from the Research Corporation. The excellent technical assistance of Sharon Elrod is gratefully acknowledged.

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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.

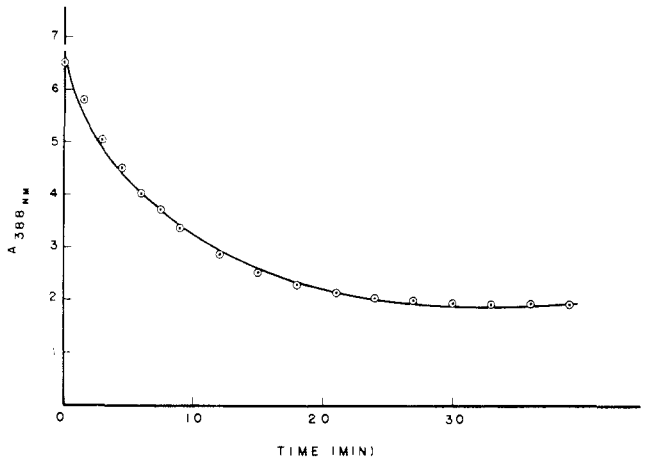
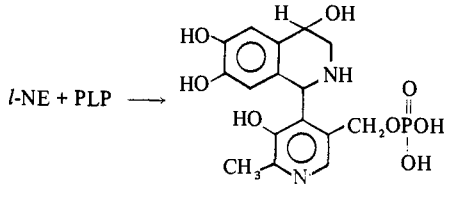


Figure 1. Time course for reaction of *l*-NE and PLP. Reaction conditions: *l*-NE concentration, 0.1 m*M*; PLP concentration, 0.1 m*M*. Phosphate buffer, 0.1 *M*; pH 7.6, 37°. Absorbance change measured at 388 nm.

between DOPA and PLP was taking place resulting in the formation of a tetrahydroisoquinoline adduct.

Similarly, we have observed that reaction of *l*-NE and PLP on a preparative scale results in formation of the tetrahydroisoquinoline (1). The ultraviolet absorption spectrum and extinction coefficients of the isolated product are consistent with the spectrophotometric changes observed on reaction of *l*-NE and PLP under conditions similar to those for COMT assay. Other spectral and elemental data are consistent with the proposed structure.



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Since the mechanism of this intramolecular condensation reaction is relevant to the overall question of COMT inhibition, a study was undertaken in an attempt to elucidate the kinetic mechanism. Spectrophotometric studies were carried out with *l*-NE in great excess over PLP and in this manner all reactions were pseudo first order. Figure 2 is a plot of k_{obsd} *vs.* [*l*-NE] at a series of pH values. From the results of Figure 2 it is apparent that (a) the overall rate of the reaction first increases with increases in [*l*-NE] and then becomes nearly independent of [*l*-NE] at its higher values; and (b) the initial increase in k_{obsd} as well as the value of k_{obsd} at high [*l*-NE] both increase with pH. These experimental results are similar to those of Bruice and Lombardo[12] for the reaction of histamine and 3-hydroxypyridine-4-aldehyde and are in accord with the mechanism of eq 1, where C and I represent carbinolamine and imine species, respectively.

$$\text{PLP} + l\text{-NE} \rightleftharpoons \text{C} \rightleftharpoons \text{I} \longrightarrow 1 \qquad (1)$$

The dependence of k_{obsd} upon [*l*-NE] at the latter's lower concentration may be attributed to the dependence of the equilibrium concentrations of C and I on [*l*-NE]. The lack of dependence of k_{obsd} upon [*l*-NE] at higher concentrations must be due to the fact that all PLP is in the form of C and I.

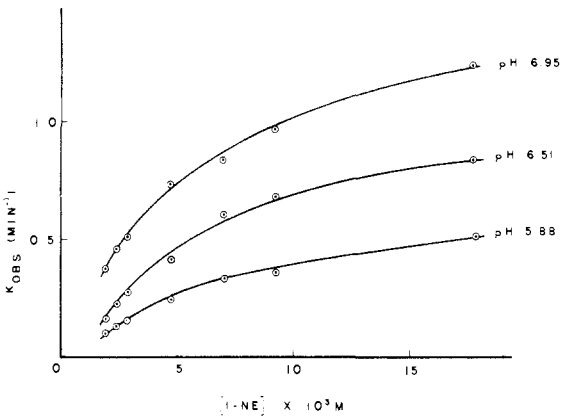


Figure 2. Dependence of the pseudo-first-order rate constant upon the concentration of *l*-NE at various pH values. Conditions are as described in the Experimental Section.

