Role of Pyridoxal 5′-Phosphate in Glycogen Phosphorylase. I. Synthesis of 3′-*O*-Methylpyridoxal 5′-Phosphate *N*-Oxide and Pyridoxal 5′-Phosphate Monomethyl Ester and the Conversion of the *N*-Oxide to Pyridoxal 5′-Phosphate by Apophosphorylase *b* from Rabbit Skeletal Muscle†

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ABSTRACT: The synthesis of 3′-*O*-methylpyridoxal phosphate *N*-oxide and of the monomethyl ester of pyridoxal phosphate are described. Both analogs bind in a ratio of 2 moles of cofactor/mole of dimer (mol wt 200,000) of apophosphorylase *b* but yield completely inactive phosphorylases. In contrast to pyridoxal phosphate *N*-oxide, these analogs are stable and unreactive with apophosphorylase. Pyridoxal phosphate *N*-oxide on reaction with apophosphorylase *b* forms pyridoxal phosphate which partially reactivates the apoprotein. In the light the deoxygenation of the *N*-oxide to the parent amine is greatly enhanced and results in partial but irreversible inactivation of the apoprotein. However, even in the dark and under nitrogen, about 20% of the stoichiometrically bound pyridoxal phosphate *N*-oxide is converted in 2 min at 30° to pyridoxal phosphate by apophosphorylase *b*. The apoprotein resolved from *N*-oxide is still 96–100% reactivatable upon reconstitution with pyridoxal phosphate. The dark reaction of apophosphorylase *b* with pyridoxal phosphate *N*-oxide is specific. Neither L-cysteine, L-methionine, L-lysine, and several other amino acids, nor egg albumin or lysozyme deoxygenate pyridoxal phosphate *N*-oxide to pyridoxal phosphate. Reduced phosphorylase *b* or holophosphorylase *b* also react with pyridoxal phosphate *N*-oxide but much slower. None of the exposed cysteinyl residues of the phosphorylase protein, which rapidly react with 5,5′-dithiobis(2-nitro)benzoic acid and iodoacetamide, are oxidized in the course of the conversion of pyridoxal phosphate *N*-oxide to pyridoxal phosphate. Adenosine-5′-P or glucose-1-P, which protect certain cysteinyl residues, did not affect the deoxygenation of pyridoxal phosphate *N*-oxide. Thus, the mechanism of the reaction and the nature of the reducing group in the protein remain obscure. The spectral properties of phosphorylase *b* containing pyridoxal phosphate *N*-oxide or pyridoxal phosphate are different, in that pyridoxal phosphate is bound to a highly hydrophobic region in the phosphorylase protein (*cf.* Shaltiel, S., and Cortijo, M. (1970), *Biochem. Biophys. Res. Commun. 41*, 594), whereas pyridoxal phosphate *N*-oxide appears to be bound to a more polar region.

Pyridoxal phosphate[1] is essential for the activity of all known α-glucan phosphorylases (Fischer *et al.*, 1970). The first phosphorylase enzyme shown to contain pyridoxal-P was rabbit skeletal muscle phosphorylase *a* (Baranowski *et al.*, 1957). Fischer and Krebs (1964) have pointed out that in rabbit muscle there is more pyridoxal-P bound to phosphorylase than to all other pyridoxal-P-dependent enzymes. Pyridoxal-P is bound to phosphorylase as an imine through its C_4-formyl group to an ε-amino group of a lysyl residue in a highly hydrophobic region of the protein (Shaltiel and Cortijo, 1970). The azomethine bond can be reduced with $NaBH_4$ but only after "de-formation" of the protein by acidification or high-salt concentrations (Fischer *et al.*, 1958; Graves *et al.*, 1965). The reduced enzyme retains about 60% of its original activity (Fischer *et al.*, 1958, 1963). This rules out a participation of the 4-formyl group of pyridoxal-P in phosphorylase catalysis, as in the case of every other pyridoxal-P-dependent enzyme (*cf.* Snell, 1958). Therefore, if pyridoxal-P should actually participate in the reaction catalyzed by the glycogen phosphorylases, functional groups other than the 4-formyl group must be involved. Illingworth *et al.* (1958), have already shown that the phosphate group of pyridoxal-P does not exchange with P_i or glucose-1-P in the course of the reaction. Recently we pointed out that the 5′-phosphate group of pyridoxal-P ($pK = 6.2$) would be a good candidate for a catalytically functional group in phosphorylase (Kastenschmidt *et al.*, 1968). This suggestion was strengthened by the results of kinetic measurements with the temperature-jump method by Ahrens *et al.* (1969) who showed that substituted hydroxypyridine 4-carbaldehydes lacking the 5′-phosphate group transfer protons in water between the ring nitrogen and the 3-oxygen mainly by an intermolecular route. On

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[1] Abbreviations used are: PN, pyridoxin or pyridoxol; PL, pyridoxal; PM, pyridoxamine. The corresponding phosphate derivatives of these compounds are PNP, PLP, and PMP, respectively. PL-N-O, etc., is pyridoxal *N*-oxide and other *N*-oxides, following IUPAC–IUB rules (1966, 1970). In addition, the following abbreviations are used: FDNB, fluorodinitrobenzene; DTNB, 5,5′-dithiobis(2-nitro)benzoic acid (Ellman's reagent); glycogen phosphorylase (EC 2.4.1.1); α-1,4-glucan: orthophosphate glucosyltransferase (EC 2.4.1.1).

introduction of the phosphate group in the 5′ position, an additional intramolecular proton transfer occurs involving three rather than two proton donor–acceptor groups. Undoubtedly, pyridoxal-P is a molecule tailored for a function as a proton shuttle (Jencks, 1969). Thus it could substitute for functional groups of amino acid side chains and participate in general acid base catalysis.

Therefore, analogs of pyridoxal-P were synthesized with all three protonatable groups modified. The 3′-*O*-methylpyridoxal-P analog had already been synthesized and was found to reactivate apophosphorylase *b* to *ca.* 25% as compared to reactivation by pyridoxal-P (Pocker and Fischer, 1969; Shaltiel *et al.*, 1969). Thus the phenolic OH group is not essential for activity. The nucleophilic pyridinium nitrogen of pyridoxal-P is a possible functional group. Bresler and Firsov (1968) have assigned a role to the ring nitrogen of pyridoxal-P in phosphorylase catalysis. Differential spectroscopy of phosphorylase *b* at high concentrations (20 mg/ml) in the presence of P_i or glucose-1-P revealed the appearance of a new maximum at 360 nm with a minimum at 330 nm. This was interpreted as indicating the formation of an ion pair between the pyridinium nitrogen of pyridoxal-P and one of the negatively charged groups of P_i or glucose-1-P. Fischer *et al.* (1970) however have stated evidence which strongly argues against this proposal. The *N*-methyl derivative of pyridoxal-P was prepared by Pocker and Fischer (1969), but does not bind to apophosphorylase (*cf.* Shaltiel *et al.*, 1969).

We, as well as Fischer and colleagues (1970) have studied the *N*-oxide derivative of pyridoxal-P prepared by the method of Fukui *et al.* (1969a). In this derivative the *N*-oxide group cannot be protonated except at pH <0.5. In contrast to *N*-methylpyridoxal-P, pyridoxal-P *N*-oxide binds to apophosphorylase *b*. Both groups found that pyridoxal-P *N*-oxide is photoreactive and that it partially reactivates apophosphorylase *b* (*cf.* Vidgoff, 1971). We now report that reactivation is due to pyridoxal-P formed from pyridoxal-P *N*-oxide in a specific reaction with apophosphorylase *b*. Although formation of pyridoxal-P is strongly light catalyzed, it also occurs in the dark and under nitrogen. Therefore we have synthesized 3′-*O*-methylpyridoxal-P *N*-oxide where protonation of the nitrogen is likewise blocked. This analog is stable and binds to apophosphorylase *b* but yields an inactive enzyme.

It is known from the work of Shaltiel *et al.* (1969), that substitution of the 5′-phosphate group of pyridoxal-P by a carboxymethyl or a sulfate group as in 5′-carboxymethyl-5′-deoxypyridoxal or pyridoxal 5′-sulfate yields on reconstitution inactive phosphorylases. The only known active analog modified at position 5 of pyridoxal-P is pyridoxal-5′-methylenephosphonate, prepared by Hullar (1969) which reactivates to about 20% (Fischer *et al.*, 1970; Vidgoff, 1971). But the phosphonate group with $pK_2 = 7.3$ (*cf.* Hullar, 1969) may still act as proton acceptor–donator group at physiological pH. In order to block the phosphate group of pyridoxal-P with $pK = 6.2$, the pyridoxal-P monomethyl ester was synthesized. It binds to apophosphorylase *b* but yields a completely inactive enzyme.

