Vitamin B_6 Analogs. Synthesis and Biological Activity of Homologs of Pyridoxal 5′-Phosphate[1]

PETER F. MÜHLRADT, Y. MORINO, AND ESMOND E. SNELL[2]

Department of Biochemistry, University of California, Berkeley, California 94720

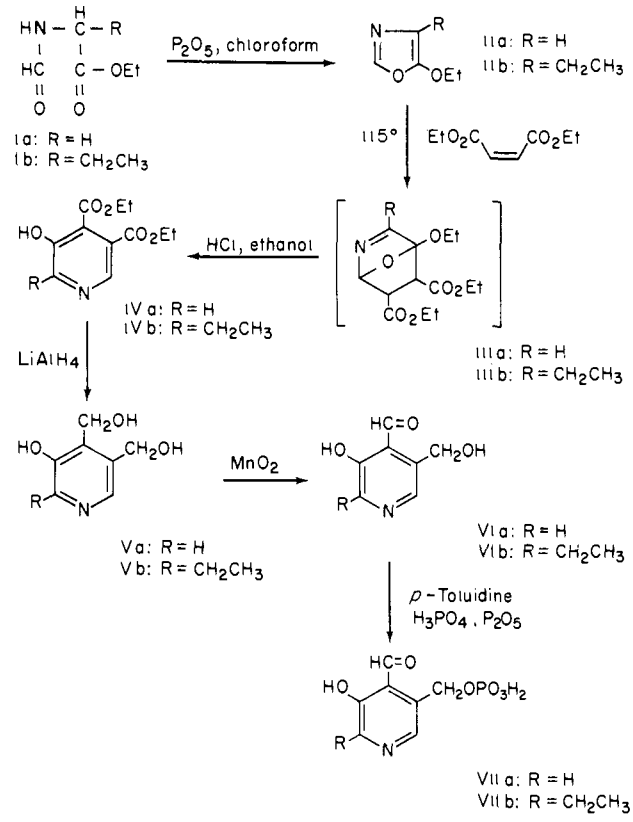
Received December 17, 1966

The synthesis from acyclic precursors of norpyridoxal 5′-phosphate (VIIa) and ω-methylpyridoxal 5′-phosphate (VIIb), compounds in which the methyl group at position 2 of pyridoxal 5′-phosphate (PLP) has been replaced by H or C_2H_5, is described. Both compounds replace PLP as a coenzyme for purified glutamate–oxaloacetate apotransaminase (GOT) of pig heart and for crystalline apotryptophanase (TPase) from *Escherichia coli*, but with varying effectiveness. VIIa is a more efficient coenzyme than PLP for GOT, as judged either by its affinity for the apoenzyme, or the maximum velocity of the reaction catalyzed by the reconstituted enzyme; VIIb is less effective than PLP for both criteria. Both VIIa and VIIb are less effective than PLP as coenzymes for TPase. The results show that the methyl group of PLP is not a prerequisite for the coenzymatic activity of this compound.

For a closer assessment of the relation of structure to function of vitamin B_6, a comparative study of the chemical and enzymological properties of analogs of pyridoxal phosphate in which the methyl group at position 2 is replaced by hydrogen or by an ethyl group is important. The latter compound has been synthesized[3] but is not currently available; the former compound has not been made. Both of the corresponding analogs of pyridoxine, 3-hydroxy-4,5-bis(hydroxymethyl)pyridine[4,5] and 2-ethyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine,[6] have been previously synthesized but only from difficultly available starting materials or in poor over-all yield. To obtain these compounds in sufficient amounts for subsequent synthesis of the corresponding analogs of pyridoxal phosphate, their synthesis *via* the more recently developed oxazole pathway[7,8] was undertaken.

The appropriate N-formyl-α-amino acid esters Ia and Ib (Scheme I) were prepared by formylation of the corresponding amino acid esters (Sheehan and Yang[9]). Formylation of the ester hydrochlorides resulted in lower yields. Cyclization of Ib with P_2O_5 in chloroform by the procedure of Karrer, *et al.*,[10] gave the oxazole IIb in 47% yield, while IIa could be prepared from Ia in only 3–5% yield under the same conditions.[11] Altered reaction conditions (see Experimental Section) gave no better results. The 5-ethoxyoxazoles IIa and IIb underwent Diels–Alder condensation with diethyl maleate to give the bicyclic compounds III, which were not isolated but were converted to the diesters IVa or IVb, respectively, with ethanolic HCl. IVa was isolated as its hydrochloride in 53% yield and IVb as the free ester in 51% yield. Reduction of the free diesters with $LiAlH_4$[12] gave the pyridoxine analogs Va and Vb which were isolated as their hydrochlorides in 30 and 68% yield, respectively. Oxidation of Va and Vb with manganese dioxide gave the corresponding analogs of pyridoxal VIa and VIb which were converted by phosphorylation of an appropriate Schiff base[13] to the corresponding analogs of pyridoxal phosphate VIIa and VIIb.

