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Pyridoxal-5′-phosphate—An inhibitor of catechol-*O*-methyltransferase *in vitro*

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NOREPINEPHRINE is enzymatically inactivated by *O*-methylation or by oxidative deamination. The former reaction is primarily extraneuronal and is catalysed by catechol-*O*-methyltransferase (COMT), a soluble magnesium-requiring enzyme which transfers a methyl group from *S*-adenosylmethionine to the catecholamine.[1] Several classes of synthetic COMT inhibitors have been characterized; substrates such as desmethylpapavarine,[2] *O*-dihydroxyphenylacetamides[3] and catechol[4] are associated with competitive inhibition of COMT. Pyrogallol[5] causes both competitive and noncompetitive[6] inhibition of the enzyme, whereas the tropolones[7] appear to inhibit COMT through chelation. Recent work has indicated that a class of compounds such as 3,5-dihydroxy-4-methoxy- and 3-hydroxy-4,5-dimethoxy-benzoic acids are associated with a mixed type of inhibition.[8]

The present communication describes inhibition of COMT *in vitro* by the naturally occurring vitamin, pyridoxal-5′-phosphate (PLP).

Female, Sprague–Dawley, 160–200 g rats were killed by a blow to the head and livers were rapidly removed and homogenized in 5 vol. of ice-cold isotonic potassium chloride solution. The homogenate was centrifuged for 30 min at 30,000 *g* in a Spinco model L centrifuge. The supernatant fraction was dialysed against 2000 vol. of 0·1 M phosphate buffer, pH 7·6, with one change over 12 hr between 0 and 4°. This enzyme preparation, with a protein concentration of approximately 25 mg/ml, was stored at −40° and was stable for weeks.

COMT was assayed by methods previously reported: 25 μl of enzyme preparation, 1 μmole magnesium chloride, 25 μl [^{14}C]-S-adenosylmethionine (specific activity of 48·5 μc/μmole at 5 μc/cm)[3] and varying amounts of norepinephrine and inhibitor were incubated for 30 min at 37° in 0·1 M phosphate buffer, pH 7·9, in a final volume of 1 ml. The reaction was terminated by addition of 0·5 ml of 0·5 M borate buffer, pH 10, and the [^{14}C]-normetanephrine formed was extracted into 10 ml of isoamyl alcohol. A 1-ml aliquot, transferred to a vial containing 1 ml ethanol and 10 ml phosphor, was counted by liquid scintillation spectroscopy in a Beckman LS250 liquid scintillation system with an efficiency for ^{14}C of approximately 75 per cent.

(−) Arterenol bitartrate (norepinephrine) and catechol were obtained from Calbiochem. Pyridoxal-5′-phosphate, pyridoxal hydrochloride, pyridoxamine dihydrochloride and pyridoxamine-5′-phosphate dihydrochloride were purchased from Sigma Chemical Company. Pyridoxine hydrochloride was obtained from Nutritional Biochemicals.

Pyridoxal-5′-phosphate, over the range of 10^{-5}M to 10^{-4}M, was associated with up to 90 per cent inhibition of COMT. This inhibition was competitive with the norepinephrine substrate with an "apparent"* K_i of $5{\cdot}39 \times 10^{-5}$M (the "apparent" K_m for norepinephrine was $1{\cdot}44 \times 10^{-4}$ M) determined by the method of Lineweaver and Burk[9] (Fig. 1). Such competitive inhibition could result from competition of PLP with norepinephrine for enzyme binding site(s) or, since PLP can form a complex with norepinephrine,[10] from competition of PLP with enzyme for the binding of norepinephrine. To investigate the latter possibility, catechol, which does not form a complex with PLP, was used as a substrate. PLP was equally as inhibitory using either catechol or norepinephrine as substrate, supporting the contention that the cofactor competes with substrate for enzyme sites. To confirm this conclusion, assays were performed to compare the inhibitory effect of PLP with and without preliminary incubation with the norepinephrine substrate for 1 hr. Complex formation involving PLP and norepinephrine increases in a hyperbolic manner with time, reaching an asymptotic plateau in approximately 1 hr.[10] Preincubation resulted in only a small increment of inhibitory activity (from 62 to 70 per cent), again indicating that direct interaction between PLP and norepinephrine is not necessary for COMT inhibition.

