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Phosphorylated Analogs of Vitamin B_6 Modified in the 5′ Position and on the Phosphate Group: Synthesis and Interaction with Pyridoxine Phosphate Oxidase and Certain Apoenzymes†

W. Korytnyk,* B. Lachmann, and N. Angelino

ABSTRACT: Representative analogs of pyridoxol-P and of pyridoxal-P have been synthesized and have been used as substrates or inhibitors of pyridoxine-P oxidase and as cofactor analogs, respectively. Pyridoxol-P and its analogs were prepared by condensation of isopropylidenepyridoxol or its 5-modified derivatives with cyanoethyl phosphate (or methylphosphonic acid) and subsequent removal of the blocking groups. Pyridoxine-P oxidase has been purified from rabbit liver by a simplified two-step chromatographic procedure and used for determination of the substrate and inhibitor activities of the analogs. Pyridoxol-P analogs in which the 5′ position was modified by extension (5′-homopyridoxol-P) and branching (5′-methylpyridoxol-P) were substrates of this enzyme, whereas replacement of a phosphate hydrogen with cyanoethyl, or of hydroxyl with methyl, abolished substrate activity. The corresponding series of pyridoxal-P analogs, obtained from the pyridoxol-P analogs by oxidation with MnO_2, were tested in three laboratories for ability to bind and activate various apoenzymes. Cofactor activity was found only in compounds with unsubstituted phosphate; but changes in the 5-methylene group also had effects, which varied with the apoenzyme studied. This structural requirement for coenzyme activity parallels to some extent the substrate specificity of the corresponding pyridoxol-P analogs with respect to pyridoxine-P oxidase. Most of the coenzymatically inactive pyridoxal-P analogs, however, were found to bind to the active sites of the apoenzymes, as was indicated by the characteristic shifts in the ultraviolet spectra.

Analogs of pyridoxal-P[1] have been used extensively as probes for the cofactor sites of a number of enzymes, such as certain transaminases, decarboxylases, and phosphorylases (Snell, 1971). Little is known about the specificity and the active sites of enzymes metabolizing vitamin B_6, such as pyridoxal phosphokinase and pyridoxine-P oxidase. The latter enzyme catalyzes the oxidation of pyridoxol-P and pyridoxamine-P to pyridoxal-P (Wada and Snell, 1961). In addition, it may regulate cellular levels of pyridoxal-P, since it is subject to considerable product inhibition (Snell and Haskell, 1971). To explore the substrate specificity of pyridoxine-P oxidase, we have simplified the earlier preparative method (Wada and Snell, 1961) and increased the purity of the enzyme.

Also, we have synthesized analogs of both pyridoxol-P and pyridoxal-P in which either the 5′-methylene or 5′-phosphate group is modified by procedures which have been applied for the first time in this area, and we have determined the substrate and inhibitor activities of the 4-alcohol analogs with this enzyme. A brief report of this study has been published

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[1] The nomenclature and abbreviations used were those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (see, *e.g., Biochem. J. 119*, 1 (1970)). The name of the enzyme pyridoxine-P oxidase has been retained as originally introduced by Wada and Snell (1961).

(Korytnyk *et al.*, 1971). Finally, we have supplied samples of the 4-aldehyde analogs to the laboratories of Drs. E. E. Snell, D. E. Metzler, and D. Graves for testing for binding and cofactor activity with the following apoenzymes: D-serine dehydratase, L-tryptophanase, L-aspartate aminotransferase, phosphorylase *b*, L-glutamic acid decarboxylase, and L-arginine decarboxylase. Both published and unpublished results from these laboratories have been summarized and compared.

Results and Discussion

Syntheses

Scheme I depicts the synthesis of pyridoxol phosphate (II) and its analogs (III, VI, IX, and XI in the middle column) as well as the pyridoxal phosphate analogs (IV, VII, X, and XII, in the right column) derived from the former by oxidation with MnO_2.[2]

The usual procedure for the synthesis of phosphorylated vitamin B_6 analogs involves protecting the aldehyde function by Schiff base formation, followed by phosphorylation of the 5-hydroxymethyl group with polyphosphoric acid (*e.g.*, Mühlradt *et al.*, 1967; Pocker and Fischer, 1969). This procedure was not readily applicable in our case, since we had encountered difficulties in obtaining 4-aldehydes of pyridoxol analogs in which the 5 position was modified, presumably because of ring–chain tautomerism (Korytnyk and Ahrens, 1971). Also, this procedure does not lend itself to the modification of the phosphate moiety.