SCHEME I



Previous studies reviewed elsewhere[14] have shown that compound Vb (ω-methylpyridoxine) does not replace pyridoxine as a vitamin for *Ceratostomella ulmi* and *Saccharomyces carlsbergensis* or for rats depleted of vitamin B_6 prior to testing the analog, but acts instead as a competitive antagonist of pyridoxine. However,

(1) Supported in part by a grant (AM-1575) from the U. S. Public Health Service.

(2) To whom inquiries should be addressed.

(3) M. Ikawa and E. E. Snell, *J. Am. Chem. Soc.*, **76**, 637 (1954).

(4) B. Van der Wal, Th. J. de Boer, and H. O. Huisman, *Rec. Trav. Chim.*, **80**, 203 (1961).

(5) S. M. Gadekar, J. L. Frederick, and E. C. de Renzo, *J. Med. Pharm. Chem.*, **5**, 531 (1962).

(6) S. A. Harris and A. N. Wilson, *J. Am. Chem. Soc.*, **63**, 2526 (1941).

(7) G. Ya. Kondratyeve and C. Huang, *Dokl. Akad. Nauk. SSSR*, **141**, 628, 861 (1961).

(8) E. E. Harris, R. A. Firestone, K. Pfister, 3rd, R. R. Boettcher, F. J. Cross, R. B. Currie, M. Monaco, E. R. Peterson, and W. Reuter, *J. Org. Chem.*, **27**, 2705 (1962).

(9) T. C. Sheehan and D. H. Yang, *J. Am. Chem. Soc.*, **80**, 1154 (1958).

(10) P. Karrer, E. Myamichi, H. C. Storm, and R. Widmer, *Helv. Chim. Acta*, **8**, 205 (1925).

(11) This is another example of the lower rate of formation and lower stability of less substituted ring compounds, as compared with alkyl substituted ones. For further examples, see L. Eliel, "Stereochemistry of Carbon Compounds," McGraw Hill Book Co., Inc., New York N. Y., 1962 ,p 196.

(12) R. G. Jones and E. C. Kornfeld, *J. Am. Chem. Soc.*, **73**, 107 (1951).

(13) M. Murakami, M. Iwanami, and T. Numata, Japanese Patent 18,749 (1965); *Chem. Abstr.*, **56**, 2069 (1966).

(14) E. E. Snell, *Vitamins Hormones*, **16**, 77 (1958).

this same compound partially replaces pyridoxine as a growth factor for tomato roots, and decreases the requirement of weanling rats for vitamin B_6 for several weeks before it becomes toxic.[15] Compound VIb, ω-methylpyridoxal, replaces pyridoxal completely for growth of lactic acid bacteria under some conditions, and acts as a vitamin B_6 antagonist under other conditions.[16] These varied activities result from the fact that ω-methylpyridoxal phosphate (VIIb) fully or partially replaces pyridoxal phosphate as a coenzyme for certain enzymes (*e.g.*, the phenylalanine α-ketoglutarate and the leucine α-ketoglutarate transaminases of *Streptococcus faecalis*, and the glutamate–oxaloacetate transaminase of rat liver) but not for others (*e.g.*, the cysteine desulfhydrase of *S. faecalis*), and thus can support or inhibit growth depending upon the growth-limiting reactions it must subserve.[16]

Compound Va (norpyridoxine) has been tested previously for its ability to replace pyridoxine as a growth factor for vitamin B_6 deficient rats[17] and for the yeast, *S. carlsbergensis*.[5,17] Like ω-methylpyridoxine, norpyridoxine is inactive for both organisms, and even at high levels does not act as a vitamin antagonist for the latter organism.[17] In striking contrast to the inactivity of norpyridoxine as a vitamin for these organisms, norpyridoxal phosphate (VIIa) was found (Table I) to be even more effective than pyridoxal phosphate as a coenzyme for the extramitochondrial glutamate–oxaloacetate transaminase of pig heart muscle, as judged either by its affinity for the apoenzyme, or the maximum velocity of the reaction catalyzed. It also