Table I. Inhibition of COMT by Tetrahydroisoquinoline (1) and Pyridoxal 5'-Phosphate[a]

Inhibitors[b]	Inhibitor concn, m*M*	% inhibition[c] *l*-Epi	*l*-NE	DHB
Tetrahydroisoquinoline (1)	0.2	52.3	33.4	11.1
	0.4	66.9	57.4	29.8
	0.8	85.8	82.8	53.0
	2.0	97.0	94.7	86.8
Pyridoxal 5'-phosphate	0.2	16.8		5.6
	0.4	19.5		11.9
	0.8	34.2		20.7
	2.0	54.3		36.6

[a] COMT was purified and assayed as described in the Experimental Section. SAM concentration, 1.0 m*M*; TES buffer concentration, 40 m*M*, pH 7.60; incubation time, 20 min. [b] Prepared as an aqueous stock solution of concentration 10 μmol/ml. [c] Expressed as per cent inhibition of O-methylation of the respective substrate.

Thus, it appears that the reaction between PLP and *l*-NE proceeds *via* rapid imine formation followed by a much slower ring-closure step. The formation of C, I, and tetrahydroisoquinoline (1) from the reaction of *l*-NE and PLP would account for the difficulties we observed in constructing linear reciprocal plots for the enzymatic data and render any such kinetic data, including that reported by Black,[1] invalid.

In Vitro Inhibition Studies. A large part of the inhibition of O-methylation of *l*-NE by PLP can be accounted for by the chemical reaction described in the previous section; however, it was still of interest to determine what effect the products of this chemical reaction have on COMT-catalyzed reactions, as well as what effect PLP itself has on the O-methylation of substrates that cannot undergo this reaction. Table I shows the degree of inhibition produced by tetrahydroisoquinoline (1) and PLP on the O-methylation of *l*-NE, *l*-Epi, and DHB. (PLP inhibition of COMT with *l*-NE as a substrate was not studied because of the complications presented by the condensation reaction.) From these data it can be readily seen that the tetrahydroisoquinoline (1) is a potent inhibitor of COMT. It should also be noted that this inhibition is much stronger than that produced by PLP. From these data it could be concluded that the majority of the inhibition observed by Black[1] with PLP was a result of (a) chemical reaction between PLP and *l*-NE resulting in removal of substrate; and (b) generation of a potent in-

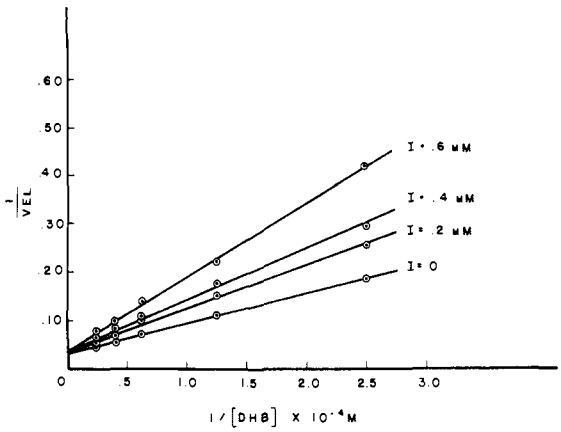


Figure 3. Reciprocal plots with DHB as the variable substrate and tetrahydroisoquinoline (1) as the inhibitor. Assay conditions as previously described.[14,15] Vel = nmol of product/mg of N Kjeldahl/min.

