# Synthesis of Analogs of Pyridoxal 5′-Phosphate*

Anna Pocker and Edmond H. Fischer

ABSTRACT: In an attempt to elucidate the role of pyridoxal 5′-phosphate in the structure and function of glycogen phosphorylase, a series of analogs of the cofactor, modified in various positions around the pyridine ring, have been synthesized. This paper reports the unambiguous synthesis of 3-*O*-methylpyridoxal, 3-*O*-methylpyridoxal oxime, 3-*O*-methylpyridoxamine, 3-*O*-methylpyridoxamine 5′-phosphate, 3-*O*-methylpyridoxal 5′-phosphate, *N*-methylpyridoxal oxime, *N*-methylpyridoxamine, *N*-methylpyridoxamine 5′-phosphate, *N*-methylpyridoxal 5′-phosphate, isopyridoxamine 4′-phosphate, and isopyridoxal 4′-phosphate. The chemical properties of these compounds including their spectral characteristics and p$K_a$ values are described. The following publication reports the enzymatic properties of these and other pyridoxal phosphate analogs in the reconstitution of muscle glycogen apophosphorylase.

The chemistry of vitamin $B_6$ and some of its phosphorylated derivatives has been the subject of continuous research directed toward the elucidation of the relationship between structure and biological activity of this class of compounds (Snell, 1958). Since PLP[1] has been found in all glycogen phosphorylases (EC 2.4.1.1) so far investigated, the question arises as to what role such a cofactor may play in glycolysis. This problem is also of importance to our understanding of the mode of action of other vitamin $B_6$ dependent systems. A number of synthetic analogs have already been examined in an effort to answer this question with respect to several PLP-containing enzymes (Illingworth *et al.*, 1958; Tomita *et al.*, 1966; Brooks *et al.*, 1966; Mühlradt *et al.*, 1967; Bocharov *et al.*, 1968; Fukui and Ohishi, 1969; Tate and Meister, 1969). In all instances, both the aldehyde and the 5′-hydroxymethyl phosphate groups appear to be involved in the binding of the cofactor to the protein and, therefore, are critical for enzymatic activity. In phosphorylase, however, the mode of action of the cofactor appears to be entirely different from that accepted for other classical PLP-containing enzymes: reduction of phosphorylase with sodium borohydride which covalently fixes the cofactor to the protein (and, therefore, irreversibly modifies the aldehyde group of PLP) leads to a molecule possessing 60% or more of maximum activity. All other usual PLP-containing enzymes in which the cofactor is directly involved in catalysis are totally inactivated by this procedure. Since apophosphorylase displays an exceptional affinity for PLP, trace contamination (1 part/100,000) of the analog by the unmodified coenzyme would lead to noticeable reactivations; therefore, particular care was exercised to ensure the purity of all the analogs described herein.

Pyridoxal was chosen as the starting material; however, direct phosphorylation of this compound was precluded since it readily forms a stable hemiacetal (Heyl *et al.*, 1951). 3′,4′-Isopropylidenepyridoxine (Korytnyk and Wiedeman, 1962) obviously could not serve as starting material for the synthesis of analogs having a blocked phenolic group, and, moreover, phosphorylation of the acetonide gives rise to products generally contaminated with small amounts of isomeric phosphates which are difficult to remove (Baddiley and Mathias, 1952). These considerations led us to follow routes similar to those of Ikawa and Snell (1954) and Iwanami *et al.* (1968), but with a few modifications (see Scheme I).

A synthesis of *N*-MePLP was reported earlier (Heyl *et al.*, 1951); however, the position of the phosphate group was not unambiguously established, the free acid was not isolated in the pure form, and yields were low. The route followed here provides an unambiguous synthesis of *N*-MePLP and may be applied to the synthesis of other intermediates of interest to this kind of study.

The present study was initiated in an effort to explore the possible involvement of the phenolic and formyl groups in the phosphorylase reaction. This paper reports the unambiguous synthesis of 3-*O*-methylpyridoxal 5′-phosphate, *N*-methylpyridoxal 5′-phosphate, and isopyridoxal 4′-phosphate, in which the formyl and hydroxymethyl phosphate groups are interchanged. The following publication describes the reconstitution of pure rabbit muscle glycogen apophosphorylases *b* and *a* with these compounds (Shaltiel *et al.*, 1969).

## Materials and Methods

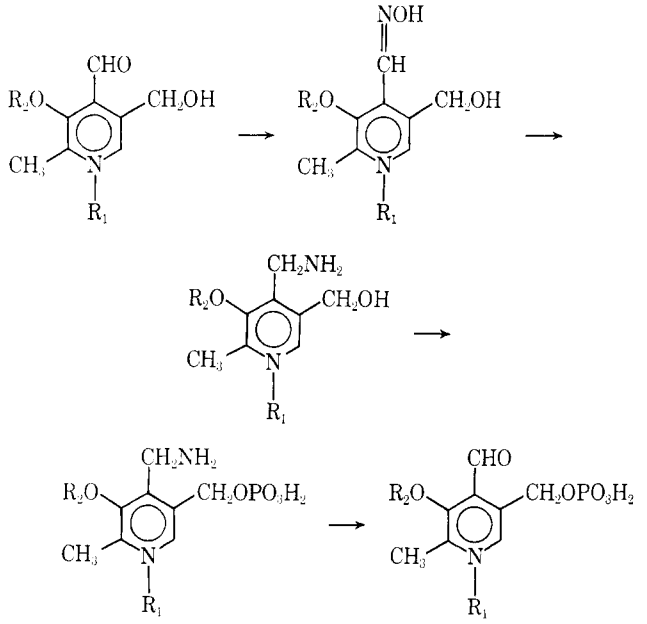
Pyridoxine and its common derivatives were purchased from Sigma and found to be satisfactory without further purification. Manganese dioxide (type A), used as a mild oxidizing agent of alcohols and amines, was prepared from manganous carbonate (Baker Analyzed) according to Harfenist *et al.* (1954); when stored in an air-tight container, it remained active indefinitely. Amberlite CG-50 cation exchanger (Mallinckrodt) was first decanted of all fines and then treated according to the procedure of Peterson *et al.* (1953). Dowex 50-X8 (200–400 mesh) was purchased from Bio-Rad and purified according to Argoudelis and Kummerow (1966). Celite analytical filter aid was obtained from Johns-Manville. High-voltage paper electrophoreses were performed on sheets of Whatman

* From the Department of Biochemistry, University of Washington, Seattle, Washington 98105. *Received August 21, 1969.* Supported by grants from the National Science Foundation (GB-3249) and from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health (AM 7902).

