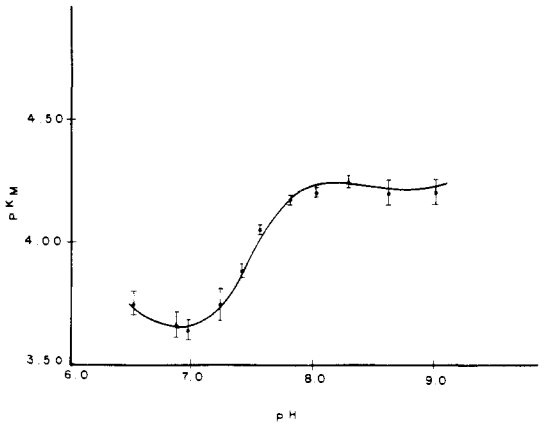


Figure 6. pK_m for DHB as a function of pH. pK_m determined as previously outlined. Data fitted to eq 1 with resulting S.E.M. shown.

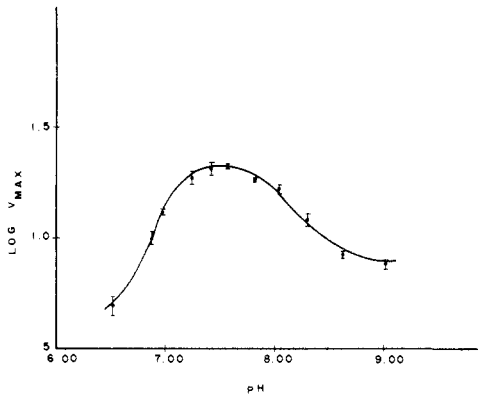


Figure 7. Log V_{max} for COMT as a function of pH. Data fitted to eq 1 with resulting S.E.M. shown.

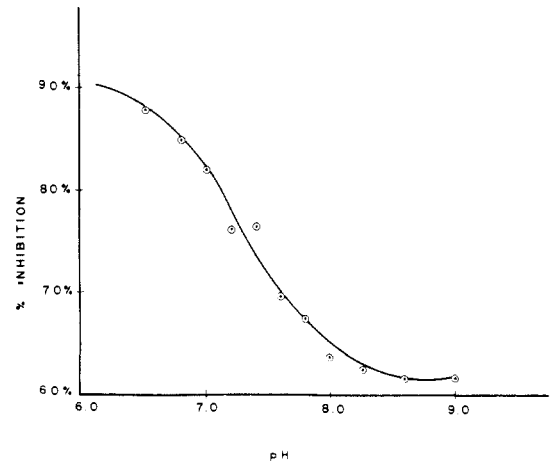


Figure 8. Per cent tropolone inhibition as a function of pH. Assay conditions are the same as in Figure 5 except tropolone concentration, 400 μM.

The effect of pH on tropolone inhibition of COMT is shown in Figure 8. Increases in pH produce a decrease in tropolone's ability to inhibit O-methylation of DHB. The observed inflection point at about pH 7.3 could indicate the ionization of a group at the binding site for tropolone; however, the situation is complicated by the fact that ionization of tropolone[23] (pK_a = 6.92) also takes place in this region. In an attempt to clarify this situation, the effect of pH on the inhibitory constants for tropolone was determined. The K_{ii} and K_{is} are summarized in Table IV. One of the more interesting observations is that at low pH (<pH 7.24) tropolone shows

Table IV. Effect of pH on Inhibition Constants for Tropolone[a]

		Inhibition constants, μM	
pH[b]	Inhibition[c]	K_{ii} ± S.E.M.	K_{is} ± S.E.M.
6.83	C		19.0 ± 4.0
7.05	C		19.2 ± 2.1
7.24	C		20.3 ± 3.7
7.42	NC	304 ± 31	25.1 ± 5.6
7.57	NC	247 ± 16	22.0 ± 1.7
7.82	NC	211 ± 48	33.2 ± 7.1
8.04	NC	180 ± 30	37.6 ± 5.3
8.30	NC	186 ± 56	39.2 ± 4.3
8.63	NC	121 ± 35	40.0 ± 6.7

[a]Kinetic conditions are the same as those outlined in Table I. [b]TES buffer. [c]Determined by inspection of reciprocal plots and if K_{ii} was statistically significant then data fitted to eq 3. C, competitive inhibition and data fitted to eq 2. NC, noncompetitive inhibition and data fitted to eq 3.