Materials and Methods

Coenzymes. Pyridoxine, pyridoxal, and pyridoxal-P are products of E. Merck AG, Darmstadt, Germany. Pyridoxamine-P was purchased from the Fluka AG, Buchs, Switzerland. ω-Hydroxypyridoxal-P was a generous gift from Dr. Saburo Fukui (Fukui *et al.*, 1969b). 3′-*O*-Methylpyridoxal-P was prepared according to Pocker and Fischer (1969). It was purified by chromatography on Dowex 50-X8 (H^+) and crystallized from hot water. 3′-*O*-Methylpyridoxal was prepared by the procedure of Heyl and Harris (1951). Pyridoxal-P *N*-oxide was synthesized following the procedure of Fukui *et al.* (1969a).

Reagents. Glucose 1-phosphate, 5′-AMP, glucose-6-phosphate dehydrogenase, phosphoglucomutase, and NADP were purchased from Boehringer, Mannheim. Activated charcoal was a product of Merck AG, Darmstadt. It was further purified by treatment with EDTA. Amberlite CG-50 I, Dowex 50-X8, and Dowex 1-X8 were obtained from Serva AG, Heidelberg, Germany. Sephadex G-25 was a product of Pharmacia, Uppsala, Sweden. Schleicher and Schüll paper no. 2043 was used for electrophoresis. MnO_2, form A, was obtained by heating $MnCO_3$ to 250° following the procedure of Harfenist *et al.* (1954). $MnCO_3$ was a product of Riedel de Haën, Hannover, Germany. [1-^{14}C]Iodoacetamide was obtained from the Radiochemical Centre, Amersham, England. Its specific activity was 50 Ci/mole. All other reagents were analytical grade. Doubly distilled water was used throughout. For detection of compounds separated by electrophoresis or thin-layer chromatography the following spray reagents were used: for pyridoxine derivatives with an unsubstituted 3-OH group (0.2%, w/v), *N*,2,6-trichlorochinonimide in methanol (Gibbs, 1927); for all derivatives with a substituted 3-OH group, Dragendorff's $[BiJ_4]^-$ reagent (Wagner *et al.*, 1961); for aldehyde (pyridoxal derivatives) (0.5% w/v), dinitrophenylhydrazine in 5% methanolic HCl; for amines (pyridoxamine derivatives) (0.25% w/v), ninhydrin in acetone.

Analytical Procedures. Thin-layer chromatography on kieselgel G was performed by the method of Stahl (1967). Ready-made plates (chromatoplates) for thin-layer chromatography were purchased from E. Merck AG, Darmstadt, and from M. Woelm, Eschwege. SiO_2 (0.08–0.2 mm) was a product of E. Merck, Darmstadt Germany. Columns of SiO_2 were used for the separation of lipophilic substances. Melting points were determined with a Linström apparatus. All melting points given are uncorrected. Elemental analyses were performed by A. Bernhardt, Elbach near Engelskirchen, and by R. Glier, Röthlein near Schweinfurt, Germany. Nuclear magnetic resonance (nmr) spectra were recorded with a Varian A60 instrument. Absorption spectra (240–500 nm) were recorded with a Zeiss PRQ 20A recording spectrophotometer. Extinction measurements at the maxima were carried out with a Zeiss PMQ II spectrophotometer. Temperature was kept constant by using jacketted thermostated cuvets.

Enzymes. Phosphorylase *b* was prepared from fresh rabbit skeletal muscle as described by Fischer and Krebs (1958) and Krebs *et al.* (1964). The enzyme was at least three-times recrystallized. It was stored at +4° under toluene vapor. Except where stated otherwise, the usual buffer was a 50 mM glycero-P–50 mM 2-mercaptoethanol–2 mM EDTA solution (pH 6.8, $\Gamma/2 = 0.14$). If required, 5′-AMP was removed by passing the enzyme over a short column (1 × 0.5 cm) of purified activated charcoal. The OD 260:OD 280 nm ratio of the AMP-free enzyme was $\leqslant 0.53$. Apophosphorylase *b* was prepared by the procedure of Shaltiel *et al.* (1966). It was collected by precipitation with $(NH_4)_2SO_4$ as described by Kastenschmidt *et al.* (1968). Reconstitution of apophosphorylase *b* with pyridoxal-P or with analogs of pyridoxal-P was carried out as described by Hedrick *et al.* (1966). Pyridoxal-P or analogs of pyridoxal-P were incubated with the

protein in 50 mM glycero-P–50 mM 2-mercaptoethanol–2 mM EDTA buffer (pH 6.8) for 30 min at 30° except where stated otherwise. Pyridoxal-P or analogs were added at varying excesses (see text). Unbound cofactors were removed by chromatography on Sephadex G-25, fine grade.

Activity Measurements. Activity assays were carried out both in the direction of glycogen synthesis and glycogen degradation. For routine assay, the release of P_i from glucose-1-P in the presence of glycogen and 5′-AMP was measured. The conditions were those described by Kastenschmidt *et al.* (1968). P_i was measured by the method of Fiske and Subbarow (1925). In some instances, the coupled assay of Helmreich and Cori (1964a) or arsenolysis of glycogen were used (*cf.* Helmreich and Cori, 1964b). Protein concentrations were determined by absorbance measurements at 280 nm using an absorbancy index (1% × cm^{-1}) of 13.2 (Kastenschmidt *et al.*, 1968). In certain instances protein concentrations were determined with the method of Lowry *et al.* (1951). Bovine serum albumin was used as reference standard.

Analyses of Enzyme-Bound Pyridoxal-P and Pyridoxal-P Analogs. Analyses of reconstituted apophosphorylase proteins for bound pyridoxal-P or analogs of pyridoxal-P involved precipitation and release of the cofactors from the protein. All concentrations of enzyme are calculated for a molecular weight of 200,000 for dimer *b* (*cf.* Cohen *et al.*, 1971). The enzyme proteins, usually 5×10^{-5} M, were precipitated with saturated neutralized $(NH_4)_2SO_4$ in the cold. The precipitate was taken up in glycero-P–2 mM EDTA–50 mM 2-mercaptoethanol buffer (pH 6.8). The suspension containing about 40–50 mg of protein/ml was then dialyzed first for 5 hr against 1 l. of 0.01 N neutral NaCl solution and then against 1 l. of double-distilled water for another 5 hr in order to remove the salt. To the dialyzed solutions containing *ca.* $1.5–2 \times 10^{-4}$ M phosphorylase protein and *ca.* $3–4 \times 10^{-4}$ M pyridoxal-P or an analog of pyridoxal-P, 1.2 N $HClO_4$, or 40% trichloroacetic acid was added to a final concentration of 0.3 N or 7%, respectively (*cf.* Baranowski *et al.*, 1957). For chromatographic and electrophoretic analyses of pyridoxal-P or pyridoxal-P analogs released from the enzyme, the acid supernatant solutions were concentrated by adsorption on a column (0.5 × 0.2 cm) of Dowex 1-X8 (formate form) and elution with a small volume of 3 M formic acid. The concentrations of pyridoxal-P or analogs usually recovered were sufficient for spectroscopic analyses. Trichloroacetic acid was removed by extraction with peroxide-free ethyl ether and $HClO_4$ was removed by precipitation with $KHCO_3$. For quantitative estimation of pyridoxal derivatives the method of Wada and Snell (1961) was used. The following extinction coefficients ($M^{-1} \times cm^{-1}$) at 410 nm of the corresponding phenylhydrazones were used: pyridoxal-P, 23,800; pyridoxal, 22,200; pyridoxal-P *N*-oxide, 20,100; 3′-*O*-methylpyridoxal-P, 25,000; 3′-*O*-methylpyridoxal-P *N*-oxide, 24,500; pyridoxal-5′-P methyl ester, 24,000.

Measurement of Pyridoxal-P Formed from Pyridoxal-P N-Oxide by Phosphorylase. For determination of pyridoxal-P and pyridoxal-P *N*-oxide, aliquots were removed from the incubation mixture after 20 min and the cofactor released from the protein by precipitation with 0.3 M $HClO_4$. To an aliquot of the supernatant fluid, the same volume of 0.5 M NaOH was added. The pyridoxal-P formed from pyridoxal-P *N*-oxide was determined from the decrease in the 295-nm absorbance of the original pyridoxal-P *N*-oxide solution. Pyridoxal-P *N*-oxide at neutral and alkaline pH has (in contrast to pyridoxal-P) a strong absorbance band at 295 nm (ϵ 9800) at pH 7.0 (*cf.* Fukui *et al.*, 1969a). Furthermore, use can be made of the differences in the molar extinction coefficients of the phenylhydrazones of pyridoxal-P and pyridoxal-P *N*-oxide.