[1] Abbreviations used are: PN, pyridoxine; PL, pyridoxal; PM, pyridoxamine; PNP, PLP, PMP, the corresponding 4′- or 5′-phosphate derivatives.

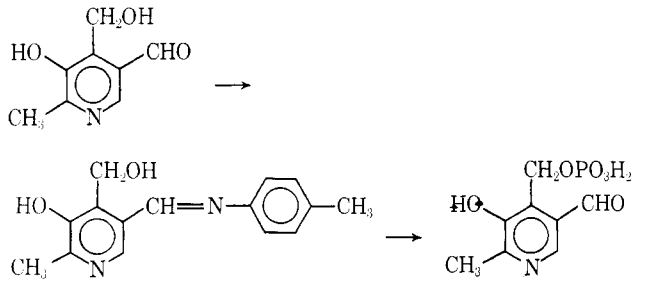
SCHEME I

a. According to Ikawa and Snell (1954)



*O*-MePLP series, $R_1 = H$; $R_2 = CH_3$
*N*-MePLP series, $R_1 = CH_3$; $R_2 = H$

b. According to Iwanami *et al.* (1968)



No. 3MM and thin-layer chromatographies on Eastman chromatogram sheets (type 6060 or 6061).

For detection of pyridoxyl derivatives, Gibbs reagent (Gibbs, 1927) was prepared by dissolving dichloroquinone chlorimide (Matheson Coleman & Bell) in isopropyl alcohol (2 mg/ml). The chromatograms were first sprayed with this reagent, then with 4 M ammonia. The phenylhydrazine reagent described by Wada and Snell (1961), diluted 1:4 in ethanol, was also used when appropriate. Aldehydes slow to react with this reagent were detected with a 0.4% solution of 2,4-dinitrophenylhydrazine in 2 N HCl. The ninhydrin reagent (0.25%) was made up in acetone.

Melting points were taken in a Thomas-Hoover apparatus; values reported are uncorrected. Solutions were flash evaporated on the Büchi Rotavapor (R) at a bath temperature not exceeding 30°. Elemental analyses were carried out by Alfred Bernhardt, Microanalytisches Laboratorium, West Germany. Ultraviolet spectra were performed on a Beckman DK-1 recording spectrophotometer. Buffers for $pK_a$ determinations were chosen on the basis of their lack of reactivity with the functional groups of the various analogs. They were as follows (Gomori, 1955): KCl–HCl buffer (0.1 M) for pH 1–2, 0.1 M citrate–sodium phosphate for pH 3–7, 0.1 M sodium phosphate for pH 7–8, and 0.1 M $NaHCO_3$–$Na_2CO_3$ for pH 8–10. Measurements were also made in 0.1 N HCl and 0.1 N NaOH. Standard stock solutions of the pure samples ($10^{-2}$ M) were diluted in the appropriate buffer to a final concentration of $10^{-4}$ M and read in 10-mm quartz cells.

Titration of the ionizable groups were carried out by the procedure of Metzler and Snell (1955) at 25°. The $pK_a$'s were determined by plotting the change in per cent absorbancy with pH. Table III in the Results section lists the over-all dissociation constants. These determinations agree with literature values given for similar structures.

## Results

*Synthesis of 3-O-Methylpyridoxal 5'-Phosphate. 3-O-*ME-PYRIDOXAL BASE.[2] 3-*O*-MePL·HCl was prepared by the method of Heyl and Harris (1951). The free base which is sparingly soluble in water was obtained in crystalline form by neutralizing the aqueous solution of the hydrochloride with sodium bicarbonate, and recrystallized from ethanol in clusters of fine needles, mp 198–200° dec. *Anal.* Calcd for $C_9H_{11}NO_3$: C, 59.66; H, 6.14; $OCH_3$, 17.14; N, 7.76. Found: C, 59.40; H, 6.13; $OCH_3$, 17.49; N, 7.64.

3-*O*-MePL base was characterized as the phenylhydrazone prepared in the usual manner. This derivative, upon recrystallization from 80% ethanol, gave long, yellow needles melting at 178–180° with decomposition. *Anal.* Calcd for $C_{15}H_{17}N_3O_2$: C, 66.42; H, 6.35; N, 15.49. Found: C, 66.18; H, 6.59; N, 15.15.

*3-O-*ME-PYRIDOXAL OXIME. 3-*O*-Me-PL·HCl (1 g) was dissolved in 50% aqueous ethanol (*ca.* 30 ml) and 2 g of hydroxylamine·HCl and 4 g of crystalline sodium acetate were added all at once. The mixture was refluxed until clear, left to stand at room temperature for 1 hr, and then chilled thoroughly, after which the oxime crystallized out in clusters of white, feathery needles, decomposing at 196°. The yield was 0.71 g or 85% based on the hydrochloride. The melting point was not raised upon recrystallization from ethanol–water. *Anal.* Calcd for $C_9H_{12}N_2O_3$: C, 55.08; H, 6.17; N, 14.28. Found: C, 54.67; H, 6.25; N, 14.72.

Both 3-*O*-MePL and 3-*O*-MePL-oxime are nonfluorescent, unlike the corresponding free 3-pyridinol derivatives.

