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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.

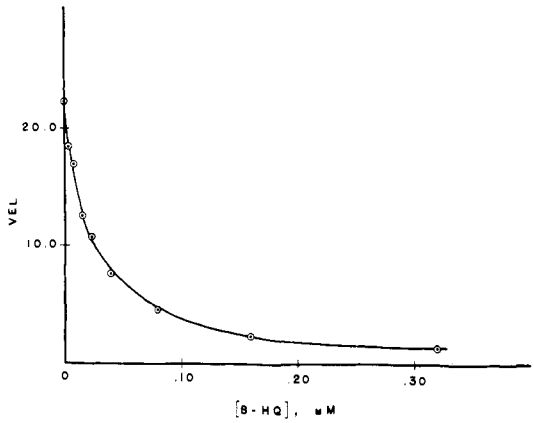


Figure 1. Initial velocity of COMT as a function of 8-HQ concentration. Assay conditions are the same as in Table II. Vel = nmol of product/mg of N Kjeldahl/min.

Table I. Interaction of 8-Hydroxyquinoline with COMT. Lack of Formation of Methylated Products

Substrate	Concn, μM	Extractable radioactivity, cpm[a]
None		165
8-Hydroxyquinoline	4.0	155
8-Hydroxyquinoline	40.0	162
8-Hydroxyquinoline	400	175
8-Hydroxyquinoline	2.0	174
DHB	2.0	12684

[a] Conditions were the same as described in Table II except the incubation time was 30 min.

a series of substituted 8-hydroxyquinolines, as well as by some previously known inhibitors of this enzymatic O-methylation. The negligible inhibitor activity observed with 8-mercaptoquinoline, 8-acetoxyquinoline, 8-aminoquinoline, and 8-quinolinesulfonic acid indicates the importance of the free 8-hydroxyl group in binding. The low inhibitor activity of 8-hydroxyquinoline *N*-oxide, as well as the complete lack of inhibition observed with various substituted *o*-aminophenols,[§] illustrates the importance of the quinoline type of nitrogen for inhibition. The requirement in this series of compounds for both the 8-hydroxyl and quinoline nitrogen for maximum inhibition of COMT illustrates the idea that another heteroatom can be substituted for one of the hydroxyls of the basic catechol molecule resulting in compounds which bind to the substrate site on the enzyme but are not themselves substrates.

Substitution of various other functional groups in the 2, 4, 5, and 7 positions on the 8-HQ molecule revealed some interesting structure-activity relationships for this series of inhibitors (Table II). Incorporation of a methyl or carboxylic acid group in the 2 position resulted in complete loss of inhibitory activity. For example, **12** produced 90.7% inhibition of COMT at a concentration of 20 μM, whereas substitution of a methyl group in the 2 position of this molecule (**13**) resulted in complete loss of activity. Substitution of an iodine (**12**, **16–18**) or bromine (**14**) atom into the 7 position appears to enhance the inhibitory activity. However, substitution of a nitro group (**19**) or carboxylic acid (**20**) in this position resulted in a decrease in activity. In the series of 5-monosubstituted 8-HQ's, it appears that electron-withdrawing groups (**4–6**, **11**) increase the inhibitory activity, whereas electron-donating groups (**7–10**) decrease activity. This idea will be illustrated more accurately later in this discussion when it is shown that the

[§] R. Borchardt, unpublished data.