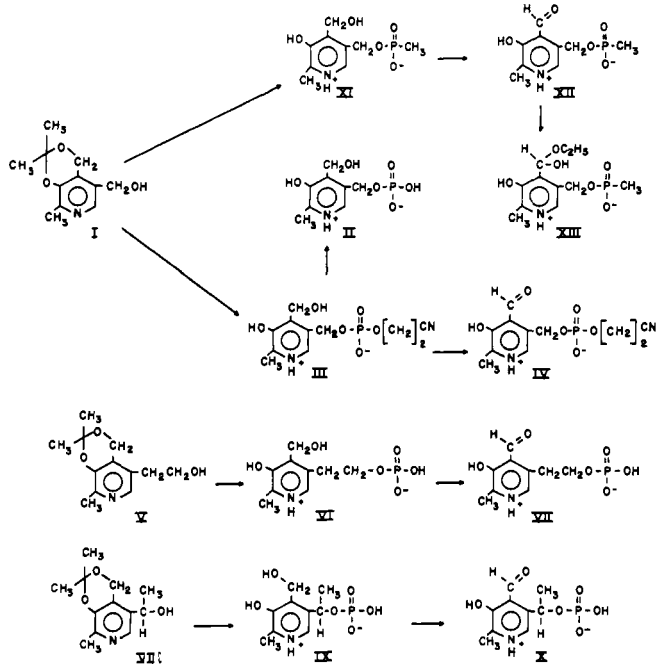
The change in strategy introduced here consists in delaying the formation of the aldehyde function until the last step. The readily available isopropylidenepyridoxol or one of its 5-modified analogs (Korytnyk *et al.*, 1967) was coupled with appropriate derivatives of phosphoric acid, using dicyclohexylcarbodiimide as the condensing agent under conditions similar to those used by Tener (1961) for blocked nucleosides. Hydrolytic removal of the isopropylidene group yielded the corresponding 5-phosphorylated analog, which was subsequently oxidized to the 4-aldehyde with MnO_2.

As Scheme I shows, isopropylidenepyridoxol (I) was condensed with cyanoethyl phosphate. The isopropylidene group was hydrolyzed with acid, and the cyanoethyl ester (III) was isolated. For the preparation of pyridoxol phosphate (II), removal of the two blocking groups was accomplished by carrying out acid and base hydrolysis in sequence. This method has been applied to the synthesis of doubly labeled pyridoxol-P (W. Korytnyk and S. C. Srivastava, unpublished results). The phosphorylated analogs were purified by ion-exchange chromatography, and characterized by ultraviolet (uv), infrared (ir), and nuclear magnetic resonance (nmr) spectroscopy (Table I). Attachment of the phosphate group to the 5-hydroxymethyl (or the modified 5-hydroxymethyl) was indicated by ^{31}P–5′-H splitting in the nmr spectra of the phosphates (Table I).

Although the phosphorylations of the 5-homo analog V and the 5′-methyl homolog VIII proceeded satisfactorily, considerable difficulties were encountered in the oxidation of pyridoxol phosphate analogs VI and IX to the corresponding aldehydes VII and X. A different method of oxidation had to be adopted for each compound individually after many unsuccessful trials.

Pyridoxol 5-methylphosphonate (XI) was obtained by condensation of methanephosphonic acid with isopropylidenepyridoxol (I) and subsequent hydrolysis. Additional confirmation of the structure was obtained by X-ray crystallography (Cole *et al.*, 1971).[3] The 4-aldehyde (XII) was obtained in good yield as a monohydrate. On crystallization from cold ethanol, it is converted to the monoethyl hemiacetal XIII.

SCHEME I



Enzymatic Studies with Pyridoxine-P Oxidase. PURIFICATION OF THE ENZYME AND SUBSTRATE ACTIVITY OF THE ANALOGS. Wada and Snell (1961; Wada, 1970) have purified pyridoxine-P oxidase approximately 65-fold from rabbit liver, treating with acid and heat and fractionating with ammonium sulfate, alumina Cγ gel, and DEAE-cellulose. We fractionated the liver extract first with Sephadex G-150 (Table II). The specific activity was increased only 5-fold. Next a DEAE-Sephadex fractionation gave a 31-fold increase in specific activity, and this fraction was used for testing substrate and inhibitor activities. No effort was made at optimizing recoveries, which netted 0.4% of the crude protein.