Carboxyamidomethylation of Phosphorylase. Holophosphorylase *b* was recrystallized from 15 mM dithioerythritol. Dithioerythritol was carefully removed by passing the enzyme solution over Sephadex G-25 equilibrated with 0.13 M KCl–0.03 M sodium glycerophosphate–1.5 mM EDTA buffer (pH 7.5) (*cf.* Battell *et al.*, 1968). The enzyme (3.5×10^{-5} M) freed from dithioerythritol was reacted in the same buffer with 1×10^{-3} M [1-^{14}C]iodoacetamide for 2 hr at 30° as described by Battell *et al.* (1968). The reaction was stopped by addition of 50 mM 2-mercaptoethanol–50 mM glycero-P buffer (pH 6.8). $NaBH_4$-reduced phosphorylase *b* was carboxyamidomethylated like native holophosphorylase *b* except that incubation with [1-^{14}C]iodoacetamide was carried out for 2.5 hr. The protein was precipitated with an equal volume of saturated $(NH_4)_2SO_4$ (pH 7.0) and separated from $(NH_4)_2SO_4$ by passage over Sephadex G-25. For radioactivity measurements, a 50-μl aliquot of the protein solution was added to 15 ml of Bray's solution and counted in glass vials in a Packard liquid scintillation spectrometer, Model 3380 (Bray, 1960). The statistical deviation from the mean was ±1%. All counts were corrected for quenching. Average counting efficiency was 30%.

Pyridoxal-P was removed from the carboxyamidomethylated proteins as described for native phosphorylase *b*. Reconstitution of the carboxyamidomethylated apoenzyme with pyridoxal-P or pyridoxal-P *N*-oxide was carried out in the usual manner.

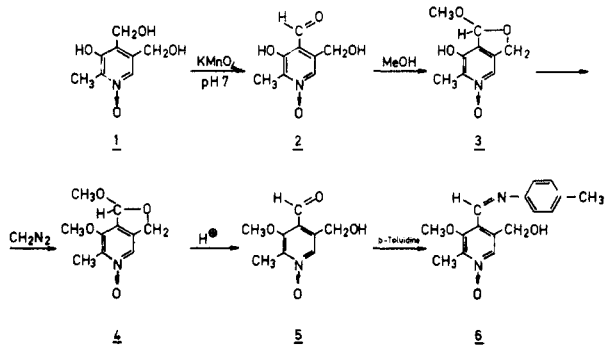
Titration of SH Groups with DTNB. Apo- or holophosphorylase *b* was treated with pyridoxal-P *N*-oxide or pyridoxal-P as described in Table III. Following this procedure, the enzymes were treated with a 20-fold molar excess of DTNB over the phosphorylase for 6 hr at 23° in 50 mM glycero-P–2 mM EDTA buffer (pH 6.8). The reaction was carried out in the dark and under nitrogen. The nitrothiophenylate anion was determined spectrophotometrically at 412 nm (*cf.* Kastenschmidt *et al.*, 1968). The reconstituted enzyme without DTNB and a solution of DTNB in buffer served as blanks.

Sedimentation velocity measurements were carried out in a Spinco Model E analytical ultracentrifuge equipped with schlieren optics. A single-sector cell with a 1.2-cm light path was used. The rotor speed was 59,780 rpm and the bar angle was 70°. The temperature (35°) was maintained constant within ±0.3°. Pictures were taken 4–24 min after the rotor had reached full speed. The schlieren diagrams were evaluated by calculating boundary movements from direct measurements with a Leitz microcomparator. $s_{20,w}$ values were corrected for the change in viscosity and density of the solvent with the temperature. The concentration of the enzyme was 2.65×10^{-5} M, in each case. All experiments were carried out in 50 mM glycero-P–50 mM 2-mercaptoethanol–2 mM EDTA buffer (pH 6.8, $\Gamma/2 = 0.14$).

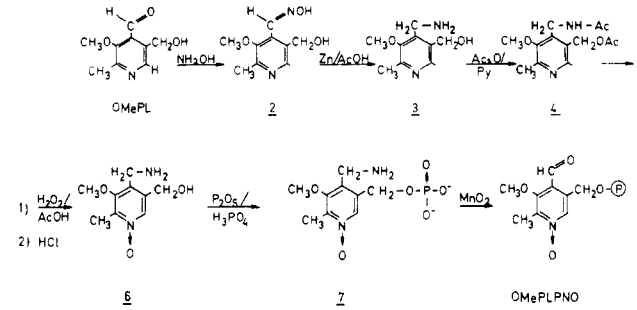
Synthetic Procedures

General Remarks. At first we tried to synthesize 3′-*O*-Me-PLP-N-O from PL-N-O. But as was the case with 3′-*O*-Me-PL we could not phosphorylate the Schiff base of 3′-*O*-Me-PL-N-O with polyphosphoric acid (*cf.* Murakami *et al.*, 1966). The lesser stability of the Schiff base of the phenolic ethers of the pyridoxal derivatives might favor condensation reactions of the aromatic aldehydes, whereby resinous high molec-

SCHEME I



SCHEME II



ular products are formed. Therefore, phosphorylation was carried out with the corresponding pyridoxamine derivatives (*cf.* Peterson and Sober, 1954).

Trichloroacetonitrile was used as the dehydrating agent for the synthesis of the pyridoxal phosphate monomethyl ester because it reacts like carbodiimide with phosphoric acid (pK_2 and pK_3) and with the monoester (pK_2), each forming imidoyl phosphate derivatives as intermediates. Since the lesser nucleophilic phosphoric acid (pK_1) does not react, the reaction leads only to the corresponding phosphodiester of PL as in the case of the pyridoxal phosphate monomethyl ester (*cf.* Cramer and Weimann, 1960, 1961). Initially we have encountered difficulties in the direct methylation of the phosphate group of PLP. Recently we have overcome these difficulties. Therefore we describe below two different procedures for the preparation of crystalline pyridoxal phosphate monomethyl ester (1) from 4′-*N*-Ac-PMP and (2) from PLP.

Preparation of 3′-O-Me-PL-N-O (*See Scheme I*). PN-N-O (**1**) was prepared from triacetylated PN by the procedure of Sakuragi and Kummerow (1959). PN-N-O (**1**) was converted to PL-N-O (**2**) by oxidation with $KMnO_4$ in neutral solution at room temperature. The methyl acetal of **2** was obtained by heating **2** with anhydrous methanol and (**3**) was reacted with diazomethane to the methyl acetal (**4**) of 3′-*O*-Me-PL-N-O. Compound **4** was purified by chromatography on SiO_2. The yield was 25%, relative to **2** (mp 158–165°). *Anal.* Calcd for $C_{10}H_{13}NO_4$: C, 56.8; H, 6.14; N, 6.63. Found: C, 56.7; H, 5.95; N, 6.58. The nmr spectra ($CDCl_3$, internal standard Me_4Si; δ in parts per million) showed 8.0, 1 H singlet of *H*-6; 6.35, 1 H singlet of 4′-CH; 5.1, 2 H singlet of 5′-CH_2; 4.1, 3 H singlet of 3′-OCH_3; 3.5, 3 H singlet of 4′-OCH_3; 2.5, 3 H singlet of 2′-CH_3. The ultraviolet (uv) spectra (nanometers) showed 258.5 (ϵ 9000) and 293 (ϵ 3300) in 0.1 N HCl, 259 (11,600) and 296 (2300) in 0.1 M phosphate buffer (pH 7), 259 (11,800), and 293 (2500) in 0.1 N NaOH. The 3′-*O*-Me-PL-N-O (**5**) was obtained from **4** by hydrolysis with HCl. *Anal.* Calcd for $C_9H_{11}NO_4 \cdot HCl$: C, 46.4; H, 5.14; N, 6.0. Found: C, 46.2; H, 5.18; N, 5.87. The uv spectra showed 259 (8200) and 293 (2900) in 0.1 M HCl, 257.5 (15,000) and 304 (7000) in 0.1 M phosphate buffer (pH 7), 259 (10,400) and 293 (2300) in 0.1 M NaOH.

Attempts to phosphorylate the Schiff base of **5** with *p*-toluidine (**6**) in 5′ position by reaction with polyphosphoric acid or $POCl_3$ were unsuccessful. Although reaction with $POCl_3$ produced (in very low yield) a material with λ_{max} near 297 nm, this material could not be crystallized.