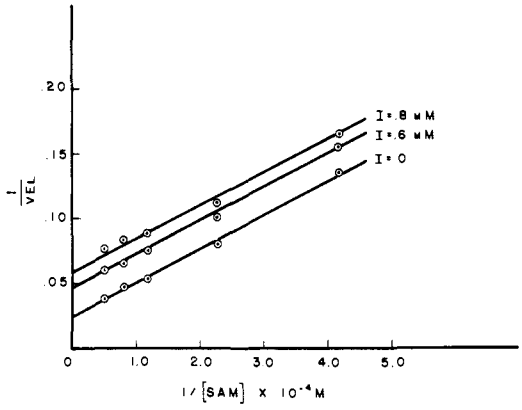


Figure 4. Reciprocal plots with SAM as the variable substrate and tetrahydroisoquinoline (1) as the inhibitor: SAM concentrations, 24–210 μM; DHB concentration, 2 mM. Vel = nmol of product/mg of N Kjeldahl/min.

hibitor in the form of the tetrahydroisoquinoline (**1**). Perhaps little of the inhibition observed by Black[1] could be accounted for by the PLP itself, since at the concentrations used the chemical reaction with *l*-NE would be nearly spontaneous.

Condensation product **1** could be classified as an alternate substrate type of COMT inhibitor. However, attempts to detect product formation with **1** were unsuccessful. Using various extraction media no detectable increase in extractable radioactive material was observed when **1** was incubated under conditions which were optimal for the O-methylation of DHB. Perhaps this can be accounted for by the high water solubility and low lipid solubility of the resulting O-methylated products.

Using reciprocal velocity *vs.* reciprocal substrate plots, the kinetic patterns for COMT inhibition by **1** were determined. As shown in Figure 3 a competitive pattern of inhibition was observed (K_{is} = 0.50 [±0.10] mM) when DHB was the variable substrate. If **1** is acting as an alternate substrate, it must be O-methylated very slowly, since accumulation of O-methylation products from **1** should result in noncompetitive kinetics as was the case observed with pyrogallol.[13] Evidence to indicate that **1** may be converted slowly to O-methylated products resulted from the observation that with poorer COMT substrates (*e.g.*, *l*-NE) we have observed noncompetitive inhibition patterns.

When SAM was the variable substrate, an uncompetitive

Table II. Role of Magnesium in the Inhibition of COMT by Pyridoxal 5'-Phosphate and Tetrahydroisoquinoline (**1**)[a]

	% inhibition[b]			
	Tetrahydroisoquinoline (**1**) concn		Pyridoxal 5'phosphate concn	
Magnesium concn, μM	1.0 mM	2.0 mM	2.0 mM	4.0 mM
4.8	22.5	62.7	48.2	71.3
12.0	27.5	68.7	46.1	69.9
48.0	36.3	76.7	48.3	71.7
120	42.0	82.0	47.8	72.5
240	50.0	82.2	47.1	73.4
480	56.0	84.0	46.5	71.4
1200	56.3	85.0	46.2	70.1

[a]COMT assay was same as those outlined in Table I except DHB concentration, 2.0 mM; incubation time, 5 min. [b]Expressed as per cent inhibition of the initial velocities for O-methylation of DHB.

Table III. Reduction of Pyridoxal 5'-Phosphate–COMT Complex[a]

DHB concn, mM	PLP concn, mM	$NaBH_4$ concn, M	Activity, cpm	% inhibition[b]
2.0			3266	
2.0	2.0		1992	39.0
2.0	2.0	0.2	3067	6.1

[a]Assay conditions: each reaction mixture was preincubated (37°, 10 min) in the absence of DHB. $NaBH_4$ was then added and incubated (0°, 20 min), after which time the reduced mixtures were assayed by adding DHB and incubating (10 min, 37°). [b]Expressed as per cent inhibition in absence of inhibitor.

pattern of inhibition was observed for **1** as shown in Figure 4. This is similar to the patterns observed with tropolones[14] and 8-hydroxyquinolines[15] and would indicate that **1** combines with a form of the enzyme after the addition of SAM.

The effect of Mg^{2+} concentration on the inhibition of COMT by **1** is summarized in Table II. As Mg^{2+} concentration is increased to an optimal concentration of 1.2 mM, inhibition with **1** increases. These observations are similar to those of Nikodejevic, *et al.*,[16] with regard to inhibition of COMT by 3,5-dihydroxy-4-methoxybenzoic acid. These data would suggest that inhibition by **1** does not result simply from chelation of free Mg^{2+}.