Michaelis constants were determined from Lineweaver–Burk plots, as shown in Figure 1. Only two pyridoxol-P analogs were found to be substrates of the enzyme: 5′-homopyridoxol-P (VI, $K_m = 0.59 \times 10^{-5}$ M) and 5′-methylpyridoxol-P (IX, $K_m = 3.10 \times 10^{-5}$ M). By comparing the values for these and pyridoxol-P (II, $K_m = 3.39 \times 10^{-5}$ M), we conclude that elongation of 5-side chain with one methylene group, as in homopyridoxol-P, increases the binding of the substrate, but "branching" by the 5′-methyl has little effect. Analogs III and XI, in which the phosphate group is modified, were found to be neither substrates nor inhibitors of the enzyme. The same applies to pyridoxol analogs in which the 5-side chain is similar, in space-filling properties, to the 5-methylene phosphate group: 5-$(CH_2)_2COCH_2Cl$, 5-$(CH_2)_2CO_2H$, and 5-$(CH_2)_2CH_2OH$ (prepared by Korytnyk, 1965, and Korytnyk and Lachmann, 1971). The 5-$(CH_2)_2CH_2OH$ analog, although inactive in regard to pyridoxine-P oxidase, was found to be a good substrate for the pyridoxine dehydrogenase of

[2] While this work was in progress, the synthesis of X by a different method was reported by Doktorova *et al.* (1969).

[3] To be published.

TABLE I: Uv and Nmr Spectra of Analogs.

Compound	Uv Spectra		Nmr Spectra				
	Solvent (N)	Max, nm (ε)	Solvent (N)	2-CH_3	4-CH_2OH or -CHO	C_6-H	Others
III	NaOH (0.1)	244 (6110) 310 (7070)	D_2O	159	303	495	5-CH_2OP, 308 (d, J = 8 cps) POC$H_2$$CH_2$CN, 237–254 (m) POC$H_2CH_2$CN, 171 (t, J = 6 cps)
IV	NaOH (0.1)	305 (1920) 388 (4940)	Me_2SO-d_6	146	625	487	5-CH_2OP, 312 (d, J = 8 cps) POC$H_2$$CH_2$CN, 230–247 (m) POC$H_2CH_2$CN, 160–190 (m)
VI	NaOH (0.1)	244 (5800) 308 (6900)	D_2O	157	304	488	5-C$H_2$$CH_2$OP, 188 (d, J = 6 cps) 5-CH_2CH_2OP, 238–258 (after decoupling: d, J = 7 cps)
VII	NaOH (0.1)	309 (1160) 392 (5700)	Me_2SO-d_6	145	628	484	5-CH_2CH_2OP: two broad peaks at 244 and 193
	HCl (0.1)	295 (5600) 340 (1800)					
	pH 7 (phosphate buffer)	327 (2700) 380 (3300)					
XI	NaOH (0.1)	242 (6270) 310 (6760)	D_2O	159	302	494	5-CH_2OP, 305 (d, J = 7 cps) PCH_3, 82 (d, J = 17 cps)
XII	NaOH (0.1)	305 (960) 390 (6110)	Me_2SO-d_6	145	728	486	5-CH_2OP, 314 (d, J = 6 cps) PCH_3, 78 (d, J = 16 cps)
IX	NaOH (0.1)	244 (6000) 309 (7000)	NaOD (1)	139	Obscured by HDO peak	463	5′-CH_3, 90 (d, J = 6 cps) 5′-H, 320 (m, partly obscured by HDO peak)
X	NaOH (0.1)	306 (1250) 392 (5940)	NaOD (1)	141	628	468	5′-CH_3, 86 (d, J = 6 cps) 5′-H, 330–365 (m)

yeast, which catalyzes the corresponding oxidation in unphosphorylated pyridoxol (W. Korytnyk and N. Angelino, unpublished results).

INTERACTION OF 4-ALDEHYDE ANALOGS WITH CERTAIN APOENZYMES. Attachment of pyridoxal-P to apoenzymes represents the next and final step of vitamin B_6 anabolism, and it was of interest to explore it by means of the pyridoxal-P analogs obtained in the present study. The interaction of pyridoxal-P and the 4-aldehyde analogs IV, VII, X, and XII with a series of apoenzymes (D-serine dehydratase, L-tryptophanase, L-aspartate aminotransferase, L-glutamic acid decarboxylase, and phosphorylase *b*) are summarized in Table III. These data were obtained in the laboratories of Drs. E. E. Snell, D. E. Metzler, and D. Graves, and some of them have been published (Dowhan and Snell, 1970; Fonda, 1971; Furbish *et al.*, 1969) and summarized by Snell (1971).