Preparation of 3′-O-Me-PLP-N-O (*See Scheme II*). The 3′-*O*-Me-PL methyl acetal (**1**) was obtained by the procedure of Heyl and Harris (1951). 3′-*O*-Me-PL oxime (**2**) was obtained from **1** rather than from 3′-*O*-Me-PL·HCl as described by Pocker and Fischer (1969). Compound **1** (5 g) was heated in 50 ml of 1 N HCl containing 10 g of $NH_2OH \cdot HCl$ for 30 min at *ca.* 100° in a water bath and then brought to pH 4.5 by addition of solid sodium acetate. The mixture was kept in the refrigerator until crystallization of **2** was complete (mp 195°, according to Pocker and Fischer (1969), mp 196°). The 3′-*O*-Me-PM (**3**), which was not isolated, was obtained from **2**. Compound **2** (5 g) was dissolved in 30 ml of glacial acetic acid and stirred with 5 g of powdered metallic Zn for 3 hr at room temperature. To make **4**, the yellow resinous residue was separated from the unreacted Zn and taken up in 30 ml of acetic anhydride. Anhydrous sodium acetate (1 g) was added and the mixture was stirred on a steam bath for 2 hr. After evaporation the residue was extracted with $CHCl_3$ and the organic layer applied to a silica gel column. Elution with a 9:1 mixture of $CHCl_3$–methanol removed the 3′-*O*-Me-PM 4′-*N*,5′-*O*-diacetate (**4**), from the column, which on addition of ether and an excess of petroleum ether (bp 30–60°), precipitated as fine needles. It was recrystallized from MeOH–Et_2O. The yield was 3.1 g (mp 112°). The nmr spectra ($CDCl_3$, internal standard, Me_4Si) showed 8.1, 1 H singlet of 6-H; 6.35, 1 H singlet (br) of 4′-NH; 5.18, 2 H singlet of 5′-CH_2; 4.4, 2 H doublet of 4′-CH_2; 3.66, 3 H singlet of 3′-OCH_3; 2.4, 3 H singlet of 2′-CH_3; 1.83 and 1.95, two 3 H singlets of 4′- and 5′-$COCH_3$.

The 4′-*N*,5′-*O*-diacetate of 3′-*O*-Me-PM (**4**) was oxidized to 3′-*O*-Me-N-O diacetate (**5**) by the same procedure used in the preparation of PN-N-O. Compound **4** (4 g), dissolved in a mixture of 50 ml of glacial acetic acid and 20 ml of a 30% H_2O_2 solution was heated for 10 hr at 70°. After the reaction mixture was concentrated *in vacuo*, the oily residue was crystallized and recrystallized from ethyl ether–petroleum ether. The yield was 2.8 g or 60% of **4** as starting material (mp 130–131°). *Anal.* Calcd for $C_{13}H_{18}N_2O_5$: C, 55.4; H, 6.4; N, 9.95. Found: C, 55.56; H, 6.62; N, 10.2.

Compound **5** (2.5 g) was deacetylated by refluxing for 70 min in a mixture of concentrated HCl (7.5 ml), ethanol (60 ml), and H_2O (32.5 ml). After evaporation, the 3′-*O*-Me-PM-N-O (**6**) was phosphorylated by the method used for the preparation of PMP (Peterson and Sober, 1954). The product was purified by ion-exchange chromatography with water as eluent. 3′-*O*-Me-PMP-N-O (**7**) was crystallized from water–acetone (mp 188–198° dec). The yield starting from **5** was 60%. The uv spectra showed 263 (ϵ 11,800) in 0.1 N HCl, 263 (12,600) in 0.1 M phosphate buffer (pH 7), and 260 (13,100) in 0.1 N NaOH. *Anal.* Calcd for $C_9H_{15}N_2O_6P \cdot H_2O$: C, 36.6; H, 5.8; N, 9.46. Found: C, 37.04; H, 6.11; N, 9.68.

To a solution of 1 g of **7** in 100 ml of water, 4 g of MnO_2 type A was added and the suspension was stirred at room temperature. After *ca.* 96 hr the ninhydrin reaction became

TABLE I: Thin-Layer Chromatography of PL Derivatives on Silica Gel (R_F Values).

PL Analogues	Solvent System: Et_2O–MeOH (6:2, v/v)	0.1% NH_3	*n*-BuOH–H_2O–HCOOH (65:15:15, v/v)
3′-*O*-Me-PL-N-O methyl acetal	0.46		
3′-*O*-Me-PL-N-O		0.57	
3′-*O*-Me-PM(Ac)$_2$	0.61		
3′-*O*-Me-PM-N-O(Ac)$_2$	0.21		
3′-*O*-Me-PMP-N-O		0.34	
3′-*O*-Me-PLP-N-O		0.48	0.23
PLP		0.7	0.33
PLP-N-O		0.91	0.20
3′-*O*-Me-PLP			0.33
ω-OH-PLP		0.66	0.24
PMP		0.57	0.04
PMP(Ac)		0.75	0.15
PM(Ac)-5′-P-ME[a]		0.73	0.19
PM-5′-P-ME[a]			0.07
PL-5′-P-ME[a]		0.32	0.43

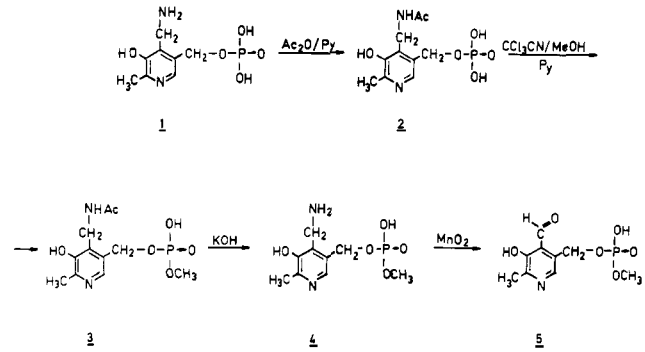
[a] 5′-PME = 5′-phosphate monomethyl ester.

negative, indicating that oxidation was complete. After removing the MnO_2 the filtrates were concentrated and applied to a Dowex 1-X8 column in the acetate form and eluted with a linear gradient of acetic acid (2–5 M). Fractions containing 3′-*O*-Me-PLP N-O (**8**) with a λ_{max} at 296 nm were further purified by chromatography on a Dowex 50-X8 column, H^+ form. Compound **8** crystallized as white prisms from water–acetone (mp 160–171° dec). *Anal.* Calcd for $C_9H_{12}NO_7P \cdot 2H_2O$: C, 34.42; H, 5.1; N, 4.46. Found: C, 34.38; H, 4.89; N, 4.84. The uv spectra showed 297 (ε 11,640), 259 (3120) in 0.1 N HCl, 296 (11,800), 259 (3140) in 0.1 N phosphate buffer (pH 7), 296 (7000), and 262 (7580) in 0.1 N NaOH. The nmr spectra (in D_2O, 2,2-dimethyl-2-silapentane-5-sulfonate (Na salt) as the internal standard, δ in parts per million) showed 10.2, 1 H singlet of C_4-CHO; 8.25, 1 H singlet of 6-H; 5.2, 2 H doublet of 5′-CH_2 (J = 7 Hz); 3.75, 3 H singlet of 3′-OCH_3; 2.55, 3 H singlet of 2′-CH_3.

The 3′-*O*-Me-PLP-N-O (**8**) was dephosphorylated by heating for 15 hr at 70° in 0.05 N HCl. The dephosphorylated compound (3′-*O*-Me-PL-N-O) was identical with the substance prepared by the different route described above (see Scheme I). Both substances were identical with respect to their behavior on thin-layer chromatography (see Table I), uv spectra, and melting point.

Blocking the phenolic OH group with a methyl group as in 3′-*O*-Me-PLP-N-O makes the derivatives less labile to decomposition in light and oxygen as compared to the PLP-N-O. Nevertheless, 3′-*O*-Me-PLP-N-O is more labile in oxygen and light than 3′-*O*-Me-PLP. λ_{max} at 296 nm of a solution of 3′-*O*-Me-PLP-N-O decreased by 5% and the band was broadened toward shorter wavelengths on standing at room temperature for 2 days when the solution was unprotected from atmospheric oxygen and light.