*3-O-*ME-PYRIDOXAMINE·2HCl. 3-*O*-MePL-oxime (0.7 g) was reduced with zinc and glacial acetic acid according to Testa and Fava (1957) and afforded 0.8 g (90%) of 3-*O*-MePM·2HCl, mp 173–176° dec. After two recrystallizations from methanol–ether the melting point was raised to 176°. *Anal.* Calcd for $C_9H_{16}Cl_2N_2O_2$: C, 42.36; H, 6.32; Cl, 27.79; N, 10.98. Found: C, 42.17; H, 6.42; Cl, 27.63; N, 10.89.

3-*O*-MePM can be readily detected on paper by its strong orange ninhydrin reaction. This color gradually turns purple on exposure to air unlike PM, which gives a stable orange spot.

*3-O-*ME-PYRIDOXAMINE 5'-PHOSPHATE·$H_2O$. 3-*O*-MePM·2HCl (0.4 g) was phosphorylated with a mixture of phosphoric

[2] Trivial names for analogs indicate substituent changes in the structure; the group modified precedes the parent compound.

acid and phosphorus pentoxide according to the method of Peterson *et al.* (1953). The product was purified by passage through an Amberlite CG-50 column (2.5 × 90 cm); the compound emerges in the second and third liter of effluent (1400–2700 ml) and can be readily detected by its ninhydrin reaction on paper which is similar to that of 3-*O*-MePM. Actually, this property can be utilized to differentiate this compound from PMP. A small amount of ninhydrin-positive polyphosphate derivative could be readily separated from the orthophosphate ester, since it emerged in the first liter of effluent. 3-*O*-MePMP was crystallized from water–ethanol mixture in white short prisms (0.4 g or 85% yield based on 3-*O*-MePM·2HCl), mp 225° dec. The compound analyzes as the monohydrate. *Anal.* Calcd for $C_9H_{17}N_2O_6P$: C, 38.57; H, 6.14; N, 10.0; P, 11.05. Found: C, 38.36; H, 6.04; N, 10.40; P, 11.63.

3-*O*-ME-PYRIDOXAL 5′-PHOSPHATE·$2H_2O$. The oxidation of 3-*O*-MePMP was achieved essentially according to the procedure of Peterson *et al.* (1953) with the following modification: 0.8 g of 3-*O*-MePMP was dissolved in 100 ml of water and 4 g of $MnO_2$–A was added all at once with stirring. At intervals, the reaction mixture was spotted on paper and tested with ninhydrin for the disappearance of the amino group and with the phenylhydrazine reagent for the appearance of the aldehyde function.

The first aldehyde spot was detected 30 min after the addition of $MnO_2$–A but it took 38 hr for the reaction to go to completion as indicated by the disappearance of the ninhydrin reaction. This should be contrasted with the very facile oxidation of PMP to PLP which is essentially complete in 15–20 min. The reaction mixture was then purified as indicated in the procedure of Peterson *et al.* (1953) and 3-*O*-MePLP was crystallized out in white, shining, prismatic plates from the concentrated solution of the effluent. A single recrystallization from a water–ethanol mixture gave the pure dihydrate derivative decomposing above 160°. The yield was 0.51 g or 57% based on 3-*O*-MePMP. *Anal.* Calcd for $C_9H_{16}NO_8P$: C, 36.37; H, 5.43; N, 4.71; P, 10.42. Found: C, 36.40; H, 5.42; N, 4.73; P, 10.09.

The purity of the material was ascertained by high-voltage electrophoresis and chromatography in several systems. In each case, the sample appeared as a single spot giving rise to a positive phenylhydrazine reaction and negative Gibbs and ninhydrin tests.

*Synthesis of N-Methylpyridoxal 5′-Phosphate.* *N*-METHYLPYRIDOXAL OXIME. Pyridoxal methochloride (*N*-MePL) was prepared from PL·HCl by the method of Heyl *et al.* (1951) and recrystallized several times until all traces of contaminating PL had disappeared as evidenced by a negative Gibbs reaction on paper.

*N*-MePL (40 g) was dissolved in 20 ml of water, and 6 g of sodium bicarbonate and 4 g of hydroxylamine·HCl were added. After 1-hr continuous stirring the bright yellow precipitate was filtered, washed repeatedly with water, then alcohol and ether, yielding 3.5 g (97%) of the oxime which decomposes at *ca.* 205°. The material was twice recrystallized from water and dried. *Anal.* Calcd for $C_9H_{12}N_2O_3$: C, 55.09; H, 6.17; N, 14.28. Found: C, 55.26; H, 6.13; N, 14.40.

When *N*-Me-pyridoxal oxime was precipitated in a sodium acetate buffer solution, as in the preparation of 3-*O*-MePL-oxime, the compound crystallized as the acetate. The free base crystallizes in two polymorphous forms.

*N*-ME-PYRIDOXAMINE·2HCl. *N*-MePL-oxime (2.2 g) was reduced with zinc powder in glacial acetic acid as described earlier for the preparation of 3-*O*-MePM. This oxime, however, is less soluble in glacial acetic acid than the corresponding pyridoxal or 3-*O*-MePL-oxime and, therefore, the reduction was carried out in a larger volume and at a water-bath temperature between 70 and 80°. The solution was decolorized with Norit and recrystallized twice from methanol–acetone yielding 2.3 g (80%) of the pure product, mp 228° dec. *Anal.* Calcd for $C_9H_{16}Cl_2N_2O_2$: C, 42.36; H, 6.32; Cl, 27.79; N, 10.98. Found: C, 42.47; H, 6.23; Cl, 27.65; N, 10.85.

On paper, it gives a negative Gibbs reaction and a strong permanent orange-red color with ninhydrin.