Table I

Comparative Coenzymatic Activities of Pyridoxal Phosphate, Norpyridoxal Phosphate, and ω-Methylpyridoxal Phosphate

Coenzyme	Glutamate–oxaloacetate apotransaminase		Apotryptophanase	
	K_m[a] μ*M*	Velocity[b]	K_m[a] μ*M*	Velocity[b]
Pyridoxal phosphate	0.15	1.0	0.71	1.0
Norpyridoxal phosphate	0.077	2.5	3.8	0.76
ω-Methylpyridoxal phosphate	0.15	0.35	11.0	0.30

[a] K_m is defined as the concentration of pyridoxal phosphate or its analogs required to yield half-maximum activity in the test system following a 15-min preincubation period with the apoenzyme. [b] The figures represent the ratio of the maximum velocity (V_m) of the reaction obtained with the specified coenzyme to that obtained with pyridoxal phosphate (PLP). V_m for PLP plus glutamate–oxaloacetate apotransaminase was 160 μmoles of oxaloacetate formed/min/mg of enzyme; for PLP plus apotryptophanase it was 25 μmoles of indole produced/min/mg of enzyme.

replaces pyridoxal phosphate as a coenzyme for the tryptophanase of *Escherichia coli*, but in this capacity is somewhat less active than the naturally occurring coenzyme. ω-Methylpyridoxal phosphate (VIIb) also activates these two enzymes but is less effective than pyridoxal phosphate (Table I).

The dispensability of the methyl group of pyridoxal for nonenzymatic model reactions that simulate the corresponding enzymatic reactions for which pyridoxal phosphate is required had been previously noted.[14,15] It is clear from the present results that the methyl group of pyridoxal phosphate likewise is not essential for coenzymatic activity, since coenzyme analogs in which this group is replaced by either hydrogen or an ethyl group retain coenzymatic activity for many apoenzymes. Modification of this group does, however, affect the shape of the coenzyme and hence its affinity for the apoenzyme. As shown earlier[16] with ω-methylpyridoxal phosphate the resulting "analog holoenzyme," for steric or other reasons, may likewise show changed affinities for its substrates.

Experimental Section

Melting points were taken on a Koller hot stage microscope and are uncorrected. Nmr spectra were determined on a Varian Associates A-60 nmr spectrometer. The chemical shifts are reported in δ values in parts per million with the Me_4Si signal as internal reference at 0 ppm. Ultraviolet spectra were taken on a Cary Model 14 spectrophotometer.

N-Formylamino Acid Esters Ia and Ib.—Free glycine ethyl ester was prepared from its hydrochloride and immediately formylated.[9] The over-all yield of N-formylglycine ethyl ester [Ia, bp 125° (2.8 mm)[18]] was 85%. Ethyl DL-α-aminobutyrate hydrochloride [mp 144–147°], prepared from α-aminobutyric acid[19] in 98% yield, was converted to the free ester [bp 166–167° (760 mm), yield 85%] and then to the N-formyl derivative [Ib, bp 114° (2.0 mm),[20] yield 80%] by these same procedures.

Anal. Calcd for $C_7H_{13}NO_3$: C, 52.82; H, 8.23; N, 8.80. Found: C, 52.81; H, 8.45; N, 8.76.

Oxazoles IIa and IIb. A. 5-Ethoxyoxazole (IIa).—P_2O_5 (30 g) was suspended in 500 ml of chloroform that had been freshly distilled over P_2O_5. To the refluxing suspension 38 g of Ia was added during a period of 30 min. After refluxing an additional 30 min, an additional 15 g of P_2O_5 was introduced; and after 30 min a second 15-g portion of P_2O_5 was added. The suspension was refluxed for a total of 5 hr, then allowed to cool to room temperature. The chloroform was decanted and discarded since it did not contain appreciable IIa.[21] The lumpy dark brown residue was dissolved by adding ice and solid Na_2CO_3 alternately so that the temperature did not rise above 5° and the pH remained at about 5. The resulting solution (about 400 ml) was extracted once with 300 ml and twice with 200 ml of ether; the extracts were dried (Na_2SO_4) and decanted, and the ether was evaporated under slightly reduced pressure. The residue was distilled under reduced pressure, the receiver being cooled by a Dry Ice–acetone mixture. For analysis, a small sample was purified by gas chromatography at 150° over a 3.90-m column containing 10% Carbowax 20 M on Chromosorb W,[22] with He as carrier: $\lambda_{max}^{95\%\ EtOH}$ 225 mμ (ε 5470); nmr spectrum (in $CDCl_3$), 3 protons as triplet at 1.44 ppm, 2 protons as quadruplet at 4.25 ppm, 1 proton as singlet at 6.30, and another as singlet at 7.26 ppm.