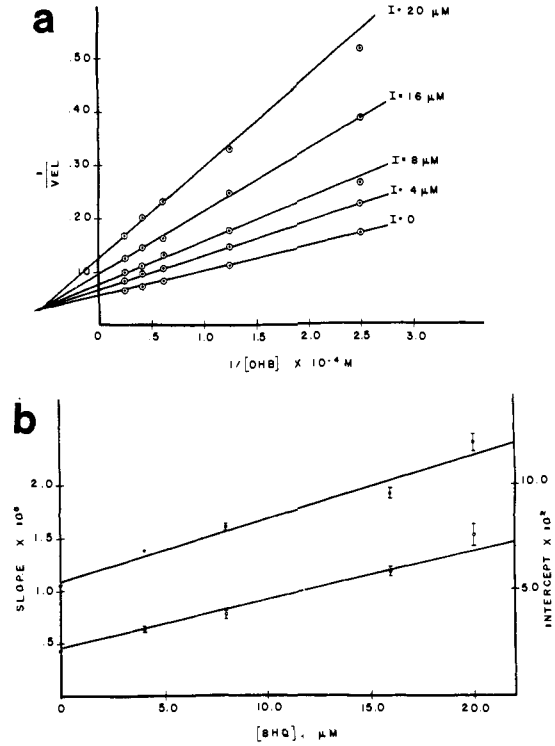


Figure 2. (a) Reciprocal plots with DHB as the variable substrate and 8-HQ as the inhibitor. Assay conditions are the same as in Table III. Vel = nmol of product/mg of N Kjeldahl/min. (b) Replots of intercepts and slopes from Figure 2a against 8-HQ concentration. Equation 1 used to calculate intercepts and slopes. Where significant ±S.E.M. are also shown. ○, slope; ●, intercepts.

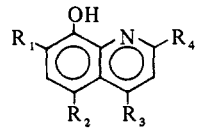
kinetic inhibition constants for these 5-monosubstituted 8-HQ's correlate well with Hammett σ. It is interesting to compare the inhibitory activity of the more active substituted 8-HQ's (**4**,**11**,**12**,**14**,**16–18**) against previously reported COMT inhibitors (**26–31**).

Kinetic Inhibition Patterns. Using reciprocal velocity *vs.* reciprocal substrate plots, the kinetic patterns for 8-HQ inhibition of the O-methylation of DHB were determined. In all cases similar patterns were also observed when *l*-NE or DHA were used as substrates, when half maximal concentrations of the nonvariable substrates were used, or when 8-hydroxy-7-iodo-5-quinolinesulfonic acid was used as the inhibitor. As shown in Figure 2a, when DHB was the variable substrate and 8-HQ the inhibitor, a noncompetitive type of inhibition was observed. Furthermore, this can be classified as linear noncompetitive since replots of the slopes or intercepts *vs.* inhibitor concentrations were linear (Figure 2b). Using eq 2 for linear noncompetitive kinetics, a K_{is} = 12.0 (±1.7) μM and K_{ii} = 17.5 (±1.6) μM were calculated. These results are similar to those obtained for tropolone inhibition[12] and would indicate that 8-HQ is probably producing its noncompetitive pattern as a result of interacting with two different form of the enzyme, one producing competitive kinetics and the other producing uncompetitive kinetics, the overall result being a noncompetitive pattern.

When SAM was the variable substrate, an uncompetitive pattern of inhibition was observed (Figure 3). This is again similar to tropolone inhibition[12] and would indicate that 8-HQ combines with a form of the enzyme after the addition of SAM.

The relationship between Mg^{2+} concentration, enzyme activity, and inhibitor activity was observed to be a complex one as shown in Figure 4. The results were again similar to

Table II. Inhibition of COMT by Substituted 8-Hydroxyquinolines[a]