As shown in Table I, PLP produced substantial inhibition of the O-methylation of *l*-Epi and DHB. Since the possibility that PLP inhibition of COMT activity could result from formation of a Schiff base with a lysine group on COMT, an attempt was made to trap such an intermediate by reduction with $NaBH_4$.[4-7] Table III shows that $NaBH_4$ reduction of a mixture of COMT and PLP in the absence of substrate results in a decrease in inhibition. This decrease probably arises from reduction of the aldehyde group of PLP which is required for inhibition. These data would tend to rule out the formation of a Schiff base intermediate with enzyme.

Table IV shows the degree of COMT inhibition produced by a series of PLP derivatives. The key functional groups involved in PLP inhibition appear to be the hydroxyl and aldehyde part of the molecule, since salicylaldehyde and 3-hydroxypyridine-4-aldehyde produce inhibition similar to that of PLP. Pyridoxal itself is a poor inhibitor because under the assay conditions used it exists primarily in a hemiacetal form.[17]

Using reciprocal plots the kinetic patterns for PLP inhibition of COMT were determined. As shown in Figure 5 a linear noncompetitive pattern of inhibition was observed when DHB was the variable substrate (K_{is} = 0.85 [±0.18] mM K_{ii} = 1.76 [±0.34] mM). When SAM was the variable sub-

Table IV. Inhibition of COMT by Pyridoxal 5'-Phosphate Derivatives[a]

Inhibitor	% inhibition[c] $[I] = 2 \times 10^{-3} M$	$[I] = 8 \times 10^{-4} M$
Pyridoxal 5'-phosphate	36.6	20.7
Pyridoxal[b]	5.1	1.2
Pyridoxine[b]	2.7	0
Pyridoxamine[b]	0	0
3-Hydroxypyridine-4-aldehyde[b]	38.1	19.5
Salicylaldehyde	34.2	17.0

[a] Assay condition as outlined in Table I except DHB concentration, 2.0 m*M*; incubation time, 20 min. [b] HCl salt. [c] Expressed as per cent inhibition of O-methylation of DHB.

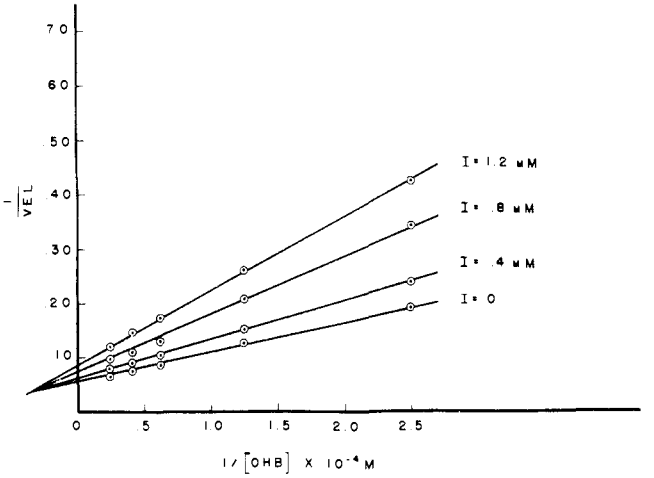


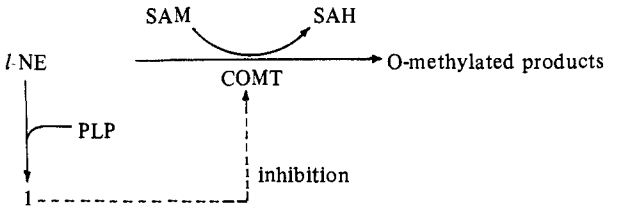
Figure 5. Reciprocal plots with DHB as the variable substrate and PLP as the inhibitor. Vel = nmol of product/mg of N Kjeldahl/min.

strate, an uncompetitive pattern of inhibition was observed as shown in Figure 6. The effect of Mg^{2+} concentration on COMT inhibition by PLP is summarized in Table II. As Mg^{2+} concentration increases the inhibition by PLP remains the same. All of these patterns are similar to those observed for tropolone[14] and 8-hydroxyquinoline[15] inhibition of COMT and indicate that PLP is inhibiting COMT by a similar mechanism and binding to similar sites.