SCHEME III



Preparation of Pyridoxal Phosphate Methyl Ester (*See Scheme III*). PMP·2HCl (1 g) (**1**) was acetylated in a mixture of equal parts (5 ml each) of pyridine and acetic anhydride for 12 hr. The volatile components were removed *in vacuo* and the remaining slightly yellow oil was taken up in 5 ml of MeOH where white microcrystals of 4′-*N*-Ac-PMP (**2**) formed immediately. The yield was 85%, mp 233° dec. To 0.8 g of **2**, dissolved in enough anhydrous pyridine, and 0.8 ml of trichloroacetonitrile (distilled over P_2O_5), 0.4 ml of anhydrous MeOH was added. The mixture was stirred and heated at 80° for 10 hr under reflux with precautions taken to exclude moisture. After removing the solvents *in vacuo*, the residual oil was taken up in 30 ml of water and separated from precipitated trichloroacetamide by filtration. The solution of 4′-*N*-acetylpyridoxamine-P methyl ester (**3**) was concentrated and applied to a Dowex 50-X8 (H^+) column. Elution by H_2O yielded **3** first and then well separated from it a small amount of unreacted **2**. Compound **3** crystallized as thick rods from water–acetone (mp 177°; yield 52% based on **2**). The nmr spectra (in D_2O, internal standard, 2,2-dimethyl-2-silapentane-5-sulfonate) showed 8.0, 1 H singlet of 6-H; 5.0, 2 H doublet of 5′-CH_2 (J = 8 Hz); 4.6, 2 H singlet of 4′-CH_2; 3.6, 3 H doublet of P-OCH_3 (J = 11 Hz); 2.6, 3 H singlet of 2′-CH_3; 2.0, 3 H singlet of CH_3CO.

Compound **3** (0.5 g) was deacetylated with 3 N KOH and left in the dark for 2 days at room temperature. After neutralization the reaction mixture was applied to a column of Amberlite CG-50 I (H^+). Elution was carried out with water. Ninhydrin-positive fractions, *i.e.*, pyridoxamine-P methyl ester (**4**), were pooled and concentrated.

Compound **4** crystallized as fine needles from MeOH–acetone. The yield was 80% based on **3** (mp 205–208°). *Anal.* Calcd for $C_9H_{16}N_2O_6P$: C, 41.2; H, 5.75; N, 10.6. Found: C, 41.62; H, 5.87; N, 10.2. The uv spectra showed 295.5 (ε 9100) in 0.1 N HCl, 252 (4800) and 325 (7900) in 0.1 N phosphate buffer (pH 7), and 245 (6600) and 308 (7900) in 0.1 N NaOH.

Compound **4** (0.5 g) was dissolved in 50 ml of water, then MnO_2-A (0.5 g) was added, and the solution was stirred at room temperature for 2 hr. Excess MnO_2-A was removed and the filtrate was concentrated *in vacuo* to about 15 ml. After addition of 0.25 g of *p*-toluidine in 0.1 N acetic acid, the Schiff base of *p*-toluidine and pyridoxal phosphate methyl ester (**5**) precipitated at pH 5–6 as yellow platelets. The Schiff base derivative of **5** was taken up in a few milliliters of MeOH and chromatographed on Sephadex-LH equilibrated with acetone. Three well-separated yellow bands appeared. The last band eluted from the column with acetone was the Schiff base derivative of **5**. The Schiff base was hydrolyzed in 2 N

KOH, applied to a column of Dowex 50-X8 (H^+) and eluted with water. Yellow fractions containing **5** were pooled and concentrated to an amorphous residue which was dissolved in a small volume of a *ca.* 1:1 methanol–dioxane mixture and acetone was added dropwise in the cold until the solution became turbid. After scratching the surface of the flask, crystals appeared and crystallization was completed over night in the refrigerator. Recrystallization was carried out from acetone–water. The yield of **5** was 40% relative to **4** as starting material (mp 112° dec). *Anal.* Calcd for $C_9H_{12}NO_6P \cdot H_2O$: C, 39.4; H, 5.09; N, 5.0. Found: C, 40.05; H, 4.87; N, 4.81. The uv spectra showed 295 (ϵ 6460) in 0.1 N HCl, 330 (1840) and 388 (4810) in 0.1 N phosphate buffer (pH 7), and 305 (800) and 388 (5800) in 0.1 N NaOH. The nmr spectra (D_2O, internal standard, 2,2-dimethyl-2-silapentanesulfonate) showed 8.15, 1 H singlet of 6-H; 6.45, 1 H singlet of 4′-CH; 5.1, 2 H doublet of 5′-CH_2 (J = 7 Hz); 3.5, 3 H doublet of P-OCH_3 (J = 11 Hz); 2.63, 3 H singlet of 2′-CH_3.

Abbreviated Procedure for the Preparation of Pyridoxal Phosphate Methyl Ester. PLP (0.5 g, dried over P_2O_5) was heated in a mixture of 30 ml of anhydrous pyridine, 1 ml of trichloroacetonitrile (distilled over P_2O_5) and 1 ml of anhydrous MeOH for 9 hr under reflux. After removing the solvents *in vacuo*, the residual oil was taken up in 25 ml of water and separated from trichloroacetamide. The filtrate was chromatographed on Dowex 50-X8 (H^+). The whole eluate was concentrated *in vacuo* and the Schiff base of *p*-toluidine and the pyridoxal phosphate methyl ester were formed as described above. After hydrolysis and removal of the *p*-toluidine the solution was neutralized and applied to a column of Dowex 1-X8 (H^+). The pyridoxal phosphate monomethyl ester was eluted from the column with an acetic acid gradient (1–3 M) and the eluate was concentrated *in vacuo*. The yellow residue was dissolved in a small volume of methanol and precipitated with acetone. The pyridoxal phosphate methyl ester crystallized in the cold over night. It was recrystallized from water–acetone. The yield was 11% relative to PLP as starting material (mp 114° dec). The product was identical with regards to the uv and nmr spectra and the melting point with the pyridoxal phosphate monomethyl ester synthesized according to Scheme III.

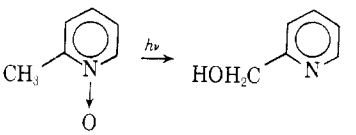
Preparation of N-Me-PLP. The *N*-Me-PLP was prepared and characterized for the first time by Pocker and Fischer (1969). We found it more convenient, however, to methylate PLP in one step to *N*-Me-PLP with dimethyl sulfate at pH 12–13 with a yield of about 60%. The *N*-Me-PLP was purified by chromatography on Dowex 50-X8 (H^+) and characterized by its uv and nmr spectra. The absorption maxima of the compound in 0.1 N HCl were at 298 and 340 nm, respectively, whereas in 0.1 M phosphate buffer (pH 7.0) or in 0.1 M NaOH the maxima were at 330 and 399 nm. Thus, the substance had the same spectral properties as the compound prepared by Pocker and Fischer (1969). By nmr spectroscopy a 3 H singlet at 4.25 ppm was found which is characteristic for the $\geqslant N^+-CH_3$ group.

Results and Discussion

Role of the N_1 Group of Pyridoxal-P

N-Me-PLP. The *N*-Me derivative of pyridoxal-P was found not to bind to apophosphorylases *b* and *a* presumably because of steric hindrance or electrostatic repulsion by the positively charged nitrogen or both (Shaltiel *et al.*, 1969). We have confirmed these findings. No aldehyde reactive with phenylhydrazine in the Wada–Snell method (1961) was recovered from an apophosphorylase *b* preparation reconstituted with a fivefold molar excess of *N*-Me-PLP followed by gel filtration on Sephadex G-25.

Pyridoxal-P N-Oxide Photoreaction. Pyridoxal-P *N*-oxide is rapidly photooxidized at neutral and alkaline pH. Major oxidation products, besides pyridoxal-P, are pyridoxic acid 5′-phosphate and an unidentified compound that migrates faster than pyridoxal-P *N*-oxide toward the anode on paper electrophoresis with formic acid (pH 2.5). This substance is colorless and has the same blue violet fluorescence as pyridoxic acid 5′-phosphate and most likely is the *N*-oxide of this compound. Photolysis of aromatic *N*-oxides has recently been used as model reaction for the study of enzymatic hydroxylation reactions. Phenols are readily hydroxylated, but even less reactive alkanes especially branched alkanes, are also hydroxylated by aromatic *N*-oxides (*cf.* Tsuchiya *et al.*, 1969; Streith *et al.*, 1967; Jerina *et al.*, 1970). An interesting reaction is the photoreaction of 2-picolin *N*-oxide to 2-hydroxymethylpyridine (*cf.* Ito and Hata, 1955; Hata, 1961). We have looked



for the corresponding ω-hydroxypyridoxal-P as a possible product both in the photoreaction of pyridoxal-P *N*-oxide and in the deoxygenation reaction of pyridoxal-P *N*-oxide with apophosphorylase *b*, but did not find any ω-hydroxypyridoxal-P. This analog is even more active than pyridoxal-P because when added at a 1:1 molar ratio it reactivates apophosphorylase *b* in the standard glycerophosphate buffer (pH 6.8) to 120% as compared to pyridoxal-P. Pyridoxal-P *N*-oxide solutions in 50 mM glycerophosphate buffer (pH 6.8) form pyridoxal-P on exposure to daylight for 60 min at room temperature. In general, more pyridoxal-P was formed in the light in dilute than in more concentrated pyridoxal-P *N*-oxide solutions. The experiments in Table II show that the pyridoxal-P formed in the light is responsible for the higher specific activity of phosphorylase reconstituted with pyridoxal-P *N*-oxide solutions preexposed to light.