Compd no.	Inhibitor[b] R_1	R_2	R_3	R_4	% inhibition[f] $[I] = 2.0 \times 10^{-5}$ M	$[I] = 4.0 \times 10^{-6}$ M
1	H	H	H	H[c]	48.0	14.9
2	H	H	H	CH_3	8.8	0
3	H	H	OH	CO_2H[d]	1.8	0
4	H	Cl	H	H[c]	68.7	31.1
5	H	NO_2	H	H[c]	50.4	16.2
6	H	SO_3H	H	H[c]	50.6	25.8
7	H	CH_3	H	H[c]	24.5	12.3
8	H	CH_2CH_3	H	H[c]	44.8	19.2
9	H	$CH_2CH_2CH_3$	H	H[c]	39.5	17.2
10	H	NH_2	H	H[c]	34.5	14.5
11	H	$C(=O)CH_3$	H	H[c]	78.9	44.6
12	I	I	H	H[d]	90.7	49.2
13	I	I	H	CH_3[e]	0	0
14	Br	Br	H	H[d]	91.2	39.8
15	Br	Br	H	CH_3[e]	0	0
16	I	Cl	H	H[e]	85.8	62.0
17	I	$C(=O)CH_3$	H	H[d]	94.7	78.0
18	I	SO_3H	H	H	94.8	77.7
19	NO_2	NO_2	H	H[d]	21.5	2.6
20	CO_2H	H	H	H[c]	0	0
21	8-Mercaptoquinoline				0	0
22	8-Acetoxyquinoline[d]				7.8	2.3
23	8-Aminoquinoline[c]				0	0
24	8-Hydroxyquinoline *N*-oxide				7.7	0
25	8-Quinolinesulfonic acid				0	0
26	Pyrogallol				16.0	8.9
27	Tropolone				15.2	3.1
28	β-Thujaplicin				45.6	11.9
29	4-Methyltropolone				52.7	27.8
30	3,4-Dimethoxy-5-hydroxybenzoic acid				20.1	8.3
31	Pyridoxal 5′-PO_4				0	0

[a] COMT was purified and assayed as previously described.[9,12] SAM concentration 1.0 m*M*; DHB concentration, 2.0 m*M*; Mg^{2+} concentration, 1.2 m*M*; TES buffer concentration, 40 m*M*, pH 7.60; incubation time, 10 min. [b] Unless otherwise noted the inhibitors were prepared as aqueous stock solution of concentrations 0.2–2.0 μmol/ml. [c] Stock solution prepared in 2 m*M* HCl. [d] Stock solution prepared in 25% DMSO–H_2O. [e] Stock solution prepared in 50% DMSO–H_2O. [f] Expressed as per cent inhibition of O-methylation of DHB. Where inhibitor was added from a stock solution prepared in aqueous HCl or aqueous DMSO, the reactions in absence of inhibitors were run with addition of a similar amount of aqueous HCl or aqueous DMSO. (The HCl or DMSO utilized showed little effect on the rate of the standard reactions.)

those observed with tropolone.[12] Increased concentrations of magnesium did not decrease the inhibition observed with 8-HQ. This provides strong evidence that simple chelation of the free Mg^{2+} by the inhibitor cannot be the mechanism by which 8-HQ inhibits COMT. This does not, however, rule out the possibility that 8-HQ binds through its chelating abilities with an enzyme bound Mg^{2+}.

In an attempt to find a correlation between the inhibitory binding constants for this series of compounds and the electronic structure, the K_{is} and K_{ii} were determined for a series of 5-monosubstituted 8-HQ's. The kinetic constants for this series, as well as the data for some 5,7-disubstituted inhibitors, are shown in Table III. As was indicated from the preliminary data in Table II, substitution of electron-withdrawing groups in the 5 position resulted in an increase in inhibition (decrease in K_{is} and K_{ii}), whereas substitution of electron-donating groups resulted in a decrease in activity (increase in K_{is} and K_{ii}). In fact, as shown in Figure 5, the K_{ii} and K_{is} both correlate reasonably well with Hammett σ producing a $\rho = 0.97$ for the K_{is} plot and $\rho = 0.78$ for K_{ii} plot. The only 5-substituted 8-HQ which does not give a reasonable correlation is the 5-NO_2 compound. This could be a result of the fact that the phenolic group for this compound would have a sufficiently low pK_a so as to be partly ionized under the conditions used for the assay. This anionic species may bind less strongly to the inhibitor site than the protonated series as was the case with tropolone.[12] The correlation with Hammett σ may then indicate that electron withdrawal is important to weaken the oxygen–hydrogen bond; however, conversion to the anionic species itself may be undersirable for binding.