All four analogs satisfy the structural requirements for nonenzymatic catalytic activity as specified by Snell (1958), and yet only the two with unsubstituted phosphate (VII and X) can activate any of the enzymes. The highest activation (24%) was obtained with 5′-methylpyridoxal phosphate in apo-D-serine dehydratase. Usually the cofactor activity was well below 10%.

In addition to appreciable cofactor activities, these two analogs also show high binding constants. For apo-D-serine dehydratase, the binding of 5′-methylpyridoxal-P is as strong as that of pyridoxal-P. Substitutents on the phosphate lower the binding constants markedly, and this depression is associ-

TABLE II: Purification of Pyridoxine-P Oxidase.

Fraction No.	Vol (ml)	Protein[a] (mg/ml)	Sp Act.[b]	Yield (%)
1	75	46	3.9	100
2	68	6.02	20.7	11.9
3	40.8	0.352	656.0	0.42
4	15.2	0.572	728.0	0.25

[a] As determined by Lowry method. [b] Expressed as nmoles/mg of protein per hr.

ated with complete loss of cofactor activity. This finding parallels the structural requirements for substrate specificity of pyridoxol-P analogs in pyridoxine-P oxidases, which also appear to require unsubstituted phosphate analogs, as we have already shown above.

Almost all analogs, when added to the apoenzymes, shift the spectrum, because the aldehyde group of the analog forms an aldimine with the ϵ-amino group of lysine within the cofactor site (Table III). By this criterion, homopyridoxal-P (VII) and even the cyanoethyl derivative IV do bind to the cofactor sites of a number of apoenzymes. This finding indicates some bulk tolerance in the 5 position. Also, all analogs that have been tested bind to apophosphorylase *b* by the criterion of spectral shift; but in no instance does the binding, which is especially strong with 5′-methylpyridoxal-P, activate the enzyme.

Molecular models of 5′-methylpyridoxal-P and of its alcohol derivative show that introduction of the 5′-methyl group restricts freedom of rotation along the C^5–$C^{5'}$ bond, thus limiting the number of conformations that the molecule can assume. In addition, the molecule now has an asymmetric center, and can exist in both D and L forms. The DL form described in this study was found to have a cofactor activity that varies from 0 (phosphorylase *b*) to 24% (D-serine dehydratase). It has been postulated (Karpeisky and Ivanov, 1966) that "classical" pyridoxal-P catalysis requires rotation along the C^5–$C^{5'}$ bond during the process of catalysis, and it could be argued that the low cofactor activity of the analog is due to the interference of the 5′-methyl group with the freedom of rotation of this bond. Nevertheless, it is equally probable that the bulk of the 5′-methyl group prevents alignment of all groups necessary for catalysis to take place, and a combination of these two effects (*i.e.*, dynamic conformation and static bulk) may account for the reduced cofactor activity of this analog. The alcohol form of the 5′-methyl analog, however, is an excellent substrate for pyridoxine-P oxidase, and the steric factors just mentioned appear to play a subordinate role in determining substrate activity for this enzyme.

These findings again illustrate the diversity of the cofactor sites of pyridoxal-P-dependent enzymes, and the specificity of pyridoxine-P oxidase as it has been determined with the newly synthesized analogs.

Experimental Section

Materials and General Methods

Pyridoxol hydrochloride was a gift (Pfizer and Co.). Ir spectra were determined with a Perkin-Elmer 457 spectrometer, uv spectra with a Perkin-Elmer 202 spectrometer, and nmr spectra