Conclusions

The present paper has attempted to clarify the mechanism by which PLP inhibits COMT. The PLP inhibition of the O-methylation of *l*-NE was shown to result from (a) a facile chemical reaction between PLP and *l*-NE resulting in removal of substrate; and (b) generation of a potent inhibitor in the form of the tetrahydroisoquinoline (**1**, Scheme I). The reaction of *l*-NE and PLP was shown to be essentially spontaneous with initial formation of carbinolamine and imine intermediates with subsequent conversion to **1**. Thus, under conditions where PLP is in excess over *l*-NE (as those reported by Black[1] in his kinetic measurements), the conversion of *l*-NE to carbinolamine and imine would be nearly spontaneous leaving little or no original substrate.

Scheme I



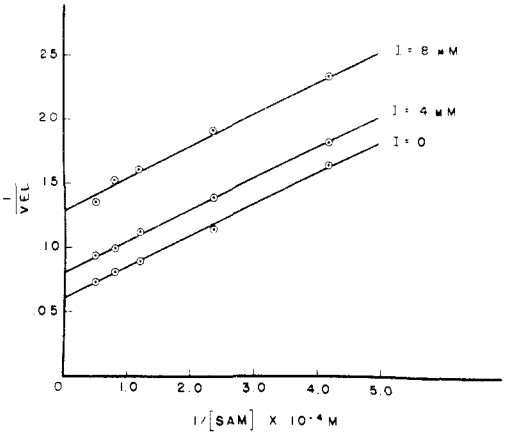


Figure 6. Reciprocal plots with SAM as the variable substrate and PLP as the inhibitor. Vel = nmol of product/mg of N Kjeldahl/min.

Inhibition of PLP-dependent enzymes[8-11] by catecholamines has previously been shown to proceed by a similar mechanism; however, to our knowledge this is the first example of PLP inhibition of an enzyme utilizing a catecholamine substrate in which the inhibition results from this dual mechanism.

For substrates not capable of undergoing the above-mentioned reaction, PLP inhibits their COMT-catalyzed O-methylation by a mechanism similar to that observed for tropolones[14] and 8-hydroxyquinolines.[15] There is no evidence to indicate the formation of a Schiff base between COMT and PLP. Inhibition appears to be simply a result of the binding of PLP to the same site as the substrate. PLP is another example of a COMT inhibitor whose effect results from substitution of a heteroatom or another functional group for one of the hydroxyls of the basic catechol system. The incorporation of an aldehyde in place of one of the hydroxyl groups (*e.g.*, salicylaldehyde) results in a compound which will bind to the substrate site but will not be converted to O-methylated products.

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. PLP (Sigma) was stored for no more than 2 days in the dark at −20° F as 10 μmol/ml of solution. The other compounds were commercially available.

Preparation of 4,6,7-Trihydroxy-1-(3'-hydroxy-5'-hydroxymethyl-2'-methyl-4'-pyridyl)-1,2,3,4-tetrahydroisoquinoline 5'-Phosphate (1). To *dl*-norepinephrine HCl (411 mg, 2.0 mmol) in 30 ml of H_2O was added pyridoxal 5'-phosphate (476 mg, 2.0 mmol) and the pH of the mixture adjusted to 7.0. The clear solution was warmed at 37° for 3 hr after which time the pH was adjusted to 5.0 and the solution concentrated to 5 ml under reduced pressure. The resulting solution was cooled overnight during which time needles of the tetrahydroisoquinoline (1) separated out. The product was recrystallized from H_2O to yield 625 mg (78%): mp 255–260° dec; nmr (DMSO-d_6) δ 8.02 (s, 1 H, C-6' CH), 7.02 and 6.91 (s, 2 H, C-5 and C-8 CH), 6.09 (s, 1 H, C-1 CH), 5.56 (m, 1 H, C-4 CH), 4.99 (m, 2 H, C-5' CH_2), 3.40 (m, 2 H, C-2 CH_2), 2.39 (s, 3 H, C-2' CH_3); uv (H_2O) λ_{max} 285 nm (ϵ 4010), 328 (7920). *Anal.* ($C_{16}H_{19}N_2O_8P \cdot 2H_2O$) C, H, N.

Kinetic Measurements. All kinetic runs were carried out at 37 ± 0.1° in glass distilled water at a calculated ionic strength of 0.3 under the pseudo-first-order conditions of a large excess of *l*-NE. Phosphate buffers were used and the pH of each solution was measured on a Corning Digital 110 pH meter.