Magnesium ion concentration plays a critical, but complicated, role in PLP inhibition of COMT. At relatively low concentrations of magnesium (10^{-4} M), no inhibition is observed. As the metal ion concentration is increased to optimal concentrations of 10^{-3} M, PLP inhibition increases to a maximum and then again decreases as increasing magnesium itself inhibits COMT (Table 1). These observations are similar to those reported by Nikodejevic *et al.*[8] with regard to inhibition of COMT by 3,5-hydroxy-4-methoxy benzoic acid derivatives. The preservation of full enzyme activity at magnesium

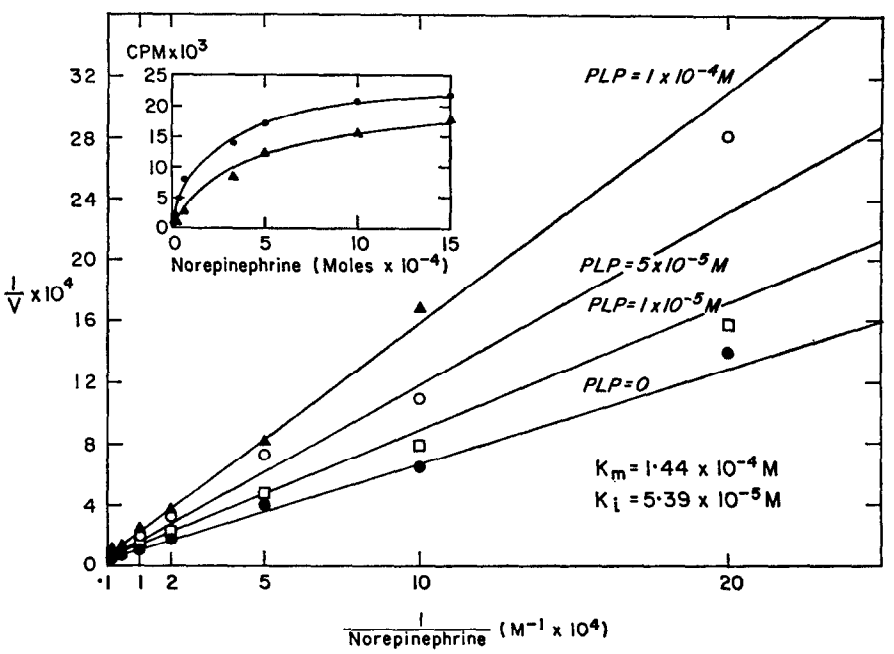

FIG. 1. Competitive inhibition of COMT by pyridoxal-5′-phosphate; double reciprocal plot. The data are plotted by the method of Lineweaver and Burk.[9] COMT assay was performed as described in the text in the presence of the indicated concentrations of pyridoxal-5′-phosphate and norepinephrine. Insert graph represents straight plot in the absence of inhibitor, and at pyridoxal phosphate concentrations of 1×10^{-4} M.

* Since assays were performed only with single concentrations of *S*-adenosylmethionine and magnesium, the term "apparent" is used.

concentrations equal to PLP concentration, the increasing inhibition at higher magnesium concentrations, and the inability of excess magnesium to overcome inhibition suggest that chelation by the vitamin is not a significant factor.

TABLE 1. INHIBITION OF COMT BY PYRIDOXAL-5′-PHOSPHATE: ROLE OF MAGNESIUM*

PLP concn (M)	Per cent inhibition			
	Magnesium chloride concn (M)			
	10^{-4}	10^{-3}	10^{-2}	10^{-1}
10^{-4}	0	36	13	15
5×10^{-5}	0	8	2	1

* COMT activity was assayed as indicated in the text at a norepinephrine concentration of 10^{-3} M. At each magnesium concentration, activity in the presence and absence of the indicated pyridoxal-5′-phosphate concentrations were compared to determine per cent inhibition.

The specificity of this inhibition was examined by comparing the reduction of enzyme activity by different analogues of vitamin B_6. Pyridoxal-5′-phosphate causes maximal inhibition. Removal of the 5′-phosphate markedly reduces inhibitory activity, as does reduction of the aldehyde group at position 4 (Table 2) when compared to *N*-substitution.

TABLE 2. SPECIFICITY OF COMT INHIBITION*

	Per cent inhibition	
	Inhibitor concn (M)	
	10^{-4}	5×10^{-5}
Pyridoxal-5′-phosphate	53	34
Pyridoxal hydrochloride	10	4
Pyridoxine hydrochloride	1	5
Pyridoxamine dihydrochloride	7	10
Pyridoxamine-5′-phosphate dihydrochloride	5	6

* Duplicate determinations of COMT activity were done as indicated in the text at a norepinephrine concentration of 10^{-4} M. Per cent inhibition was estimated by comparing enzyme activity in the presence of the indicated inhibitor concentrations to that in the absence of inhibitor.

The mechanism of inhibition has not been defined. Although competition of pyridoxal phosphate with norepinephrine for enzyme binding site(s) is suggested, the data are equally consistent with a mechanism of competition of pyridoxal phosphate with S-adenosylmethionine. It is of interest that pyridoxal-5′-phosphate, the catalytically active form of vitamin B_6, serves as an inhibitor of another enzyme, COMT. These findings may imply that vitamin coenzymes function as regulator molecules through enzyme inhibition as well as activation. However, preliminary studies indicate that COMT activity *in vivo* is not reduced by injection of pyridoxine hydrochloride.

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