competitive kinetics with respect to DHB, whereas at higher pH the kinetic pattern becomes noncompetitive. The decrease in inhibitory ability with increasing pH (Figure 8) results from an increase in K_{is} for tropolone as shown in Table IV. The increase in K_{is}, we believe, is attributable to ionization of the inhibitor rather than dissociation of a functional group on the enzyme. Evidence to support this comes from data obtained on a series of 8-hydroxyquinolines which inhibit COMT by the same mechanism and bind to the same sites as tropolone.[16] 8-Hydroxyquinoline, which does not itself have a dissociation constant in this pH range, does not show a change in K_{is} with pH.[16] The decrease in the inhibitory ability and the increase in K_{is} with increasing pH may reflect the fact that the protonated tropolone species binds better to the enzyme than the anionic species. The decrease in K_{ii} with increasing pH, however, may partly reflect a dissociation of a group on the enzyme, since a similar decrease was observed with 8-hydroxyquinoline.[16]

Conclusions

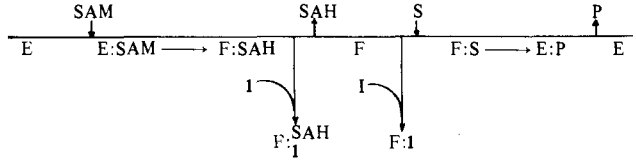
At the present time the kinetic mechanism of COMT appears to be the subject of considerable controversy. Flohe and Schwabe[12,13] using initial rate data proposed a "rapid equilibrium random" type mechanism for COMT. From very preliminary initial rate data Jarrott[14] has also proposed either an ordered or a random mechanism. However, initial rate data, product inhibition data, and binding studies by Creveling and coworkers strongly suggest a "ping-pong" mechanism.‡ Consequently, this study was undertaken in an attempt to utilize dead-end inhibitors to assist in clarification of this controversy. In the discussion of these results relative to the mechanism of COMT we have intentionally failed to include the magnesium ion. This was justified on the basis that preliminary reports on COMT have indicated that the interaction between substrates and divalent cations occurs on the enzyme surface, so that the metal must be binding prior to the substrates.[24] It is also unrealistic in most cases to expect the metal to combine with and leave the enzyme during each catalytic cycle.[11b] Thus, it has been assumed in this discussion that the metal binds prior to SAM or catechol substrate and, if held at a saturating level in kinetic studies, would have no effect on tropolone inhibition patterns.

One of the more interesting results obtained from this study relative to the mechanism of COMT was the observation that tropolones are uncompetitive inhibitors with respect to SAM. Uncompetitive dead-end inhibition is characteristically produced by combination of the inhibitor with a

form of the enzyme that occurs after combination of the variable substrate and before release of all the products.[11a] The results with tropolone suggest that it is combining to a form or forms of the enzyme after the addition of SAM. This purely uncompetitive pattern with tropolone *vs.* SAM would effectively rule out the rapid equilibrium random mechanism and an ordered mechanism (where catechol substrate added first), since both cases should show a noncompetitive pattern with SAM. This leaves only an ordered mechanism or a ping-pong mechanism (where in both cases SAM added first to the enzyme). If one assumes binding of tropolone to only one form of the enzyme, then both of these mechanisms would show an uncompetitive pattern with SAM. However, this assumption would make it difficult to explain the noncompetitive pattern with varying catechol substrate.

The noncompetitve pattern observed, when DHB, *l*-NE, or DHA were the variable substrates, and the variation of this noncompetitve pattern with pH would suggest tropolone was adding to two different forms of the enzyme, one producing competitive and the other uncompetitive kinetics. The overall result is the observed noncompetitive pattern. This type of pattern would be difficult to explain by the ordered mechanism, since combination of tropolone with two different forms of the enzyme would result in destruction of the purely uncompetitive kinetics with varying SAM. The tropolone inhibition data would best support a ping-pong mechanism as proposed by Creveling and coworkers.‡ A similar mechanism has recently been proposed for histamine *N*-methyltransferase.[25] The data obtained for tropolone inhibition of COMT would best fit a hybrid ping-pong mechanism similar to that proposed for oxaloacetate transcarboxylase.[26] Scheme I shows the mechanism proposed for