Anal. Calcd for $C_5H_7NO_2$: C, 53.09; H, 6.29; N, 12.38. Found: C, 52.93; H, 6.34; N, 12.50.

The yield of IIa was not improved by variations in reflux time between 1.25 and 6 hr or by conducting the reaction at room temperature for 3 days instead of at reflux temperatures. When a 2% solution of Ia in chloroform containing 10% of $SOCl_2$ or $POCl_3$ was refluxed for 3 hr, no IIa was detected in the reaction mixture.[23]

(15) B. Sandman and E. E. Snell, *Proc. Soc. Exptl. Biol. Med.*, **90**, 63 (1955).

(16) J. Olivard and E. E. Snell, *J. Biol. Chem.*, **213**, 203, 215 (1955).

(17) G. Pol and H. J. Klein Obbink, *Rec. Trav. Chim.*, **80**, 217 (1961).

(18) D. E. Metzler, M. Ikawa, and E. E. Snell, *J. Am. Chem. Soc.*, **76**, 648 (1954).

(19) E. Fischer, *Ber.*, **34**, 433 (1901).

(20) R. G. Jones, *J. Am. Chem. Soc.*, **71**, 651 (1949), reports bp 110° (3 mm) for this product.

(21) Reaction mixtures were tested for the presence of IIa as follows. When P_2O_5 was the cyclizing agent, the $CHCl_3$ was decanted and P_2O_5 was decomposed with cold 1 *N* NaOH or Na_2CO_3. The alkaline solution was extracted with ether, the ether was removed *in vacuo*, and the amount of IIa in the residue was estimated spectrophotometrically (λ_{max} 225 mμ) in 95% ethanol. When the cyclizing agents were soluble in chloroform, $CHCl_3$ was removed from an aliquot *in vacuo* without heating, and the residue was dissolved in 1 *N* NaOH or Na_2CO_3 and then treated with ether as described above.

(22) We thank Dr. Ellen Gorenchaik for this operation.

TABLE II

ABSORPTION MAXIMA AND MOLAR ABSORBANCE OF ANALOGS OF PYRIDOXAL AND PYRIDOXAL PHOSPHATE[a]

Compound	λ_{max}, mμ (ε) 0.1 N HCl	pH 7.0[b]	0.1 N NaOH	In acid phenylhydrazine[c]
ω-Methylpyridoxal (VIb)	289 (8900)	318 (8400)	239 (8300) 300 (5700) 392 (1800)	410 (21,500)
Pyridoxal	288 (9100)	317 (8800)	240 (8300) 300 (6100) 390 (1700)	410 (23,000)
Norpyridoxal (VIa)	283 (6400)	313 (4200)	241 (8900) 299 (5000) 383 (500)	415 (18,500)
ω-Methylpyridoxal 5′-phosphate (VIIb)[a]	295 (8200) 335 (1500)	389 (5800)	390 (6900)	410 (24,000)
Pyridoxal 5′-phosphate[a]	293 (7200) 334 (1300)	388 (5500)	389 (6600)	410 (24,500)
Norpyridoxal 5′-phosphate (VIIa)[a]	290 (4700) 328 (900)	381 (2900)	382 (4300)	415 (21,000)

[a] Quantitative determinations of organic phosphate showed 99% of the theoretical for pyridoxal phosphate, 98% for ω-methylpyridoxal phosphate, and 83% for norpyridoxal phosphate. The true molar absorbancies of the latter compound, therefore, may be somewhat higher than those tabulated. [b] In 0.1 *M* potassium phosphate buffer. [c] Procedure of Wada and Snell[25] for determination of pyridoxal and pyridoxal phosphate.