*N*-ME-PYRIDOXAMINE 5′-PHOSPHATE. *N*-MePM·2HCl (1.5 g) was phosphorylated as described earlier to yield 1.3 g (84%) of *N*-MePMP. The compound crystallized from water and ethanol in long, prismatic, white needles. It is light sensitive, oxidizes readily in air when dry, but is relatively stable when kept in solution in the dark and at low temperature. It analyzes as the inner salt. *Anal.* Calcd for $C_9H_{15}N_2O_5P$: C, 41.22; H, 5.77; N, 10.67; P, 11.81. Found: C, 41.06; H, 5.85; N, 10.51; P, 12.01.

On paper it gives a negative Gibbs reaction and a strong, permanent orange-red color with ninhydrin, similar to that of *N*-MePM.

*N*-ME-PYRIDOXAL 5′-PHOSPHATE. All steps in the preparation and purification of this compound must be carried out in the dark.[3] Recrystallized *N*-MePMP (0.4 g) was dissolved in 10 ml of water; the solution was cooled in an ice bath and 0.4 g of $MnO_2$–A added with stirring. The amine oxidizes rapidly as shown by the disappearance of the ninhydrin reaction within 10 min. The reaction was immediately stopped by filtration of the catalyst through a Celite pad and the pad rinsed with a total volume of 750 ml of water (or until the effluent was only faintly yellow). The pooled filtrate and washings were then flash evaporated to a volume of 10 ml and purified by passage through an Amberlite CG-50 column (80 × 2.5 cm).

*N*-MePLP appeared in the first 500 ml of effluent. The preparation was contaminated by small amounts of various fluorescent compounds and was therefore reapplied to the resin under nitrogen in order to minimize possible oxidation and polymerization processes. The aldehyde-positive fractions were then pooled and once more concentrated to a yellow oil which crystallized out slowly in short, thick, bright yellow rods upon standing in the freezer, mp 96–98°. This compound gives a negative Gibbs test and contains 1.5 molecules of water of crystallization. However, when run on electrophoresis, it appears to be still contaminated by trace amounts of a more acidic fluorescent material. *Anal.* Calcd for $C_9H_{12}NO_6P \cdot 1.5H_2O$: C, 37.50; H, 5.25; N, 4.86; P, 10.75. Found: C, 37.68; H, 5.43; N, 4.74; P, 10.41.

*N*-MePLP crystallizes from an ethanol–water mixture with 1 mole of water and 1 mole of ethanol in clusters of bright yellow, short prisms. Its oxime decomposes at 225°.

*Synthesis of Isopyridoxal 4′-Phosphate.* ISOPYRIDOXAMINE

[3] Colored compounds were readily generated during the purification of *N*-MePLP, particularly when the samples were exposed to light. This phenomenon was attributed to a light-mediated dimerization or polymerization reaction, as already described for PLP itself (Morrison and Long, 1958). *N*-MePLP appeared to be particularly sensitive to this process.

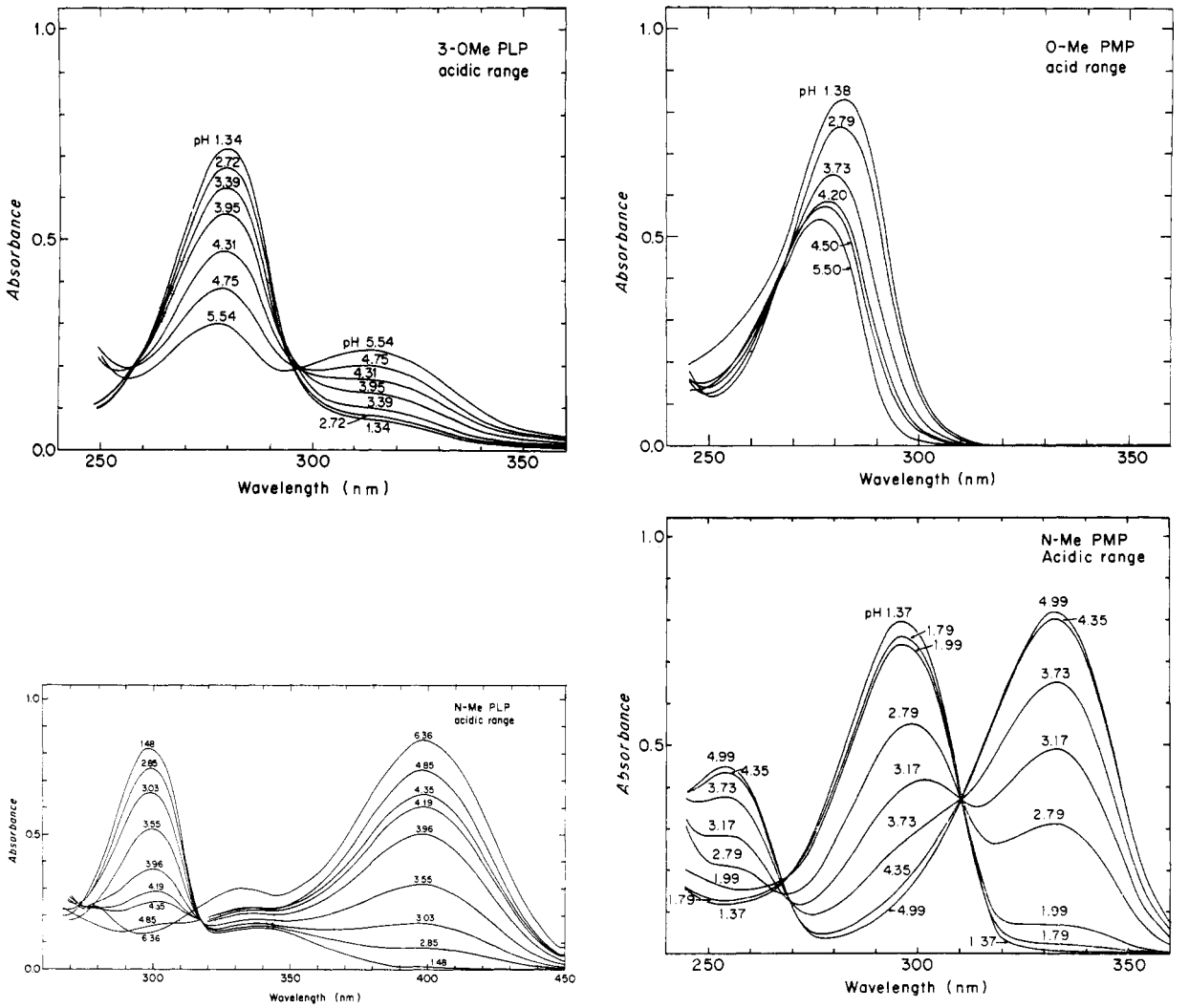



FIGURE 1A: Spectrophotometric titration curves of 3-*O*-MePLP, 3-*O*-MePMP, *N*-MePLP, and *N*-MePMP. Conditions are indicated under Methods.