Scheme I



COMT based on the inhibition studies reported in this paper. This mechanism might involve an intermediate methylated form of the enzyme (F) and thus allow for independent binding of substrates to two distinct sites on the enzyme. Thus, tropolone (I) could be producing its inhibition by combining with form F:SAH and form F producing the uncompetitive pattern observed with SAM and noncompetitive pattern observed with substrate. Data obtained on a series of 8-hydroxyquinolines would also support this mechanism.[16]

Experimental Section

Materials. SAM-$^{14}CH_3$ (New England Nuclear, 55.0 mCi/mmol) was diluted to a concentration of 10 μCi/ml and stored at −20°F. SAM iodide (Calbiochem) was stored as a 0.01 *M* solution in 0.001 *N* HCl at −20°F. *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES, Sigma) buffers were prepared as 0.2 *M* stock solutions. The remaining compounds were obtained from commercial sources or synthesized by routine methods.

Purification of COMT. COMT was purified from rat liver (male, Sprague-Dawley, 180–200 g) according to the method of Nikodejevic, *et al.*[7] The material was purified through the calcium phosphate gel step resulting in a preparation which contained 3.53 mg of protein per milliliter with a specific activity of 60.67 nmol/mg of N Kjeldahl/min with 3,4-dihydroxybenzoic acid as substrate. This represented a 280-fold purification from the crude supernatant.

Measurement of Enzyme Activity. Kinetic experiments were carried out in 15-ml screw cap culture tubes and the basic incubation mixture contained the following components (in micromoles) added in this sequence: water, so that final volume was 0.25 ml; substrate (variable); inhibitor (variable); *S*-adenosyl-L-methionine (variable); 0.05 μCi of *S*-adenosyl-L-methionine-^{14}C; magnesium chloride (variable); dithiothreitol (1.0); *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) buffer, pH variable (10); and the enzyme preparation (0.025 ml) containing 3.53 mg of protein per milliliter. The reaction was started by the addition of enzyme and incubated for 3–5 min at 37°. When dihydroxybenzoic acid was used as substrate the reaction was stopped by addition of 0.1 ml of 1.0 *N* HCl and the mixture extracted with 10 ml of a toluene–isoamyl alcohol mixture (7:3). After centrifugation, an aliquot (5 ml) of the organic phase was transferred to a scintillation vial, a dioxane-based phosphor solution (10 ml) added, and the radioactivity measured in a Beckman LS-150 scintillation spectrophotometer. The results were corrected for blank values obtained by carrying out the reaction without substrate. When *l*-NE was used as substrate, the reaction was stopped by the addition of 0.25 ml of 0.5 *M* borate buffer, pH 10, and a mixture of toluene–isoamyl alcohol (3:2) used for extraction. When 3,4-dihydroxyacetophenone was used as substrate, the reaction was stopped by addition of 0.1 ml of 1.0 *N* HCl and toluene was used for extraction.

Similar inhibition and kinetics of inhibition were obtained with these substrates in both Tris and phosphate buffers. Enzyme activity was expressed as nanomoles of product formed per milligram of N Kjeldahl per minute.

Data Processing. Reciprocal velocities were plotted graphically against reciprocals of the substrate concentrations. In all cases a reasonably linear relationship was obtained. These data were then fitted to eq 1 using a least-squares method and assuming equal variance for the velocities.[27] All calculations were performed on a Hewlett-Packard 2100A digital computer using a FORTRAN IV program[15] which provided values of K, V, K/V, $1/V$, and the standard errors of their estimates. Slopes (K/V) and intercepts ($1/V$) were then plotted graphically against the inhibitor concentration. All replots were linear. On the basis of the replots the proper pattern was chosen for each experiment and all the data points were fitted to the appropriate equation.[28] Data fitting linear competitive inhibition were fitted to eq 2 and for linear noncompetitive inhibition to eq 3.

$$v = VA/(K + A) \quad (1)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (2)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (3)$$

Acknowledgment. The author gratefully acknowledges support of this project by a University of Kansas General Research Grant and partial support by a Health Sciences Advancement Award (FR06147) granted to the University of Kansas by the National Institutes of Health and a grant from the Research Corporation. The excellent technical assistance of Sharon Elrod is gratefully acknowledged.