B. 4-Ethyl-5-ethoxyoxazole (IIb).—P_2O_5 (100 g) was suspended in 1500 ml of freshly distilled, dry $CHCl_3$. To the refluxing suspension, 102.7 g of Ib was added during a period of 1 hr. One hour later a 50-g portion of P_2O_5 was introduced, and after 2 hr another 50 g of P_2O_5 was added. The suspension was refluxed for a total of 5 hr. After standing overnight at room temperature, the chloroform was siphoned off from the insoluble lumps of P_2O_5, concentrated to 150 ml, and extracted with a little concentrated aqueous NaOH. The insoluble P_2O_5 was dissolved by slowly adding NaOH pellets and ice so that the temperature was kept at 0–5° and the pH at 3–5. After solution was complete, the NaOH extract of the $CHCl_3$ layer was added and the pH brought to 9. The alkaline solution was extracted once with 500 ml and three times with 300 ml of ether. The ether extracts were combined with the chloroform layer and concentrated at atmospheric pressure until the boiling point began to rise above 70°. The residue was distilled under vacuum; the oxazole IIb distilled at 71–73° (20 mm); yield 46.0 g (47%); d^{24} 1.002; $\lambda_{max}^{95\%\ EtOH}$ 228 mμ (ε 4160); nmr spectrum (no solvent), 6 protons as two superimposed triplets at 1.19 and 1.32 ppm, 2 protons as a quadruplet at 2.50 ppm, 2 protons as a quadruplet at 4.24 ppm, 1 proton as a singlet at 7.69 ppm.

Anal. Calcd for $C_7H_{11}NO_2$: C, 59.56; H, 7.85. Found: C, 59.63; H, 7.38.

Pyridinedicarboxylic Acid Esters IVa and IVb. A. Diethyl 3-Hydroxypyridine-4,5-dicarboxylate (IVa) Hydrochloride.—IIa (13.3 mmoles) was mixed with 2 equiv of diethyl maleate and heated in a round-bottom flask with an air-cooled condenser for 5 hr at 115°. After cooling, 13 ml of dry HCl (1.14 *N*) in absolute EtOH was added and the mixture was heated on a steam bath for 20 min. By cooling to −20° and adding ether, 1.03 g of IVa·HCl precipitated. From the mother liquor two further crops of 0.69 and 0.24 g could be collected; total yield, 1.95 g (53%). A small sample, recrystallized from ethanol for analysis, melted at 109–114°.

Anal. Calcd for $C_{11}H_{14}ClNO_5$: C, 47.91; H, 5.02; Cl, 12.86. Found: C, 46.97; H, 5.10; Cl, 13.80.

B. Diethyl 2-Ethyl-3-hydroxypyridine-4,5-dicarboxylate (IVb).—IIb (71.8 g) was mixed with 172 g (160 ml) of freshly distilled diethyl maleate and kept at 120° for 5 hr in a stoppered flask. After cooling, 130 ml of dry HCl (4 *N*) in absolute ethanol was added slowly. The temperature rose to 80° and was held there for 20 min. The mixture was cooled and extracted twice with 150 ml of water. The extracts were neutralized as soon as they were separated from the organic layer. The combined extracts were brought to pH 4 with a concentrated aqueous solution of metaphosphoric acid and the diester IVb was extracted twice with 250 ml and twice with 150 ml of a chloroform–ethanol (3:2) mixture. The extracts were dried (Na_2SO_4) then decanted, and the volatile solvents were removed *in vacuo*. The residue was fractionated in high vacuum, and the fraction boiling between 145 and 150° (1.6–1.7 mm) was collected; yield 69.5 g (51%), d^{25} 1.166, λ_{max}^{EtOH} 299 mμ (ε 5150).

Anal. Calcd for $C_{13}H_{17}NO_5$: C, 58.42; H, 6.41; N, 5.24. Found: C, 58.38; H, 6.38; N, 5.38.