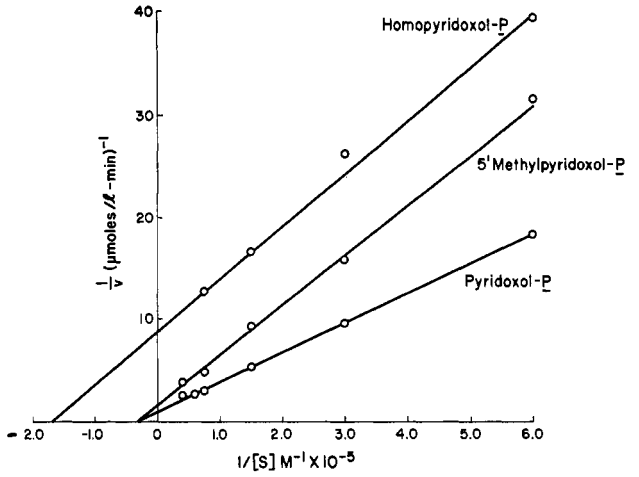


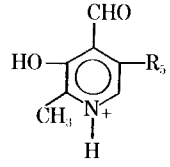
FIGURE 1: Lineweaver–Burk plots for substrate of pyridoxine-P oxidase.

with a Varian A-60A instrument. Positions of peaks are expressed in cycles per second from Me_4Si as internal standard. Assignments of peaks were made on the basis of previous work (Korytnyk and Ahrens, 1970). Thin-layer chromatography (tlc) was used routinely as described, and spots were detected either by spraying with Gibbs' reagent, through use of the phenylhydrazine reagent (Ahrens and Korytnyk, 1970), or both. An Amberlite CG-50 cation exchanger was used as described by Peterson *et al.* (1953).

Sephadex G-150 and DEAE-Sephadex A-50 were supplied by Pharmacia Fine Chemicals, Inc., and were prepared as recommended by the manufacturer, using an upward-flow technique. The columns were likewise supplied by the same company. Enzyme activity was determined by the method of Wada and Snell (1961), with only minor modifications. To the enzyme solution (0.2 ml of fraction 4 or a larger volume for less pure enzyme), pyridoxol-P (0.15 μmole) was added, and the solution was adjusted to 3.0 ml with 0.2 M pH 8.0 Tris buffer. Flasks were stoppered, and were incubated at 37° in a shaker bath for 30 min. The reaction was stopped by the addition of 0.3 ml of 100% (weight/volume) of trichloroacetic acid. After the precipitate was centrifuged out, 2.0 ml of the supernatant was diluted with 1.8 ml of water. Then 2 ml of phenylhydrazine solution (1.0 g of decolorized and recrystallized phenylhydrazine hydrochloride in 50 ml of 10 N H_2SO_4) was added, the solution was allowed to stand for 10 min at room temperature, and the absorption was determined at 410 mμ against an appropriately prepared blank.

A. Syntheses. PYRIDOXOL 5′-PHOSPHATE (II). α^4,3-*O*-Isopropylidenepyridoxol (I; 420 mg, 2 mmoles, prepared by the method of Korytnyk and Wiedeman, 1961) and 2-cyanoethyl phosphate (520 mg, 3.5 mmoles, prepared by the method of Tener, 1961) were dissolved in dry pyridine (8 ml). *N*,*N*′-Dicyclohexylcarbodiimide (1.25 g, 6 mmoles) was added, and the solution was stirred at room temperature for 5 hr, moisture being excluded (drying tube). Addition of 2 ml of water, with stirring for another hour, produced dicyclohexylurea, which was filtered off, and the filtrate was evaporated *in vacuo*. The residue was dissolved in acetonitrile, leaving additional undissolved dicyclohexylurea, which was filtered off, and the filtrate was evaporated. The oily reaction product was dissolved in water and extracted with $CHCl_3$ in order to remove any unreacted starting material. The aqueous layer was brought to pH 2 with 2 N HCl.

TABLE III: Interaction of Pyridoxal-P and Its Analogs with Apoenzymes.