Formation of Pyridoxal-P from Pyridoxal-P N-Oxide by Apophosphorylase b in the Dark. Solutions of pyridoxal-P *N*-oxide in glycerophosphate buffer (pH 6.8) were stable when kept in the dark and in the absence of oxygen. Neither pyridoxal-P nor any other compound besides pyridoxal-P *N*-oxide was found by thin-layer chromatography. We have stored pyridoxal-P *N*-oxide in substance in the dark for 1.5 years without any detectable decomposition or formation of pyridoxal-P. Therefore, the pyridoxal-P formed upon incubation of apophosphorylase with pyridoxal-P *N*-oxide under conditions strictly excluding light and oxygen must result from a reaction of the pyridoxal-P *N*-oxide with apophosphorylase itself. All subsequent experiments were carried out in the dark and in the absence of oxygen. Reconstitution of apophosphorylase *b* with pyridoxal-P *N*-oxide at 30° and at a molar ratio of 1.0–1.25. yielded an enzyme preparation with an activity ranging from 20 to 25% of that of pyridoxal-P reconstituted phophorylase *b* (see Figure 1). Although, when more pyridoxal-P *N*-oxide is added more pyridoxal-P is formed, the ratio of pyridoxal-P formed:pyridoxal-P *N*-oxide added actually declines. This suggests that the deoxygenation of pyridoxal-P is limited by the availability of sites. Accordingly, at higher molar ratios of pyridoxal-P *N*-oxide to apophosphorylase *b*, the formation of active en-

TABLE II: Formation of PLP on Exposure of PLP-N-O to Light.[a]

Preexposure of PLP-N-O	Act. of Phosphorylase *b* Reconstituted with PLP-N-O (μmoles/min per mg)
Dark, 60 min, room temperature	12.0
Light, 60 min, room temperature	18.9
Light, 60 min, room temperature[b]	18.7

[a] PLP-N-O (7.1×10^{-5} M) was dissolved in 50 mM glycerophosphate–2 mM EDTA buffer (pH 6.8). Reconstitution of apophosphorylase *b* (3.5×10^{-5} M) with the PLP solutions was carried out in the dark for 30 min at 30°. Activity was measured in the routine assay. [b] Assay carried out in the presence of 0.05 M 2-mercaptoethanol.

zyme does not further increase but rather declines (see Figure 1). This suggested to us that pyridoxal-P *N*-oxide phosphorylase itself is inactive and that the active enzyme is formed from apophosphorylase and that part of pyridoxal-P *N*-oxide which is converted to pyridoxal-P. In a large-scale experiment, the only compounds resolved from the enzyme and detected by thin-layer chromatography were pyridoxal-P and pyridoxal-P *N*-oxide. As was to be expected, the pH–activity profiles of the enzymes obtained either by reconstitution of apophosphorylase *b* with pyridoxal-P or pyridoxal-P *N*-oxide are therefore identical (Figure 2). Apophosphorylase *b* preparations reconstituted with pyridoxal-P *N*-oxide at a molar ratio of 1:1 had specific activities of 11–14 μmoles of $P_i \times mg^{-1} \times min^{-1}$ under standard assay conditions at pH 6.8. Incubation of pyridoxal-P *N*-oxide phosphorylase *b* (5×10^{-5} M) with 1×10^{-4} or 2×10^{-4} M pyridoxal-P for 30 min at 30° prior to assay did not further increase the activity.

Reactivation of apophosphorylase *b* with pyridoxal-P *N* oxide at 30° was complete in 2 min. In the same time, the formation of pyridoxal-P was also complete (see Figure 3). As shown in Figure 3, reduced phosphorylase *b* with pyridoxamine-P irreversibly bound to the protein also reacts with pyridoxal-P *N*-oxide. Native holophosphorylase *b* (not shown in Figure 3) reacted like reduced phosphorylase *b*. However, compared to apophosphorylase *b*, reduced or holophosphorylase *b* forms pyridoxal-P from pyridoxal-P *N*-oxide initially much slower. Moreover, even when the reaction finally reached completion, only half as much pyridoxal-P was formed from pyridoxal-P *N*-oxide by reduced or holophosphorylase *b*. This suggests that the deoxygenation does not take place, exactly or exclusively at the same site to which pyridoxal-P is bound in the active enzyme.

The Reductant in the Deoxygenation of Pyridoxal-P N-Oxide by Apophosphorylase b. Aromatic *N*-oxides are often used in preparative organic chemistry to facilitate heterocyclic substitution reactions, especially in position 4 of the aromatic ring. The oxygen is usually removed by strong electrophiles such as PCl_3, $SOCl_2$, triphenylphosphite, etc. (see Hamana, 1955),[2] but according to Relyea *et al.* (1962),

[2] As cited in E. Ochiai (1967).

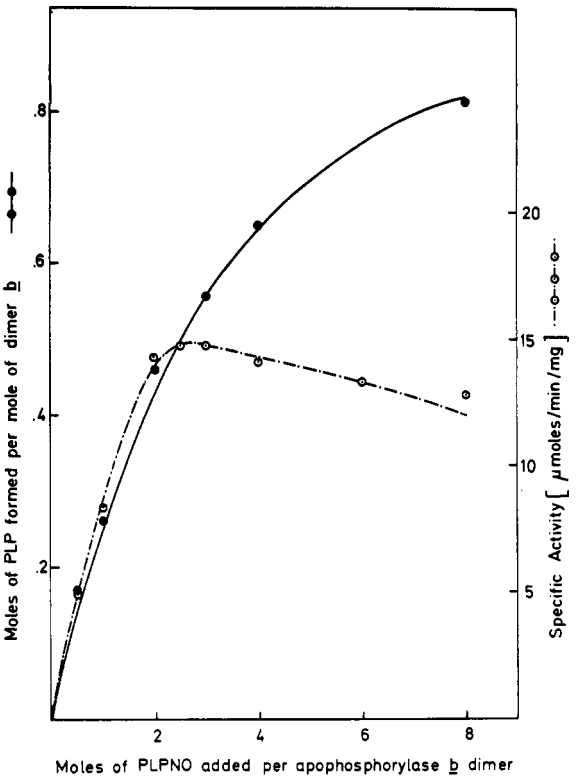


FIGURE 1: Formation of pyridoxal-P from pyridoxal-P *N*-oxide by apophosphorylase *b*. Apophosphorylase *b* (5×10^{-5} M) was incubated with PLP-N-O at the molar ratio indicated for 20 min at 30° in 50 mM glycerophosphate–2 mM EDTA buffer (pH 6.8). The reaction was carried out in the dark and under nitrogen.

sulfhydryls are also capable of deoxygenating aromatic *N*-oxides. An example is the deoxygenation of 2-picoline *N*-oxide to 2-picoline by 1-butanethiol. Traynelis and Yamauchi (1969) reported that aromatic *N*-oxides oxidize dimethyl sulfoxides to dimethyl sulfones. An electron attracting group in the para position enhances this reaction.

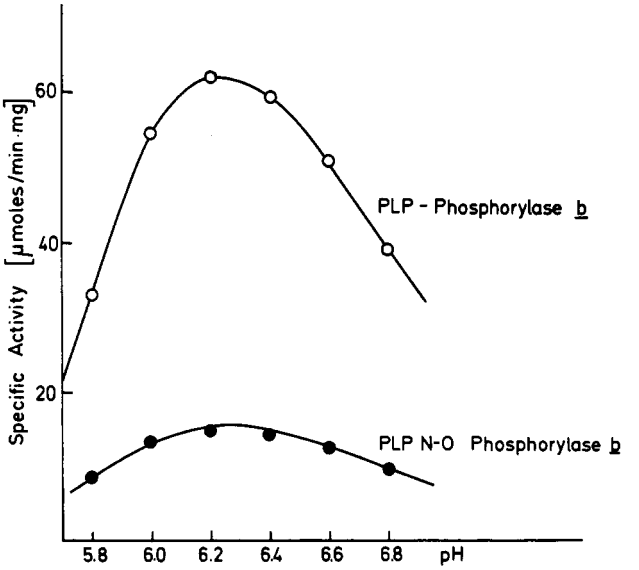


FIGURE 2: Activity–pH profiles of pyridoxal-P and pyridoxal-P *N*-oxide phosphorylase *b*. Apophosphorylase *b* (3.5×10^{-5} M) was reconstituted with a 1.5-fold molar excess of PLP or PLP-N-O.