Pyridoxine Analogs Va and Vb. A. 3-Hydroxy-4,5-bis(hydroxymethyl)pyridine (Va) Hydrochloride.—A solution of IVa·HCl (1.91 g) in 10 ml of water was adjusted to pH 6.5 with Na_2CO_3, then extracted four times with 15-ml portions of ether, the pH of the aqueous phase being adjusted after each extraction to 5–6.5 with 1 *M* H_3PO_4. The ether extracts were dried (Na_2SO_4), the ether was removed *in vacuo*, the residue was redissolved in a little chloroform, and the last traces of moisture were removed with the $CHCl_3$ by azeotropic distillation. The resulting crystalline free base IVa (1.52 g) was dissolved in 50 ml of absolute ether and added in a period of 10 min to a refluxing suspension of 1 g of $LiAlH_4$ in 200 ml of ether. The mixture was refluxed for 2 hr and then left at room temperature overnight. Excess $LiAlH_4$ was destroyed with ethyl acetate, 40 ml of a mixture of ice and water was added, and the ether was removed on a steam bath. The precipitate of $Al(OH)_3$ was filtered off and discarded after extensive washing with hot water. The filtrate was adjusted to pH 6 with CO_2 gas and evaporated to dryness *in vacuo*. It was redissolved in absolute ethanol–$CHCl_3$ (2:1) and the solvents were evaporated *in vacuo*. This procedure was repeated once. Material that was insoluble in the solvent mixture was discarded, and the filtrate was evaporated to a dry foam. Va could not be crystallized as the hydrochloride from ethanolic HCl and ether. The hydrochloride was reconverted to the free base by dissolving the syrup in a little water, adding $NaHCO_3$, and exhaustively extracting with absolute EtOH. The extracts were taken down to a thin syrup, which was applied to a column containing 45 g of SiO_2 (Merck, 0.05–0.02 mm). Va was eluted by 15–20% methanol in $CHCl_3$ and crystallized as the hydrochloride from ethanolic HCl and ether, then recrystallized from methanol–ether; yield 382 mg (29%), mp 133–135°.[23]

Anal. Calcd: C, 43.88; H, 5.26; N, 7.31. Found: C, 43.76; H, 5.25; N, 7.28.

B. 2-Ethyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine (Vb) Hydrochloride.—$LiAlH_4$ (10 g) was dissolved in 1500 ml of boiling anhydrous ether. IVb (12.9 g) diluted with 100 ml of ether was added during a period of 1 hr so that the mixture continued to reflux. After refluxing for 5 hr, the excess $LiAlH_4$ was carefully destroyed with 40 ml of ethyl acetate. Ice (300 g) was gradually added to the mixture, and the yellow precipitate was filtered off and discarded after washing four times with 200 ml of boiling water. The ether layer was discarded and the combined aqueous extracts were concentrated to 250 ml. CO_2 was bubbled through for 30 min and the solution was evaporated to dryness *in vacuo*. The residue was extracted once with 100 ml and again with 50 ml of boiling absolute ethanol. The extracts were evaporated to a syrup which crystallized readily on the addition of 250 ml of 4 *M* dry ethanolic HCl; yield 7.23 g (68%), mp 186–191°.

Pyridoxal Analogs VIa and VIb.—The preparation of 2-ethyl-3-

(23) Van der Wal, *et al.*,[4] report 130–135° dec.

hydroxy-4-formyl-5-hydroxymethylpyridine (ω-methylpyridoxal) (VIb) by oxidation of Vb with MnO_2 has been described.[3] 3-Hydroxy-4-formyl-5-hydroxymethylpyridine (norpyridoxal) (VIa), was prepared by an analogous procedure.[3,6] To 100 mg of Vb·HCl was added 2 ml of a suspension of washed MnO_2 prepared[24] from 78 mg of $KMnO_4$. The mixture was held at pH 3.0 by occasional additions of 10% H_2SO_4 (0.20 ml total). After 3 hr at room temperature it was heated at 50° for 7 min. $NaHCO_3$ was then added to pH 6.4, and the precipitate was discarded. Methanol (*ca.* 11 ml) was added and the precipitate was centrifuged out. The supernatant solution was concentrated to a small volume under nitrogen, then streaked onto thin layer plates of silica gel G (Merck). After development with $CHCl_3$–methanol (5:1), the yellow zone from each chromatogram was collected and extracted with methanol, and the methanol was evaporated under nitrogen. The crude, crystalline free base was recrystallized from acetone–HCl to yield 51 mg (5%) of VIa·HCl. The substance has no melting point but chars at 130°. Like pyridoxal, it readily forms an oxime. Its spectrum resembles those of pyridoxal and ω-methylpyridoxal, but the absorption maxima are shifted and show substantially different extinction values (Table II).