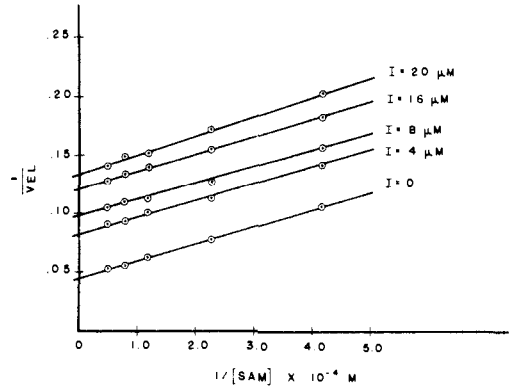


Figure 3. Reciprocal plots with SAM as the variable substrate and 8-HQ as the inhibitor. Assay conditions outlined in Experimental Section except SAM concentration, 24–210 μM. DHB concentration, 2 m*M*. Vel = nmol of product/mg of N Kjeldahl/min.

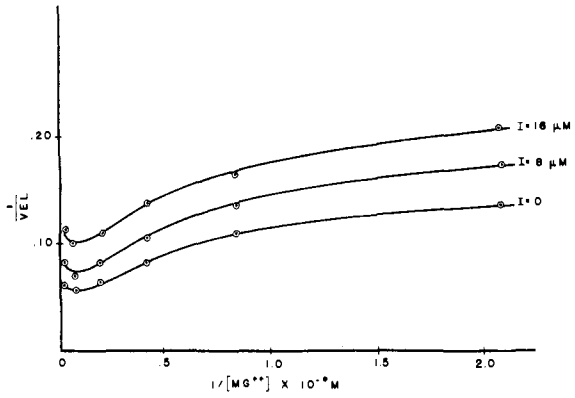


Figure 4. Reciprocal plots with Mg^{2+} as the variable component and 8-HQ as the inhibitor. Assay condition outlined in Experimental Section except SAM concentration, 1.0 m*M*. DHB concn, 2 m*M*. Vel = nmol of product/mg of N Kjeldahl/min.

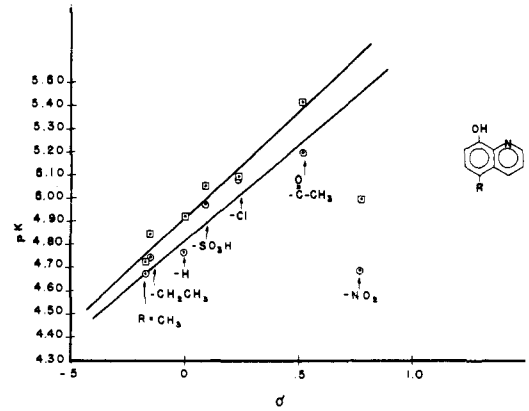
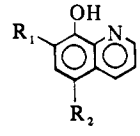


Figure 5. Plots of pK_{ii} and pK_{is} *vs.* Hammett σ. Assay conditions same as in Table III. σ values taken from D. H. McDaniel and H. C. Brown, *J. Org. Chem.*, **23**, 420 (1958). Correlation coefficients >0.95. pK_{is} = ⊡. pK_{ii} = ⊙.