The rates of cyclization were measured spectrophotometrically by following the decrease in absorbance at 388 nm, using a Gilford

Model 240 spectrophotometer equipped with an automatic sample-changing accessory. Reactions were initiated by adding a given amount of *l*-NE from a stock solution to a 2.5×10^{-4} *M* solution of PLP in the appropriate buffer and decreases in absorption at 388 nm were recorded continuously until no further change was observed. Pseudo-first-order rate constants were calculated on a Hewlett-Packard 2100A digital computer, using a program designed to calculate a least-squares evaluation of a plot of $\ln (A_{\text{Inf}} - A_o/A_{\text{Inf}} - A_T)$ *vs.* time. The correlation coefficients were usually greater than 0.999.

Purification and Assay of COMT. COMT was purified from rat liver (male, Sprague Dawley, 180–200 g) and assayed according to the methods previously described.[14,15] Kinetic data processing was also achieved as previously described.[14,15]

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Synthesis of New Bis(1-aziridinyl) Phosphinate Alkylating Agents Containing *O*-Phenyl *N*-Phenylcarbamate Side Chains†,[1]

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In view of the previously observed unusually high therapeutic indices of certain aromatic carbamate nitrogen mustards against the Walker 256 tumor, suggesting the possibility of selective uptake of these agents by the tumor cells, several new compounds were synthesized in which the same *O*-phenyl *N*-phenylcarbamate side chains were linked to bis(1-aziridinyl)- and bis(2,2-dimethyl-1-aziridinyl)phosphinyl alkylating groups. The syntheses of these compounds, requiring selective reactions of bifunctional molecules, are described. The biological test results do not show any increase in the therapeutic indices of these alkylating agents and thus do not support the suggested "selective carrier" action of the *O*-phenyl *N*-phenylcarbamate moiety.

In a previous study[2] of the structure-activity relationships of a variety of aromatic nitrogen mustards and aziridine-type alkylating agents, it was observed that the "carbamate mustards" (synthesized by Owens, *et al.*[3,4]) and the 2,2-dimethylaziridine derivatives (synthesized in our laboratory[5-7]) represented two striking classes of selective-acting alkylating agents. It was suggested that the unusually high therapeutic indices shown by the carbamate mustards against Walker carcinosarcoma 256 in rats may be due to the action of their *O*-phenyl *N*-phenylcarbamate moieties as "selective carrier" structures, capable of mediating preferential "uptake" of these cytotoxic agents by the tumor cells. On the other hand, the favorable antitumor spectra of the 2,2-dimethylaziridine derivatives have been attributed to the unique chemical reactivity pattern of their alkylating functions.[2,8-10]

It appeared, therefore, of interest to synthesize new compounds in which 2,2-dimethylaziridine-containing alkylating functions are linked to *O*-phenyl *N*-phenylcarbamate carrier structures similar to those of the "carbamate mustards," hoping that a combination of the favorable properties of both types of antitumor agents may be achieved. Furthermore, it was of interest to test our hypothesis concerning the extraordinary selectivity of the carbamate mustards against the Walker 256 tumor by synthesizing agents in which the same carrier structures are linked to an alkylating function other than nitrogen mustard. For the latter purpose, it seemed more appropriate to employ the ring-unsubstituted bis(1-aziridinyl)phosphinyl group as the alkylating function, since this is considerably more reactive with nucleophiles than the corresponding 2,2-dimethylaziridine derivative and is more similar in both structure and pharmacologic properties to the nitrogen mustard moiety.[8]

The present paper describes the successful syntheses of bis(1-aziridinyl)phosphinyl and bis(2,2-dimethyl-1-aziridinyl)phosphinyl analogs **4**, **5**, **15**, and **16** corresponding to both the "carbamate" and "reverse carbamate" series[2] of the aromatic carbamate nitrogen mustards and of two additional derivatives, **19** and **20**, in which the two phenyl groups of the side chain are linked by OCH_2 rather than carbamate moieties. The latter were designed to permit estimation of the "structurally nonspecific" component of the effect of the long lipophilic side chains on the activities of these agents. Finally, our unsuccessful attempts aimed at the syn-

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