Pyridoxal Phosphate Analogs VIIa and VIIb.—ω-Methylpyridoxal phosphate (VIIb) has been prepared previously.[3] Norpyridoxal phosphate (VIIa) was prepared by phosphorylation of the Schiff's base formed between norpyridoxal and *p*-toluidine.[13] For this purpose, 155 mg of Va was oxidized with MnO_2 as described in the preceding section. After neutralization with $NaHCO_3$ and centrifugation, the supernatant solution was mixed with 120 mg of *p*-toluidine hydrochloride. The Schiff's base, which precipitates immediately, was dissolved in chloroform–ethanol (2:1), the solution was dried (Na_2SO_4), and the solvent was removed under vacuum. Recrystallization from methanol–$CHCl_3$ gave 60 mg of the crystalline, yellow Schiff's base which decomposed at 180°. This was mixed in a small centrifuge tube with 0.6 ml of a homogeneous mixture (1.1:1.0) of 85% H_3PO_4 and P_2O_5. The tube was stoppered tightly, and heated 5 hr at 45°. The resulting syrup was mixed with 0.6 ml of 0.1 *N* HCl, heated 15 min at 60° to hydrolyze pyrophosphates, and adjusted to pH 3.0 with 50% NaOH while cooling in ice. The thick precipitate which formed was collected by centrifugation, dissolved in cold 2 *N* NaOH and diluted to 20 ml with cold water. After extracting twice with 10 ml of chloroform–ethanol (2:1) to remove *p*-toluidine, the solution was adjusted to pH 5.5 with concentrated HCl then poured onto a column of the anion exchanger, Dowex 1-X8 (200–400 mesh), in its formate form.

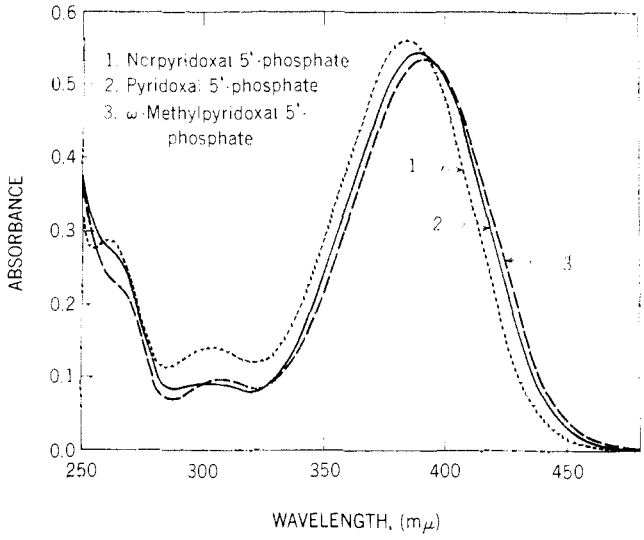


Figure 1.—Comparative spectra of pyridoxal phosphate and its analogs in 0.2 *N* KOH; pyridoxal phosphate and ω-methylpyridoxal phosphate, 1 μmole/ml. The sample of norpyridoxal phosphate showed approximately 0.7 the absorbance of pyridoxal phosphate at 390 mμ; its concentration was adjusted upward for comparative purposes.

The column was developed with 0.25 *N* formic acid; norpyridoxal phosphate is recognized by its absorption (in alkali) at 390 mμ, where neither Va nor VIa absorbs. Fractions containing it were concentrated by freeze drying to a syrup, from which crystalline norpyridoxal 5′-phosphate (VIIa) was obtained on trituration with ethanol; yield 14 mg. The spectrum of the product resembles that of pyridoxal phosphate and of ω-methylpyridoxal phosphate (Figure 1, Table II).

Enzymatic Testing Procedures.—Commercially available glutamate–oxaloacetate transaminase (Boehringer, 180 units/mg) was freed of its coenzyme by the procedure of Wada and Snell.[25] Crystalline apotryptophanase was prepared[26] from *E. coli* B/117-A. Both enzymes were incubated with the test coenzymes for 15 min prior to determining their activity by the cited procedures.[25,26] The results are shown in Table I.

(24) M. Visconti, C. Eberhart, and P. Karrer, *Helv. Chim. Acta*, **34**, 1831 (1951).

(25) H. Wada and E. E. Snell, *J. Biol. Chem.*, **237**, 127 (1962).

(26) W. A. Newton, Y. Morino, and E. E. Snell, *ibid.*, **240**, 1211 (1965).