Enzymes	PLP	PLP Analogs			
		XII, $R_5 = CH_2OP(O^-)CH_3$	IV, $R_5 = CH_2OPO(CH_2)_2CN$ (O⁻)	X, $R_5 = CH(CH_3)OPOH$ (O⁻)	VII, $R_5 = (CH_2)_2OPOH$ (O⁻)
Apo-D-serine dehydratase[a]	$K = 3.5 \times 10^{-8}$ M; shift in spectrum (aldimine formation)	$K = 1.9 \times 10^{-5}$ M; shift in spectrum; no cofactor activity	$K = 4.1 \times 10^{-6}$ M; shift in spectrum; slight cofactor activity due to hydrolysis	$K = 3.5 \times 10^{-8}$ M; 24% cofactor activity; shift in spectrum (aldimine formation)	$K = 5 \times 10^{-7}$ M; K_m for substrate: 2.5×10^{-3} M; 7.6% cofactor activity
Apotryptophanase[a]	$K = 7 \times 10^{-7}$ M; shift in spectrum	$K = 1.6 \times 10^{-3}$ M; no shift in spectrum; no cofactor activity	$K = 1.3 \times 10^{-3}$ M; no shift in spectrum; no cofactor activity	Not determined	Not determined
Apoaspartate aminotransferase[b]	Shift in spectrum	Shift in spectrum	Shift in spectrum; some cofactor activity due to hydrolysis	Shift in spectrum; 3% cofactor activity	Shift in spectrum; 1.3% cofactor activity
Apophosphorylase[c]		Shift in spectrum	Shift in spectrum	Shift in spectrum; inhibitor to the reconstitution of apoenzyme with PLP	Not determined
Apoglutamic acid decarboxylase[b]	Shift in spectrum	Apparently little binding	Apparently little binding	Shift in spectrum; 3% cofactor activity	Shift in spectrum; 5% of cofactor activity
Apo-L-arginine decarboxylase[a]	$K = 2.7 \times 10^{-8}$ M	Not determined	Not determined	Not determined	$K = 0.86 \times 10^{-6}$ M; 11% cofactor activity

[a] Dr. E. E. Snell, personal communication; activities of pyridoxal phosphates (PLP), XII, IV, and X in apo-D-serine dehydratase reported by Dowhan and Snell (1970). [b] Dr. D. E. Metzler, personal communication, and Furbish *et al.* (1969) and Fonda (1971). [c] Dr. D. Graves, personal communication. Apoenzyme was prepared according to Shaltiel *et al.* (1966).

After standing overnight, the solution was evaporated *in vacuo*, and the residue was dissolved in 1 N LiOH (20 ml) and was refluxed for 1 hr while the pH was maintained well above pH 10. The solution was filtered, evaporated to a small volume, and applied to a Bio-Rad AG50W X8 column in the H^+ form. Water eluted the phosphoric acid, and 2 N HCl the phosphorylated compound, which was contaminated with LiCl. Gibbs-positive fractions were combined, evaporated, and partially dissolved in ethyl alcohol. Undissolved LiCl was filtered off, evaporated, dissolved in a small amount of H_2O, and applied to an Amberlite CG-50 H^+ column, which was eluted with water. The Gibbs-positive fractions were combined (except the first fraction), filtered, evaporated *in vacuo*, and crystallized from MeOH. The yield of pyridoxol phosphate was 350 mg (70%).

PYRIDOXOL 2-CYANOETHYL 5′-PHOSPHATE (III). The procedure for pyridoxol 5′-phosphate was followed as described until the chloroform extraction step. The aqueous layer was kept at room temperature for 2 days to hydrolyze the isopropylidene group.

Water was removed *in vacuo*, and the oily residue was crystallized from a MeOH–EtOH mixture. The product, mp 188° dec, was obtained in 34% yield (300 mg from 630 mg of α^4,3-*O*-isopropylidenepyridoxol).

Anal. Calcd for $C_{11}H_{15}N_2O_6P$: C, 43.72; H, 5.00; N, 9.27. Found: C, 43.57; H, 5.14; N, 9.34.

PYRIDOXAL 2-CYANOETHYL 5′-PHOSPHATE (IV). To a solution of pyridoxol 2-cyanoethyl 5′-phosphate (100 mg, 0.33 mmole) in H_2O (2 ml) was added MnO_2 (300 mg, 3.3 mmoles, prepared according to the method of Mancera *et al.*, 1953), and the suspension was stirred at room temperature for 45 min. The MnO_2 was filtered (using Celite), and the filtrate was adjusted to pH 7.4 with Amberlite CG-50 (Na^+ form). After filtration and washing with water, the solution was applied to an Amberlite CG-50 column (H^+ form, Peterson *et al.*, 1953) and eluted with water. The fractions that contained the pure material (as determined by tlc) were combined and evaporated. The residue (70 mg, 70%) was a yellow powder, which analyzed as the monohydrate.

Anal. Calcd for $C_{11}H_{13}N_2O_6P \cdot H_2O$: C, 41.51; H, 4.75; N, 8.80. Found: C, 42.06; H, 5.12; N, 7.74.

1-C^5′-HOMOPYRIDOXOL PHOSPHATE (VI). The procedure followed was exactly as described for pyridoxol phosphate. Starting with 500 mg (2.2 mmoles) of V, 480 mg (82%) of VI was obtained.