Table III. Inhibition Constants for Substituted 8-Hydroxyquinolines[a]



Inhibitor		Inhibition constants, μM[b]	
R_1	R_2	K_{ii} ± S.E.M.	K_{is} ± S.E.M.
H	H	17.5 ± 1.6	12.1 ± 1.7
H	Cl	8.35 ± 2.30	8.39 ± 1.15
H	NO_2	20.9 ± 6.7	10.1 ± 3.9
H	SO_3H	10.8 ± 1.3	8.88 ± 1.86
H	CH_3	21.4 ± 4.3	19.2 ± 5.2
H	CH_2CH_3	18.2 ± 3.9	14.5 ± 2.7
H	$C(=O)CH_3$	6.46 ± 1.02	3.91 ± 0.83
I	SO_3H	1.18 ± 0.13	0.89 ± 0.16
I	$C(=O)CH_3$	2.32 ± 0.54	0.95 ± 0.21

[a] COMT was purified through the calcium phosphate gel step.[9,12] Specific activity of 60.47 nmol of product/mg of N Kjeldahl/min. Assay conditions: DHB concentrations, 40–400 μM; SAM concentration, 1.0 m*M*; TES buffer, pH 7.60; inhibitor concentrations, 4.0–20 μM. [b] Data fitted to eq 2.

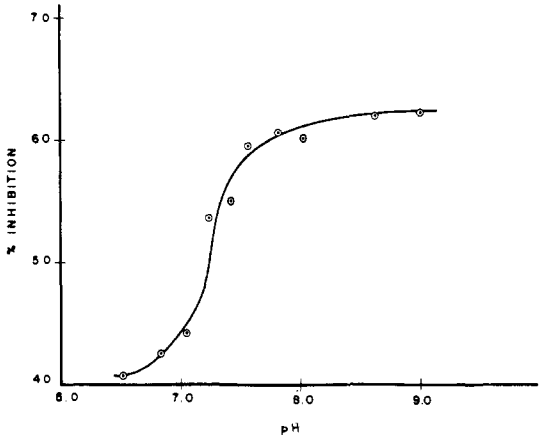


Figure 6. Per cent 8-HQ inhibition as a function of pH. Assay conditions the same as in Table II. 8-HQ concentration = 40 μM.

The data in Table III also indicate that incorporation of a a halogen atom into the 7 position increases inhibitor activity. For example, substitution of an iodine in the 7 position of 8-hydroxy-5-quinolinesulfonic acid and 8-hydroxy-5-quinolyl methyl ketone produces inhibitors which bind 4–10 times better than the parent compounds. The relative inhibitory activity of this series of compounds compared to previously reported inhibitors can be seen by a comparison of 8-hydroxy-7-iodo-5-quinolinesulfonic acid which has a binding constant 25 times greater than that of tropolone.

Effect of pH on Kinetic Constants. Useful information about functional groups at the binding site on an enzyme can be obtained by studying the effect of pH on the kinetic inhibition parameters for a specific inhibitor. Figure 6 shows the effect of pH on 8-HQ's ability to inhibit COMT-catalyzed methylation of DHB. An inflection point at about pH 7.4 was observed. Since 8-HQ itself has no ionizable groups in this pH region, this inflection point could be attributed to the dissociation of a group present at the binding site of the inhibitor. In fact, this inflection point is similar to the one observed for the binding of the substrate to the active site of the enzyme.[12]

To determine if this inflection in the pH profile of 8-HQ is a result of changes in K_{ii} or K_{is} or both, the effect of pH on these parameters was determined and is summarized in Table IV. The increase in inhibitory activity with increases in pH appears to be a result of a decrease in K_{ii}. However, K_{is} (within experimental error) remains constant with changes in pH. These results are similar to those observed for tropolone,[12] where the same decrease in K_{ii} was observed. In the case of tropolone, however, a significant increase in K_{is} was also observed which we attributed to the ionization of the inhibitor itself rather than a functional group on the enzyme. To provide further evidence to support this hypothesis, as well as to determine if tropolone and 8-HQ are binding to similar sites, a study of the kinetics of multiple inhibition of COMT by 8-HQ and tropolone was conducted using the procedures of Yonetani and Theorell.[14] These studies were carried out at pH 6.53 where the two inhibitors are essentially competitive with respect to DHB. As shown in Figure 7, a series of parallel straight lines were obtained when reciprocal velocities were plotted *vs.* tropolone concentrations at varying concentrations of 8-HQ. The slope of the lines remained constant with varying 8-HQ; however, the intercepts were a linear function of 8-HQ. This provides evidence that at pH 6.53, 8-HQ and tropolone are competing for the same site on the enzyme. Since the competitive kinetic portion of 8-HQ inhibition (K_{is}) showed no change with pH, and since 8-HQ and tropolone compete for this same site, the change in K_{is} observed for tropolone probably reflects the dissociation of inhibitor itself rather than a group on the enzyme.