4′-PHOSPHATE. At first, an attempt was made to prepare isoPLP by phosphorylation of isoPM followed by oxidation of the amine. isoPM was prepared by reduction of isoPL oxime with zinc in glacial acetic acid according to the procedure of Brooks *et al.* (1966). This material (2 g) was dissolved in 10 ml of the phosphorylating reagent, kept for 2 hr at 60°, and treated as already described. The precipitated crude isoPM 5′-phosphate was passed through a column of Amberlite CG-50 (2.5 × 60 cm) and fractions giving a yellow ninhydrin color were pooled and evaporated to a small volume. Crystallization was induced by addition of methanol and chilling; recrystallization from water–methanol yielded a pure product (0.7 g, 30% of theoretical) as the monohydrate. *Anal.* Calcd for $C_8H_{15}N_2O_6P$: C, 36.09; H, 5.68; N, 10.52; P, 11.63. Found: C, 35.91; H, 6.01; N, 10.48; P, 11.83. Unfortunately, $MnO_2$ oxidation of isoPMP did not occur at room temperature; on the other hand, warming the reaction mixture to 60° led to extensive hydrolysis of the 4′-hydroxymethyl phosphate group with the appearance of unidentified decomposition products. It was therefore decided to prepare isoPLP by direct phosphorylation of the *p*-toluidine Schiff base of isoPL. isoPM as well as isoPMP gave with ninhydrin a yellow color which slowly turned to purple.

ISOPYRIDOXAL–*p*-TOLUIDINE SCHIFF BASE. Pure isoPL (0.5 g, mp 184–186° dec) was dissolved in the smallest volume of 95% ethanol (*ca.* 5 ml) with heating. Recrystallized *p*-toluidine (0.4 g) was then added and the yellowish solution was left to stand at room temperature for 1 hr. The solvent was then evaporated and the residue recrystallized twice from tetrahydrofuran: yield 0.5 g or 70% of theoretical mp 192–195° dec.

ISOPYRIDOXAL 4′-PHOSPHATE. Phosphorylation of the Schiff base was carried out according to the procedure of Iwanami *et al.* (1968) with the following modifications. isoPL–*p*-toluidine Schiff (1 g) was left to stand at room temperature for 24 hr in 10 ml of polyphosphoric acid, then warmed gently for 1 hr to 35–40°; the Schiff base dissolves very slowly to give a bright yellow solution. The reaction mixture was cooled and 5 ml of 0.1 N HCl was added to hydrolyze the polyphosphates and the Schiff base; the reaction occurred almost instantaneously. The hydrolysis mixture was diluted to 20 ml and applied to a Dowex 50-X8 column (2.5 × 40 cm); elution was