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Catechol *O*-Methyltransferase. 2. *In Vitro* Inhibition by Substituted 8-Hydroxyquinolines

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The transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to a catechol substrate by the enzyme catechol *O*-methyltransferase (COMT) (E.C. 2.1.1.6) has been found to be inhibited *in vitro* by various substituted 8-hydroxyquinolines. A study of the structure-activity relationships of a series of substituted 8-hydroxyquinolines was carried out and from this study have evolved some of the most potent *in vitro* inhibitors of COMT yet reported (*e.g.*, 7-iodo-8-hydroxyquinoline-5-sulfonic acid; $K_i = 8.93 \times 10^{-7} M$). Rate studies indicate that these inhibitors are linear noncompetitive with respect to catechol substrate and uncompetitive with respect to *S*-adenosyl-L-methionine; a complex relationship exists with respect to magnesium. From the noncompetitive rate data were calculated the inhibition constants for the slope (K_{is}) and intercept (K_{ii}). Variation of these inhibition contants with structural changes on the basic 8-hydroxyquinoline molecule was determined. For 5-substituted compounds a good correlation with Hammett σ was observed. The ability of 8-hydroxyquinoline to inhibit COMT was found to increase with increasing pH which could be attributed to a decrease in K_{ii}. K_{is} was found to remain constant over the pH range of 6.8–8.3. By studying the kinetics of inhibition of combinations of tropolone and 8-hydroxyquinoline, evidence was obtained to indicate these inhibitors bind at least partly to the same site on the enzyme.

The extraneuronal inactivation of norepinephrine is dependent upon the enzyme catechol *O*-methyltransferase (COMT) (E.C. 2.1.1.6).[1,†] The inhibition of this route of catecholamine metabolism has been the subject of considerable research, interest resulting in the identification of several classes of synthetic[2–9] and natural inhibitors.[10] Some of these inhibitors have proven useful in ascertaining the relative importance of COMT in the metabolism of norepinephrine,[11] as well as in the elucidation of the mechanism of methyl transfer.[12,‡]

A potential class of COMT inhibitors which has obtained little attention are those in which another heteroatom has been substituted for one of the hydroxyls in the basic catechol molecule. An example of this type of inhibitor is 8-hydroxyquinoline (8-HQ) which Ross and Haljasmaa[4] reported was 50% more potent than pyrogallol as an *in vitro* inhibitor of mouse brain COMT. This inhibition did not appear to be a direct result of 8-HQ's metal chelating abilities. Since this particular class of compounds has not been extensively investigated for their ability to inhibit COMT, it was felt that a structure–activity relationship study and an investigation of the kinetics of inhibition of this class of inhibitors could provide useful information about the enzyme. It is also important to note that specific compounds in this general class (chiniofon, vioform, diodoquin) have been used in the chemotherapy of amebiosis.[13] COMT inhibition *in vivo* by these therapeutic agents may be relevant to toxicity and side effects observed with these compounds. The present paper reports the results of a study on the *in vitro* inhibition of COMT by substituted 8-HQ's.

Results and Discussion

In Vitro Inhibition Studies. The effect of varying concentrations of 8-HQ on the initial velocity of the COMT catalyzed O-methylation of DHB is shown in Figure 1. A K_i = *ca.* $2 \times 10^{-5} M$ was obtained for 8-HQ as compared to a $K_m = 9.0 \times 10^{-5} M$ for the substrate DHB. Similar inhibition was observed with *l*-NE and DHA as substrates. The inhibition by 8-HQ was completely reversible in that enzymatic activity was recovered after dialysis or gel filtration on Sephadex G-25. 8-HQ, being structurally related to catechol, conceivably might serve as a substrate for COMT and be converted to an *O*-methyl derivative. This was not the case and no detectable methylation product was formed in the presence of the enzyme under conditions which were optimal for the O-methylation of DHB (Table I).

Table II shows the degree of COMT inhibition produced by

†Abbreviations used are: SAM, *S*-adenosyl-L-methionine; DHB, 3,4-dihydroxybenzoic acid; *l*-NE, *l*-norepinephrine; DHA, 3,4-dihydroxyacetophenone; 8-HQ, 8-hydroxyquinoline; COMT, catechol *O*-methyltransferase (E.C. 2.1.1.6); K_{is}, inhibition constant for the slope; K_{ii}, inhibition constant for the intercept.

‡C. R. Creveling, unpublished data.