Table IV. Effect of pH on Inhibition Constants for 8-Hydroxyquinoline[a]

pH[b]	Inhibition constants, μM[c]	
	K_{ii} ± S.E.M.	K_{is} ± S.E.M.
6.83	39.0 ± 3.4	10.2 ± 4.1
7.05	29.4 ± 6.5	10.1 ± 2.0
7.24	27.3 ± 3.6	12.0 ± 3.1
7.42	22.0 ± 3.1	15.0 ± 5.2
7.57	17.2 ± 1.6	12.0 ± 1.7
7.82	20.0 ± 4.1	15.1 ± 5.0
8.04	19.0 ± 3.7	14.0 ± 4.9
8.30	16.5 ± 4.3	10.7 ± 5.4

[a]Kinetic conditions same as those outlined in Table II. [b]TES buffer. [c]Data fitted to eq 2.

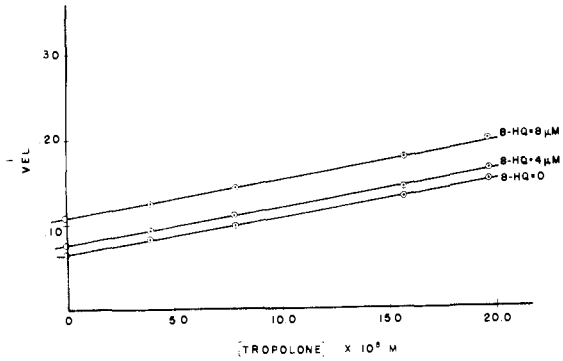


Figure 7. Reciprocal velocity *vs.* tropolone concentration with varying 8-HQ concentration. Assay conditions are the same as in Table III except pH 6.53. Vel = nmol of product/mg of N Kjeldahl/min.

Conclusions

The present paper has attempted to delineate the structure–activity relationship requirements, as well as the kinetic mechanism for inhibition of COMT by 8-HQ's *in vitro*. From a structural standpoint there appears to be a strict requirement for the 8-hydroxyl group, the quinoline nitrogen, and the lack of substitution in the 2 position. Substitution of halogen in the 7 position increases inhibitory activity, whereas substitution of a nitro group or carboxylic acid group in this position decreases activity. Incorporation in the 5 position of electron-donating groups decreases activity, whereas an electron-withdrawing group in this position increases activity. Further proof for this electronic effect is seen by the correlation of binding constants with Hammett σ producing a positive ρ value. A strongly electron-withdrawing group in the 5 position, however, may decrease activity because of an increase in the amount of the less reactive anionic species.

Rate studies and competition studies have shown that 8-HQ inhibits COMT by the same mechanism and competes for the same sites as tropolone. The kinetics of 8-HQ inhibition support the proposed "ping-pong" mechanism for COMT,[12] since the observed inhibition patterns could result from 8-HQ binding to the two different forms of the enzyme. The K_{ii} parameters are in general 15–210 times less than those for tropolones, indicating that the 8-HQ's bind much stronger to this site, whereas the K_{is} constants are only 2–25 times less than the tropolones. The biggest difference in these two classes of inhibitors appears to be the binding associated with the intercept effect (K_{ii}).