Anal. Calcd for $C_9H_{14}NO_6P$: C, 41.07; H, 5.36; N, 5.32. Found: C, 41.26; H, 5.33; N, 5.03.

1-C^5′-HOMOPYRIDOXAL PHOSPHATE (VII). A solution of VI (150 mg, 0.51 mmole) in H_2O (4 ml) was stirred with MnO_2 (1.2 g, prepared according to the method of Harnfeist *et al.*, 1954) for 150 min at room temperature, light being excluded. After adjustment of the pH to 7.2 (Amberlite CG-50, Na^+ form), the solution was filtered (Celite filter aid), evaporated to a small volume, and applied to an Amberlite CG-50 (H^+) column. The column was eluted with water, and the uv spectra of the fractions were determined. Appropriate fractions were combined, filtered, and evaporated, and were coevaporated with H_2O. The solid yellow residue weighed 40 mg (26%) after drying.

Anal. Calcd for $C_9H_{14}NO_7P$: C, 38.72; H, 5.05; N, 5.02. Found: C, 38.40; H, 4.71; N, 4.88.

4′,3-*O*-ISOPROPYLIDENEPYRIDOXOL (VIII). α^4,3-*O*-Isopropylideneisopyridoxal (3.1 g, 15 mmoles, prepared according to the method of Korytnyk *et al.*, 1964) in Et_2O (10 ml) was added for 30 min to a stirred solution of MeMgCl (22 mmoles) in ether (20 ml). After being stirred for 15 min at room temperature, the solution was refluxed for 2 hr. The solution was poured into ice-cold ammonium chloride solution, and was extracted with ether. The ether solution was shaken with $NaHSO_3$ and $NaHCO_3$ solutions and with H_2O. Then the ether was evaporated, and the oil that remained was crystallized from ether–petroleum ether (bp 30–60°). The yield was 2.5 g (75%), mp 121° (Korytnyk *et al.*, 1967, reported mp 125–128°).

DL-5′-METHYLPYRIDOXOL 5′-PHOSPHATE (IX). Compound VIII (600 mg, 4 mmoles) was reacted with 2-cyanoethyl phosphate as has been described for the preparation of II. The product was purified by ion-exchange chromatography (Bio-Rad AG50W X8) and eluted with 0.5 N HCl. The fractions were combined, filtered, and evaporated. The oily residue was dissolved in a mixture of methanol and acetonitrile, yielding 230 mg of the product. The mother liquor was evaporated, taken up in H_2O, and rechromatographed, yielding an additional amount of material (80 mg); the total yield was 310 mg (48%).

Anal. Calcd for $C_9H_{14}NO_6P$: C, 41.07; H, 5.36; N, 5.32. Found: C, 40.79; H, 5.37; N, 5.21.

DL-5′-METHYLPYRIDOXAL PHOSPHATE (X). A solution of IX (90 mg, 0.35 mmoles) was dissolved in H_2O (10 ml) and stirred for 100 min with MnO_2 (800 mg, prepared according to the method of Harnfeist *et al.*, 1954). The pH of the reaction mixture was adjusted to 7.3 with Amberlite CG-50 (Na^+) resin, and was filtered (Celite filter aid). The filtrate was concentrated, and was chromatographed with an Amberlite CG-50 column (wrapped in black paper), as described earlier. The combined fractions were filtered and evaporated. The oily residue was heated with acetonitrile containing a few drops of H_2O. The oil changed to a yellow solid material (60 mg, 63%).

Anal. Calcd for $C_9H_{14}NO_7P$: C, 38.73; H, 5.05; N, 5.02. Found: C, 39.05; H, 5.03; N, 4.75.

PYRIDOXOL 5′-METHYLPHOSPHONATE (XI). Methanephosphonic acid was prepared as follows. To an aqueous solution of the barium salt of methylphosphonic acid (4.62 g, 20 mmoles; prepared according to the method of Holy, 1967), H^+ resin (Bio-Rad, AG50W X-8; 200–400 mesh) was added until the solution became clear. The solution was chromatographed with the same resin. Elution with water, and evaporation of combined fractions *in vacuo*, yielded 1.85 (96%) of methanephosphonic acid.