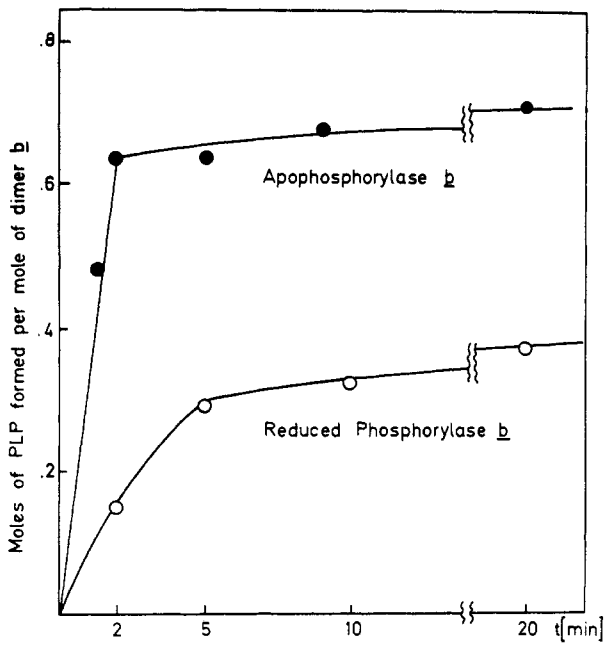


FIGURE 3: Time course of pyridoxal-P formation from pyridoxal-P *N*-oxide by apo- and reduced phosphorylase *b*. Apophosphorylase *b* and reduced phosphorylase *b* (5×10^{-5} M each) were incubated in 0.5 ml of 50 mM glycerophosphate–2 mM EDTA buffer (pH 6.8) with a twofold molar excess of PLP-N-O in the dark and under nitrogen. The reaction was stopped at the times indicated on the abscissa by the addition of 0.9 ml of 0.3 N $HClO_4$.

This also applies to pyridoxal-P *N*-oxide which readily oxidizes dimethyl sulfoxide, forming dimethyl sulfone and pyridoxal-P as shown by Vidgoff (1971). On the other hand, sulfhydryls which are rather easily oxidized such as cysteine, dithiothreitol or 2-mercaptoethanol or egg albumin (which has many cysteinyl residues) react extremely slowly with pyridoxal-P *N*-oxide. Even after 12-hr incubation of pyridoxal-P *N*-oxide with 100 mM cysteine in glycerophosphate buffer (pH 6.8) at 50°, no measurable amounts of pyridoxal-P were formed. Lysozyme, when added at comparable or even higher concentration than apophosphorylase *b* did not deoxygenate pyridoxal-P *N*-oxide. L-histidine, L-lysine, L-tyrosine, L-serine, L-tryptophan, L-methionine, or ε-aminocaproic acid added in excess over the pyridoxal-P *N*-oxide did not react. With all these compounds, an almost instantaneous appearance of the 415-nm band indicated the formation of an azomethine bond between the 4-formyl group of pyridoxal-P *N*-oxide and an ε-amino and/or an α-amino group. Thus the formation of an azomethine bond alone is not responsible for the deoxygenation of pyridoxal-P *N*-oxide. This points to a rather specific role of the phosphorylase protein in the deoxygenation of pyridoxal-P *N*-oxide and poses the following question. Which amino acid side chain of phosphorylase is specially involved in the reduction of pyridoxal-P *N*-oxide to pyridoxal-P? First, we have considered a cysteinyl residue of the phosphorylase protein as the reductant for the deoxygenation of pyridoxal-P *N*-oxide. There are slow- and fast-reacting SH groups in the phosphorylase protein (*cf.* Battell *et al.*, 1968; see also Shaltiel and Zaidenzaig, 1970). Reaction of the former with a variety of sulfhydryl reagents causes inactivation, disruption of quarternary structure and formation of monomers. Chemical modification of the fast-reacting groups neither affects catalytic activity nor quarternary structure of phosphorylase *b*.

Battell *et al.* (1968) found that iodoacetamide at low concentration reacts rapidly without loss of activity with only two SH groups per dimer *b*. Kastenschmidt *et al.* (1968) and Gold and Blackman (1970) titrated three and four such groups per dimer *b* with DTNB and FDNB, respectively. Pyridoxal-P *N*-oxide phosphorylase *b* and pyridoxal-P phosphorylase *b* were reacted in glycerophosphate buffer (pH 6.8) with DTNB (Ellman, 1959), using the procedure of Kastenschmidt *et al.* (1968). When light and oxygen were strictly excluded, no loss of DTNB-reactive SH groups was observed with either the pyridoxal-P or the pyridoxal-P *N*-oxide phosphorylase (see Table III). After exposure of pyridoxal-P *N*-oxide phosphorylase *b* to daylight for 20 min about 1.7 SH groups/dimer *b* became unreactive with DTNB. This explains why we consistently found in previous experiments that for each mole of pyridoxal-P formed, 1 mole of SH (*i.e.*, about 0.8 sulfhydryl/dimer *b*) became unreactive with DTNB (*cf.* Ehrlich *et al.*, 1971). In the former experiments photooxidation must have occured during sampling and transfer of the reaction mixture to a Sephadex column for the removal of unbound cofactor. It may be noted from Table III, that the phosphorylase protein treated with pyridoxal-P *N*-oxide in the dark did not suffer an irreversible change by its reaction with pyridoxal-P *N*-oxide. Holophosphorylase reconstituted from the apoprotein of the pyridoxal-P *N*-oxide phosphorylase had about the same specific activity (59.9 μmoles $\times$ $min^{-1} \times mg^{-1}$) as a holophosphorylase which was reconstituted from the apoprotein of a reconstituted pyridoxal-P phosphorylase *b* (61 μmoles $\times$ $min^{-1} \times mg^{-1}$). This is in contrast to the behavior of apo- or holophosphorylase *b* treated with pyridoxal-P *N*-oxide in the light, which were to 49 and 17%, respectively, irreversibly denatured. This process could not be reversed by incubation with SH compounds, but pyridoxal-P bound to phosphorylase considerably protected it against photodenaturation by pyridoxal-P *N*-oxide. Pyridoxal-P *N*-oxide may still serve as a specific reagent which interacts with groups which are important for catalytic activity. Jori *et al.* (1971) have recently shown that photooxidation with a suitable specific protein reagent may give useful information on structure–function relationships in an enzyme.

The experiments with iodoacetamide which are shown in Table IV confirm the results obtained with DTNB. The activity measurements indicate that even more pyridoxal-P was formed in the dark from pyridoxal-P *N*-oxide by the carboxyamidomethylated than by the native apophosphorylase *b*. Moreover, carboxyamidomethylation of 4 SH groups/dimer *b* of reduced phosphorylase *b* did not decrease the amount of pyridoxal-P formed from pyridoxal-P *N*-oxide, although the reduced phosphorylase *b* had lost by carboxyamidomethylation almost 40% of its original activity. Thus, SH groups of phosphorylase *b* which rapidly react with iodoacetamide or DTNB cannot be the reductants for the deoxygenation of pyridoxal-P *N*-oxide. Avramovic-Zikic *et al.* (1970) reported that 5′-AMP protects the SH group in the acidic peptide A from carboxyamidomethylation and drastically reduces the rate of alkylation of peptide N. Neither 5′-AMP (2×10^{-3} M) nor glucose-1-P (2.5×10^{-2} M) added alone or together prevented the loss of DTNB titratable SH groups in pyridoxal-P *N*-oxide phosphorylase. This would seem to exclude also the participation of these cysteinyl residues in the reaction with pyridoxal-P *N*-oxide. Hence, so far our search for an SH group in the phosphorylase protein which could reduce pyridoxal-P *N*-oxide to pyridoxal-P has not been successful.

TABLE III: Effect of Light on the Reaction of PLP-N-O with SH Groups of Phosphorylase *b*.[c]

Cofactors Used for the Initial Reconstitution	Conditions for Initial Reconstitution	DTNB-Reactive SH Groups/Mol Wt 200,000	Change	Act. (μmoles × min^{-1} × mg^{-1})	Change[a] (%)	Act. of the Apoenzyme on Second Reconstitution with PLP (μmoles × min^{-1} × mg^{-1})	Change[a] (%)
PLP	Dark (40 min)	5.3	0	63.0		61.0	−3.2
PLP	Dark (20 min) and light (20 min)	5.1	−0.2	60.2	−6.7	58.0	−7.9
PLP-N-O	Dark (40 min)	5.1	−0.2	11.1		59.9	−5.0
PLP-N-O	Dark (20 min) and light (20 min)	3.6	−1.7	8.9	−19.9[b]	31.0	−49.3
PLP Phosphorylase *b* reacted with PLP-N-O	Dark (20 min) and light (20 min)			49.1	−22.0	52.5	−16.7

[a] The change in activity is relative to that of the PLP phosphorylase reconstituted in the dark (63 μmoles × min^{-1} × mg^{-1}) with the exception of [b] where the change in activity is relative to that of the PLP-N-O phosphorylase (11.1 μmoles × min^{-1} × mg^{-1}. [c] Apophosphorylase *b* (2.5×10^{-5} M) was reconstituted initially under the conditions specified with 1×10^{-4} M PLP or PLP-N-O, respectively. The last entry refers to holophosphorylase *b* (2.5×10^{-5} M) treated with 1×10^{-4} M PLP-N-O. Aliquots of the reconstituted enzymes and holophosphorylase *b* were resolved from PLP and PLP-N-O and the apoenzymes were reconstituted again for 40 min in the dark with PLP prior to treatment with DTNB. All incubations were under nitrogen.