As with tropolones[12] the inhibitory activity of 8-HQ was shown to be sensitive to pH resulting in increases in activity with increases in pH. This effect was different than that observed for tropolone, where a decrease in activity was observed with increase in pH. The reason for the difference is readily apparent if one looks at the magnitude of K_{ii} and the changes in this parameter with pH for 8-HQ. The increase in 8-HQ activity with increasing pH can be attributed completely to the K_{ii} effect and this appears to be a result of dissociation of an ionizable group on the enzyme causing an increased binding of the inhibitor.

This class of inhibitors has been utilized to point up the fact that another heteroatom or another functional group can be substituted for one of the hydroxyl groups of the catechol substrate resulting in inhibitors of COMT which bind reversibly and more tightly to the enzyme than the substrate itself. Other examples of this approach will be the subject of future communications.

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 following compounds were commercially available from the indicated sources: 1, 3, 4, 5, 12–14, 20, 24–28 (Aldrich); 11, 15–17, 19 (Alfred Bader); 2, 18, 22, 23 (Baker); 6, 31 (Sigma); 29, 30 (Regis).

8-Mercaptoquinoline. Commercially available quinoline-8-sulfonyl chloride (1.83 g, 8.0 mmol) was reduced according to the procedure of Lee[15] utilizing $LiAlH_4$ (0.55 g, 14.5 mmol) to yield 0.64 g (44%), mp 57–58.5° (lit.[15] mp 58–59°).

5-Amino-8-hydroxyquinoline. 8-Hydroxy-5-nitroquinoline (1.90 g, 10 mmol) was reduced by catalytic hydrogenation with PtO_2 in EtOH at 25° under atmospheric pressure. The catalyst was removed by filtration and the solvent was removed. The resulting brown solid was crystallized (C_6H_6) to yield 1.21 g (75%), mp 143–145° (lit.[16] mp 145–146°).

5-Alkyl-8-hydroxyquinolines. The appropriate 4-*N*-alkyl-2-aminophenol[17] (1.5 g), concentrated sulfuric acid (4 ml), and glycerol (2.0 ml) were mixed and heated at 140–150° for 0.5 hr; then arsenic pentoxide (1.5 g) was added and heating continued for another 4 hr. The cooled mixture was diluted with H_2O, excess Na_2CO_3 was added, and the crude products were isolated by continuous extraction with $CHCl_3$. Evaporation of the solvent and purification by sublimation afforded pure products: 8-hydroxy-5-methylquinoline, 0.32 g (17%), mp 118–120° (lit.[17] mp 121–122°); 5-ethyl-8-hydroxyquinoline, 0.25 g (15%), mp 102–104° (lit.[17] mp 105–106°); 8-hydroxy-5-propylquinoline, 0.48 g (28%), mp 59–60° (lit.[17] mp 57–58°).

Purification and Assay of COMT. COMT was purified from rat liver (male, Sprague–Dawley, 180–200 g) according to the methods previously described.[9,12] Enzyme preparations utilized for kinetic experiments had a specific activity of 60.47 nmol of product/mg of N Kjeldahl/min for DHB as substrate. Enzyme assayed according to previously described procedures.[9]

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

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

to eq 1 using a least-squares method and assuming equal variance for the velocities.[18] All calculations were performed on a Hewlett-Packard 2100 A digital computer using a FORTRAN IV program[19,20] 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 concentrations. All replots were linear. Data fitting linear noncompetitive inhibition were fitted to eq 2.