A solution of this acid in 20 ml of aqueous pyridine was prepared and was stored at 2°; 2.4 ml of this solution (250 mg, 2.6 mmoles) was coevaporated twice with pyridine and dissolved in pyridine (7 ml), and isopropylidenepyridoxol (I, 420 mg, 2 mmoles) and *N*,*N*′-dicyclohexylcarbodiimide (850 mg, 4 mmoles) were added. The reaction mixture was stirred at room temperature for 20 hr. Water (1 ml) was added, and stirring was continued for another hour. Dicyclohexylurea was filtered off, and the filtrate was evaporated *in vacuo*. The residue was taken up in a small amount of acetonitrile, and was filtered again to remove the urea derivative.

The filtrate was diluted with water, and was extracted three times with chloroform to remove any untreated starting material, and the aqueous solution was kept standing for 2 days at room temperature to hydrolyze the isopropylidene group. After evaporation, the oily residue was treated with acetonitrile, and the material crystallized, yielding 310 mg (63%) of XI. The compound was recrystallized from methanol; mp 188° dec.

Anal. Calcd for $C_9H_{14}NO_5P$: C, 43.73; H, 5.71; N, 5.67. Found: C, 43.44; H, 6.00; N, 5.74.

PYRIDOXAL 5′-METHYLPHOSPHONATE (XII). To a solution of XI (150 mg, 0.6 mmole) in water (4 ml), MnO_2 (540 mg, prepared as described by Mancera *et al.*, 1953) was added, and the mixture was stirred for 40 min at room temperature. After filtration (Celite filter aid), the pH of the filtrate was adjusted to 7.2 with Amberlite CG-50 (Na^+). After filtration, the solution was evaporated to a small volume, applied to an Amberlite CG-50 (H^+) column, and eluted with water. Fractions containing the pure aldehyde were combined, filtered, and evaporated *in vacuo*, yielding 120 mg (80%) of the aldehyde.

Anal. Calcd for $C_9H_{12}NO_5P \cdot H_2O$: C, 41.07; H, 5.36; N, 5.32. Found: C, 41.56; H, 5.00; N, 5.01.

The aldehyde just described, when dissolved in EtOH, gave a crystalline ethyl hemiacetal (XIII) in 50% yield (mp 121° dec).

Anal. Calcd for $C_{11}H_{18}NO_6P$: C, 45.37; H, 6.23; N, 4.89. Found: C, 45.10; H, 6.33; N, 4.62.

The hemiacetal was readily cleaved to the original aldehyde with dilute HCl.

B. Purification of Pyridoxine-P Oxidase. The livers of freshly slaughtered rabbits were cut into small pieces, which were kept frozen at −10 to −15° until used. Frozen rabbit liver (30 g) was thawed at 0–4°, and was homogenized with 90 ml of 0.01 M phosphate buffer (pH 8) for 5 min in a Waring Blendor. The homogenate was chilled in an ice bath for 30–40 min, and was centrifuged at 70,000*g* for 4 hr. The clear red supernatant was filtered through one layer of lintless cloth (Miracloth) to remove large particles of fatty material (fraction 1, Table I). A Sephadex G-150 column (bed volume was 5 × 73 cm) was prepared, using 0.2 M pH 8.0 phosphate buffer as the eluent. Approximately 75 ml of fraction 1 was applied. The column was run at an operating pressure of approximately 15 cm for about 2 days, and 5-ml fractions were collected. Fractions with the highest enzyme activity were dark red. They were combined (fraction 2), and were brought to 80% saturation with solid ammonium sulfate. After stirring at 0° for 30 min, the precipitate was collected by centrifugation, and was resuspended in 0.2 M pH 8.0 phosphate buffer, giving a total volume of 12 ml. This solution was dialyzed overnight against 2 l. of 0.2 M pH 8.0 phosphate buffer, and was filtered through cloth.

An ion-exchange column (DEAE-Sephadex A50, bed volume 2.5 × 92 cm) was prepared as recommended, using 0.2 M pH 8.0 phosphate buffer, which was also used as the eluent. The dark red solution from step 2 was applied to the column, and 3-ml fractions were collected for 1.5 days. The red fractions that came first were inactive, and only the later fractions, which were colorless, contained the enzyme. These fractions were combined (fraction 3), and were used without further purification for substrate activity determinations. A slight additional purification was achieved when the combined fractions were lyophilized to one-fourth to one-half of the original volume, dialyzed against 3 l. of 0.2 M phosphate buffer (pH 8.0), and finally filtered through a small piece of cloth (fraction 4).

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