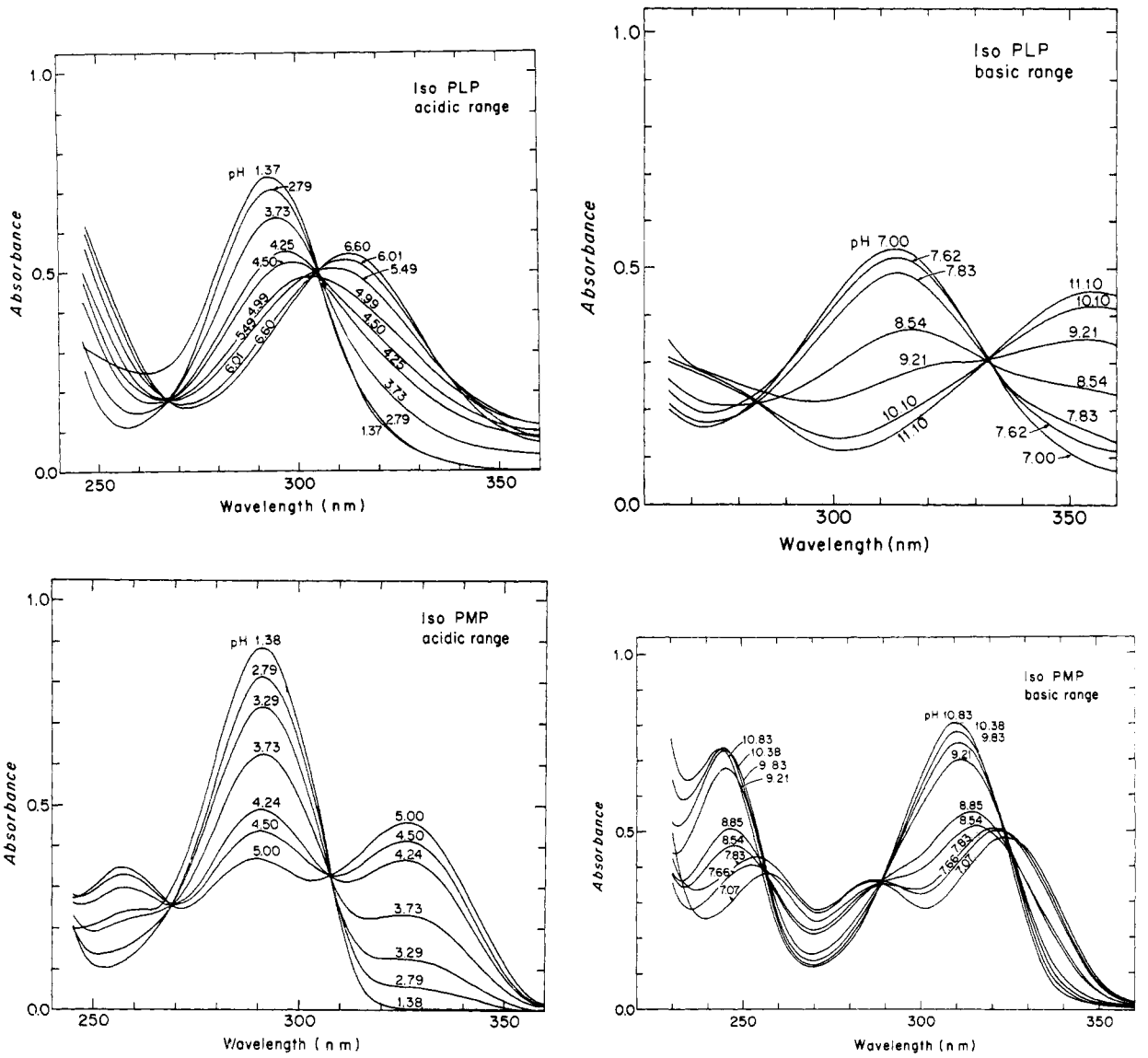



FIGURE 1B: Spectrophotometric titration curves of isoPLP and isoPMP. Conditions are indicated under Methods.

carried out with water (30 ml/hr), fractions giving a positive Gibbs test were pooled and concentrated, and the compound crystallized in short white prisms. isoPL-4′-phosphate readily hydrolyzes in warm water to isoPL and phosphoric acid but it could be further purified by an additional passage through Dowex 50 ($H^+$). The material gives a yellow phenylhydrazone with an absorbance maximum at 378 nm; yield was 0.25 g or 25% based on isoPL–*p*-toluidine Schiff base. *Anal.* Calcd for $C_8H_{16}NO_6P \cdot 0.5H_2O$: C, 37.51; H, 4.32; N, 5.46; P, 12.09. Found: C, 38.05; H, 4.55; N, 5.26; P, 11.21.

*Separation and Detection of O- and N-Substituted Pyridoxyl Derivatives.* Thin-layer chromatography on silica gel sheets was applied at each stage of the synthesis to establish the purity of the intermediates. $R_f$ values in three different solvent systems are listed in Table I. The *O*- and *N*-methylated derivatives do not react with the Gibbs reagent, and no other specific and sensitive color test could be found; therefore, detection relied on the use of general functional group reagents such as phenylhydrazine for the aldehyde group and ninhydrin for the primary amino group. *N*-Me derivatives could be detected by their strong fluorescence or by the yellow color produced in alkaline medium in the presence of acetone (Siegel and Blake 1962). This latter procedure could not be applied for the detection of the 3-*O*-methylated aldehydes because of their poor reactivity.

*Spectral Characteristics and Dissociation Constants.* Infrared spectra of all new compounds were taken in Nujol mulls and KBr pellets; none showed marked absorption in the C=O stretch region indicating that the formyl group is hydrated or masked. Likewise, the C=N stretch band of the corresponding oximes was difficult to identify.

On the other hand, ultraviolet absorption spectra were determined to confirm the structure and spectral characteristics of the compounds synthesized in this study. Only the spectrophotometric titration curves of the phosphorylated derivatives are illustrated here (Figure 1). Similar spectra as functions of pH were taken for all other analogs described herein; they have been deposited with the National Auxiliary Publications

TABLE 1: Detection of Pyridoxal Derivatives on Silica Gel Sheets.[a]

| | A | B | C |
|---|---|---|---|
| PL | 0.52 | 0.65 | 0.77 |
| PM | 0.27 | 0.49 | 0.79 |
| 3-*O*-MePL | 0.65 | 0.59 | 0.86 |
| 3-*O*-MePM | 0.18 | 0.29 | 0.83 |
| *N*-MePL | 0.17 | 0.42 | 0.30 |
| *N*-MePM | 0.09 | 0.26 | 0.15 |
| PLP | 0.37 | 0.36 | 0.13 |
| PMP | 0.08 | 0.25 | 0.17 |
| 3-*O*-MePLP[b] | 0.33 | 0.26 | 0.24 |
| 3-*O*-MePMP | 0.29 | 0.23 | 0.15 |
| *N*-MePLP | 0.08 | 0.17 | 0.06 |
| *N*-MePMP | 0.03 | 0.09 | 0.03 |

[a] Solvent A: 1-butanol–acetone–acetic acid–5% ammonia–water (35:25:15:15:10, v/v), solvent B: *t*-butyl alcohol–water–88% formic acid (65:15:15, v/v), and solvent C: *t*-amyl alcohol–acetone–water–concentrated ammonia (40:35:20:5, v/v). [b] *O*-MePLP is more readily separated from PLP by electrophoresis at pH 3.6, 2000 V for 55 min; and $R_{PLP}$ of 0.53 is obtained. Visualization of the spots on paper is carried out with the 2,4-dinitrophenylhydrazine reagent.

Service (ASIS) from which they may be obtained.[4] Table II lists the absorption maxima and molar absorbancy coefficients of the analogs in aqueous solution and at three pH values. *O*-Me-PLP and isopyridoxal 4′-phosphate, in contrast to PLP and *N*-MePLP, lack an absorption band in the visible range of the spectrum. This stresses once more the effect of the neighboring free phenolic group on the spectral characteristics of pyridoxal derivatives.