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

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Catechol *O*-Methyltransferase. 3. Mechanism of Pyridoxal 5′-Phosphate Inhibition

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In an attempt to clarify the mechanism by which pyridoxal 5′-phosphate (PLP) inhibits the COMT-catalyzed O-methylation of *l*-norepinephrine (*l*-NE), enzyme kinetic studies on purified COMT and nonenzymatic studies have been carried out. Since difficulty was encountered in constructing linear reciprocal plots for PLP inhibition of COMT, nonenzymatic studies were carried out which revealed that a facile chemical reaction was taking place between *l*-NE and PLP resulting in the formation of a tetrahydroisoquinoline (**1**). Kinetic studies have established that the nonenzymatic reaction proceeds *via* rapid imine formation followed by a much slower ring closure step. This tetrahydroisoquinoline (**1**) was shown to account for the majority of the enzyme inhibition observed with PLP. Using substrates which are not capable of undergoing this side reaction, PLP was shown to be a very poor inhibitor of COMT, and its mechanism of inhibition was similar to that observed for tropolone and 8-hydroxyquinoline. Thus, it appears that the inhibition of COMT-catalyzed O-methylation of *l*-NE by PLP can be accounted for by three separate mechanisms: (1) removal of the substrate by a chemical reaction between *l*-NE and PLP; (2) production of a potent inhibitor **1**; and (3) weak inhibition by PLP itself.

Of possible significance relative to the nature of the active site of the enzyme catechol *O*-methyltransferase (COMT)[†] was the recent report[1] that pyridoxal 5′-phosphate (PLP) inhibited the COMT-catalyzed O-methylation of *l*-norepinephrine (*l*-NE). This was of interest because PLP is the prosthetic group of many aminotransferases, deaminases, and other enzymes concerned with reactions involving amino acids.[2] In these enzymes PLP is present as a Schiff base linked to the ε-amino group of a lysine residue.[3] PLP has also been widely used as a functional group reagent since it combines with lysine residues in a number of enzymes that do not require this coenzyme for catalytic activity, including AMP deaminase,[4] glutamic dehydrogenase,[5] rabbit muscle aldolase,[6] and phosphofructokinase.[7]

Interestingly, various PLP-dependent enzymes, including dopa decarboxylase,[8] tyrosine aminotransferase,[9,10] and pyridoxal kinase,[11] have been shown to be inhibited by catecholamines. This inhibition results from the reaction of PLP and the catecholamine resulting in the formation of tetrahydroisoquinoline adducts.[8,10] Inhibition is observed because of competition between the catecholamine and the enzyme for the coenzyme PLP.

In an attempt to determine if PLP is inhibiting COMT by combination with the enzyme or reaction with the substrate, an investigation of the mechanism of PLP inhibition of this enzyme was carried out. The present paper reports the results of this study.

Results and Discussion

Chemistry. Since difficulty was encountered in constructing linear reciprocal velocity *vs.* reciprocal substrate plots for PLP inhibition of COMT-catalyzed O-methylation of *l*-NE, spectrophotometric studies were carried out which revealed that a facile reaction was taking place between *l*-NE and PLP. Incubation of *l*-NE and PLP, under conditions approximating those of COMT assay, resulted in a rapid increase in absorbance at 328 nm and a simultaneous decrease at 388 nm. Figure 1 shows a plot of $A_{388\ nm}$ *vs.* time indicating that the reaction is 93% complete after 20 min at 37°. Other investigators studying the inhibition of catecholamines on PLP-dependent enzymes, including dopa decarboxylase[8] and tyrosine aminotrasferase,[9,10] have observed similar spectrophotometric changes and resulting product formation between PLP and catecholamines. Axelrod and Black[9] incorrectly proposed that the product was a reversible complex formed from two molecules of PLP and one molecule of the catecholamine. Later Fellman and Roth[10] studying DOPA inhibition of tyrosine aminotransferase showed that a reaction

[†]Abbreviations used are: SAM, *S*-adenosyl-L-methionine; DHA, 3,4-dihydroxyacetophenone; DHB, 3,4-dihydroxybenzoic acid; *l*-NE, *l*-norepinephrine; *l*-Epi, *l*-epinephrine; PLP, pyridoxal 5′-phosphate; COMT, catechol *O*-methyltransferase (E.C. 2.1.1.6); K_{is}, inhibition constant for the slope; K_{ii}, inhibition constant for the intercept.