## Discussion

In reconstitution experiments involving rabbit muscle apophosphorylase, it is essential that all analogs tested be rigorously freed of PLP contamination since under the conditions used for these experiments (Hedrick *et al.*, 1966, and following publication) as little as $10^{-9}$ mole of the natural cofactor would bring about noticeable reactivation. Great care was therefore taken to purify not only the final phosphorylated derivatives, but also each intermediate prior to its further transformation. As indicated above, both the *O*- and *N*-methylated derivatives do not react with Gibbs reagent, while PLP and isoPLP give a clearly positive response at concentrations as low as $10^{-5}$ M; consequently, all compounds obtained (except isoPLP) were tested at $10^{-2}$ M concentration. A negative Gibbs test was taken to indicate less than 0.1% contamination by the nonsubstituted derivative. As indicated under Results, characteristic ninhydrin reactions could serve to distinguish many amine derivatives.

[4] For this supplementary material, order NAPS Document 00735 from ASIS National Auxiliary Publications Service, c/o CCM information Corp., 903 3rd Ave., New York, N. Y. 10022, remitting $1.00 for microfiche or $3.00 for photocopies.

The spectral properties of all synthetic derivatives modified in the 1 and 3 positions were determined as functions of pH and compared to those of the known standards, namely, pyridine, 3-pyridinol, and *N*-Me-3-pyridinol. The same was done for the isopyridoxyl derivatives. As expected, shifts in wavelength and intensity observed for the 3-*O*-MePL derivatives were similar to those shown by pyridine (and different from those of 3-pyridinol), whereas the *N*-methylated derivatives displayed spectral behavior characteristic for *N*-Me-3-pyridinol; compounds of the two series can thus be readily distinguished. A similar behavior was observed with isoPLP.

In general, 3-pyridinols exist in any one of four spectrally distinct species, namely, the zwitterionic, anionic, cationic, and neutral forms; it can be domonstrated empirically that the wavelength number observed for these species decreases in the same order (Nakamoto and Martell, 1959). Whereas pH-dependent shifts in absorption are often diagnostic of changes affecting chromophores, this is not the case with pyridoxal derivatives where hydration of the aldehyde group or formation of the internal hemiacetal both contribute to the fine structure of the spectrum. Since an equilibrium exists between the free and substituted forms of the carbonyl group, interpretation of titration curves for pyridoxal and its *O*- and *N*-methyl derivatives, and isopyridoxal, becomes difficult. Nevertheless, $pK_a$ determinations in these latter instances are instructive (see Table III). Thus, it is often assumed that the zwitterionic, rather than the neutral form, predominates when the conjugate acid of pyridoxal is neutralized in aqueous medium, and hence, that the $pK_a$ observed refer to the removal of the phenolic proton. However, 3-*O*-MePL has no phenolic proton and yet its $pK_a$ is 4.55 (Metzler and Snell, 1955), similar to that of pyridoxal itself and *N*-MePL (Johnston *et al.*, 1963). On the other hand, the loss of a proton from the *N*-Me derivatives cannot lead to a neutral species and consequently, the $pK_a$ of such derivatives must reflect the ionization of the phenolic group. It will be noticed that the $pK_a$ of *N*-MePL and isoPL are also similar to that of pyridoxal. These determinations would imply that under conditions normally employed for the reconstitution of phosphorylase, *N*-MePLP and isoPLP would be present in the zwitterionic form while 3-*O*-MePLP would be in the neutral form; as seen in the following publication (Shaltiel *et al.*, 1969) only the latter analog restored partial enzymatic activity.

It will be noted that isoPMP displays a maximum absorption at 287 nm around neutrality. Nakamoto and Martell (1959) described that 2-hydroxymethyl-3-hydroxypyridine present an absorption peak at 286 nm in acid and at 283 nm around neutrality; these were ascribed to the hydrogen-bonded, cyclic, cationic, and uncharged forms, respectively. It is difficult to envision here a cyclic derivative similar to the one described by Nakamoto and Martell (1959) since the hydroxymethyl residue adjacent to the phenolic group in position 3 is blocked.

Another difference between the *O*- and *N*-methylated derivatives of pyridoxal can be seen in their relative rates of oxidation by $MnO_2$. Whereas oxidation of *N*-MePMP reaches completion in 9–10 min (as compared with 15 min for PMP), that of 3-*O*-MePMP requires as much as 36 hr. Likewise, isoPMP undergoes extremely slow $MnO_2$ oxidation and a positive ninhydrin reaction is still observed after 64 hr. Substitution of the phenolic group results in a marked decrease in pho-

TABLE II: Absorption Maxima and Molar Absorbancies of Pyridoxal Analogs and Derivatives.[a]

| Compounds | 0.1 N HCl | | 0.1 M NaP buffer (pH 7) | | 0.1 N NaOH | |
|---|---|---|---|---|---|---|
| | $\lambda_{max}$ (nm) | $\epsilon \times 10^{-3}$ | $\lambda_{max}$ (nm) | $\epsilon \times 10^{-3}$ | $\lambda_{max}$ (nm) | $\epsilon \times 10^{-3}$ |
| 3-*O*-MePL | 285 | 8.5 | 275 | 4.9 | 273 | 4.5 |
| 3-*O*-MePM | 282 | 7.6 | 275 | 4.9 | 270 | 4.5 |
| *N*-MePL | 292 | 8.1 | 321 | 8.5 | 318 | 8.7 |
| | | | 255 | 5.4 | 253 | 5.1 |
| *N*-MePM | 295 | 8.0 | 331 | 8.9 | 328 | 9.1 |
| | | | 255 | 4.8 | 255 | 4.6 |
| 3-*O*-MePLP | 279 | 7.1 | 313 | 2.7 | 313 | 1.9 |
| 3-*O*-MePMP | 282 | 8.0 | 275 | 5.4 | 270 | 4.9 |
| *N*-MePLP | 340 | 1.3 | 398 | 7.0 | 400 | 6.4 |
| | 298 | 7.5 | 331 | 2.7 | 329 | 2.2 |
| *N*-MePMP | 295 | 8.2 | 331 | 9.1 | 327 | 9.4 |
| | | | 252 | 3.6 | 252 | 4.4 |
| isoPLP | 293 | 7.3 | 313 | 5.4 | 356 | 4.4 |
| | | | 221 | 14.9 | 239 | 19.2 |
| isoPMP | 291 | 8.8 | 324 | 4.6 | 309 | 8.2 |
| | | | 285 | 3.5 | 243 | 7.6 |
| | | | 258 | 3.6 | | |

[a] Samples prepared for elemental analysis and diluted to $10^{-4}$ M were used for these measurements.

TABLE III: Acid Dissociation Constants of Pyridoxal Derivatives Determined from Spectrophotometric Data.

| Compound | $pK_a$[a] | Compound | $pK_a$ |
|---|---|---|---|
| 3-Pyridinol | 4.86[b] | | |
| | 8.72 | | |
| 3-*O*-Me-pyridinol | 4.88[b] | | |
| *N*-Me-pyridinol | 4.86[b] | | |
| PL | 4.20[c] | PLP | 4.66[d] |
| | 8.66 | | 8.69 |
| PM | 3.31[d] | PMP | 3.69[d] |
| | 7.90 | | 8.61 |
| 3-*O*-MePL | (4.75)[c] 4.55 | 3-*O*-MePLP | 4.15 |
| 3-*O*-MePM | 3.40 | 3-*O*-MePMP | 3.60 |
| *N*-MePL | 3.90 | *N*-MePLP | 3.70 |
| *N*-MePM | 2.95 | *N*-MePMP | 3.00 |
| isoPL | 4.00 | isoPLP | 4.25 |
| | | | 8.80 |
| isoPM | | isoPMP | 3.75 |
| | | | 8.60 |

[a] The dissociation constants measured spectrophotometrically during titration from acid to base. The $pK_a$'s of the phosphate groups are not determined by this procedure and therefore not listed. [b] Literature values taken from Albert (1963). [c] Determined by Metzler and Snell (1955). [d] Perrin (1965).

tosensitivity; obviously, the vicinal phenolic group is catalytically involved in these reactions.

As indicated above, *N*-MePL-oxime was obtained in two crystalline forms. When freshly prepared, it precipitated in long, yellow, prismatic needles, while after two or three recrystallizations from water, rhombohedral crystals were obtained which did not revert to their original habit. It is possible that the two crystalline forms represent the *syn* and *anti* isomers of the oxime; the tridimensional structure of the stable, rhombohedral form was determined by X-ray crystallography (A. C. Bloomer and L. H. Jensen, unpublished results).

## Acknowledgment

The authors are indebted to Dr. David E. Metzler for most helpful comments and suggestions regarding this manuscript.

References


Albert, A. (1963), *in* Physical Methods in Heterocyclic Chemistry, Katritzky, A. R., Ed., New York, N. Y., Academic, p 79.

Argoudelis, C. T., and Kummerow, F. A. (1966), *Biochemistry 5*, 1.

Baddiley, J., and Mathias, A. P. (1952), *J. Chem. Soc.*, 2583.

Bocharov, A. L., Ivanov, V. I., and Karpeisky, M. Ya. (1968), *Biochem. Biophys. Res. Commun. 30*, 459.

Brooks, H. G., Laasko, J. W., and Metzler, D. E. (1966), *J. Heterocyclic Chem. 3*, 126.

Fukui, S., and Ohishi, N. (1969), *Arch. Biochem. Biophys. 130*, 584.

Gibbs, H. D. (1927), *J. Biol. Chem. 72*, 649.

Gomori, G. (1955), *Methods Enzymol. 1*, 138.

Harfenist, M., Baverley, A., and Lazier, W. A. (1954), *J. Org. Chem. 19*, 1608.

Hedrick, J. L., Shaltiel, S., and Fischer, E. H. (1966), *Biochemistry 5*, 2117.

Heyl, D., and Harris, S. A. (1951), *J. Am. Chem. Soc. 73*, 3434.

Heyl, D., Luz, E., Harris, S. A., and Folkers, K. (1951), *J. Am. Chem. Soc. 73*, 3430.

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

Illingworth, B., Jansz, H. S., Brown, D. H., and Cori, F. C. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 1180.

Iwanami, M., Numata, T., and Murakami M. (1968), *Bull. Chem. Soc. Japan 41*, 161.

Johnston, C. C., Brooks, H. G., Albert, J. D., and Metzler, D. E. (1963), *in* Chemical and Biological Aspects of Pyridoxal Catalysis, Snell, E. E., Fasella, P. M., Braunstein, A., and Rossi Fanelli, A., Ed., New York, N. Y., Pergamon, p 69.

Korytnyk, W., and Wiedeman, W. (1962), *J. Chem. Soc.*, 2531.

Metzler, D. E., and Snell, E. E. (1955), *J. Am. Chem. Soc. 77*, 2435.

Morrison, A. L., and Long, R. F. (1958), *J. Chem. Soc.*, 211.

Mühlradt, P. F., Morino, Y., and Snell, E. E. (1967), *J. Med. Chem. 10*, 341.

Nakamoto, K., and Martell, A. E. (1959), *J. Am. Chem. Soc. 81*, 5857.

Perrin, D. (1965), Dissociation Constants of Organic Bases in Aqueous Solutions, Butterworth, London.

Peterson, E. A., Sober, H. A., and Meister, A. (1953), *Biochem. Prepn. 3*, 29.

Shaltiel, S., Hedrick, J. L., Pocker, A., and Fischer, E. H. (1969), *Biochemistry 8*, 5189.

Siegel, F. P., and Blake, M. I. (1962), *Anal. Chem. 34*, 397.

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

Tate, S. S., and Meister, A. (1969), *Biochemistry 8*, 1056.

Testa, E., and Fava, F. (1957), *Chimia 11*, 310.

Tomita, I., Brooks, H. G., and Metzler, D. E. (1966), *J. Heterocyclic Chem. 3*, 178.

Wada, H., and Snell, E. E. (1961), *J. Biol. Chem. 236*, 2089.