TABLE IV: Reaction of PLP-N-O with Carboxyamidomethylated Phosphorylase *b*.[d]

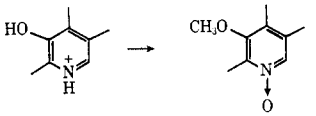
Preparations	PLP Formed from PLP-N-O (Mole/Mole)[a]	Sp Act.[c] (μmoles × min^{-1} × mg^{-1})
PLP-phosphorylase *b*		62.1
Carboxyamidomethylated[b] PLP-phosphorylase *b*		67.0
PLP-N-O phosphorylase *b*		12.4
Carboxyamidomethylated[b] apophosphorylase *b* treated with a twofold molar excess of PLP-N-O		19.3
Reduced PLP-phosphorylase *b* treated with a twofold molar excess of PLP-N-O	0.26	43.6
Carboxyamidomethylated[c] reduced PLP-phosphorylase *b* treated with a twofold molar excess of PLP-N-O	0.28	26.8
Reduced PLP-phosphorylase *b* treated with a fourfold molar excess of PLP-N-O	0.42	43.1
Carboxyamidomethylated[c] reduced PLP-phosphorylase *b* treated with a fourfold molar excess of PLP-N-O	0.36	26.6

[a] Moles of PLP formed per mole of dimer *b*. [b] By radioactivity measurements, 1.2 carboxyamidomethylated SH groups were found per dimer (see Methods). [c] By radioactivity measurements, 4.0 carboxyamidomethylated SH groups were found per dimer (see Methods). [d] Holophosphorylase *b* was first carboxyamidomethylated with [1-^{14}C]iodoacetamide according to Battell *et al.* (1968) and then resolved from PLP. The carboxyamidomethylated apoprotein was then reconstituted with PLP-N-O or with PLP at a twofold molar excess. Apophosphorylase obtained from untreated native holophosphorylase *b* served as control. $NaBH_4$-reduced phosphorylase *b* was treated like holophosphorylase *b*. All experiments with PLP-N-O were carried out in the dark and in the absence of oxygen.

It has not been excluded, however, that the reducing group is only reactive with pyridoxal-P *N*-oxide in phosphorylase monomers. Quite likely the explanation of the specific reaction of the phosphorylase with pyridoxal-P *N*-oxide will have to wait for the complete elucidation of the primary and of the three-dimensional structure of phosphorylase. This work is being actively pursued (*cf.* Fischer *et al.*, 1970).

Spectral Properties of Pyridoxal-P N-Oxide Phosphorylase b. Native pyridoxal-P phosphorylase *b* at pH 7 has major and minor absorption maxima at 333 and 425 nm, respectively

TABLE V: Stoichiometry of Binding of PLP and Analogs of PLP to Apophosphorylase *b*.[a]

Analog	Type of Modification	Moles of Analog Added/ Mole of Apoenzyme[b] (Molar Ratio)	Moles of Analog Bound/ Mole of Apoenzyme[b] (Molar Ratio)	Sp Act. (μmoles/ mg per min)	Extent of PLP Reactivation of Apophosphorylase *b* after Release of Analog (or PLP) (%)	$s_{20,w}$ (S)
PLP		2:1	1.85–2.2	65	96	8.3
3-*O*-Me-PLP-N-O		10:1	1.75–1.9	0	96	8.1
PL-5′-P-ME	5′-$CH_2OPO(OH)_2$ → 5′-$CH_2OPO(OH)OCH_3$	5:1	1.9–2.2	0	96	8.4

[a] The cofactors were determined by the method of Wada and Snell (1961). [b] Calculated as dimer *b* of mol wt 200,000.

(Figure 4). The ratio (1% cm^{-1}) $A_{333}:A_{425}$ = 13.3. From the data of Shaltiel and Cortijo (1970), it may be deduced that this spectrum is like that of a Schiff base derivative of pyridoxal-P in a 95–100% dioxane solution. Thus pyridoxal-P appears to be bound to a highly hydrophobic region in the phosphorylase protein. The corresponding absorbancy ratio for pyridoxal-P *N*-oxide phosphorylase *b* reconstituted with 2 moles/mol wt 200,000 of apophosphorylase *b* is 1.67 (*cf.* Figure 4). Subtraction of the absorbancy which is due to the 20–25% pyridoxal-P formed from pyridoxal-P *N*-oxide reduced this ratio to 1.45–1.40, respectively.

A similar low value as found with pyridoxal-P *N*-oxide phosphorylase *b* is obtained with the pyridoxal-P *N*-oxide *n*-hexylamine Schiff base in a 75–80% dioxane–water solution. This suggested that pyridoxal-P *N*-oxide in contrast to pyridoxal-P is bound to a more polar environment in phosphorylase *b*. Moreover it suggested the possibility that the azomethine bond is reducible by $NaBH_4$ even at neutral pH and in the absence of deforming agents (*cf.* Fischer *et al.*, 1958, 1963). This was indeed the case and will be reported in the following paper (Pfeuffer *et al.*, 1972) in which are also given additional experiments which show a higher reactivity of pyridoxal-P *N*-oxide phosphorylase *b* as compared with pyridoxal-P phosphorylase *b* toward another nucleophile, *i.e.*, L-cysteine at neutral pH.[3]

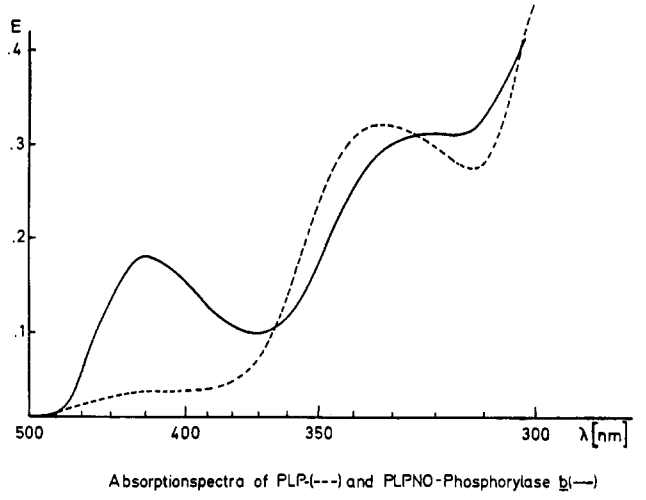


FIGURE 4: Absorption spectra of pyridoxal-P and pyridoxal-P *N*-oxide phosphorylase *b*. The concentrations were 3.7 × 10^{-5} and 2.5 × 10^{-5} M for PLP- and PLP-N-O phosphorylase *b*, respectively. The buffer was 50 mM glycerophosphate–2 mM EDTA (pH 6.8). The temperature was 23° and the light path 1 cm (see also Vidgoff, 1971).

3′-O-Methylpyridoxal-P N-Oxide. Although these results make it very likely that the partial reactivation of apophosphorylase *b* by pridoxal-P *N*-oxide results from the pyridoxal-P formed rather than from pyridoxal-P *N*-oxide itself, we nevertheless thought it desirable to search for a stable analog of pyridoxal-P with an unprotonatable nitrogen in the pH range in which phosphorylase is active and that is still bound to apophosphorylase; 3′-*O*-methylpyridoxal-P *N*-oxide met these requirements. Apophosphorylase *b* reconstituted with 3′-*O*-methylpyridoxal-P *N*-oxide sediments as a homogeneous symetrical peak with a similar $s_{20,w}$ value (8.1 S) as apophosphorylase *b* reconstituted with pyridoxal-P. 3′-*O*-Methylpyridoxal-P *N*-oxide is stable and unreactive with the apoenzyme. Upon resolution of 3′-*O*-methylpyridoxal-P *N*-oxide phosphorylase, 1.8–1.9 moles of 3′-*O*-methylpyridoxal-P *N*-oxide per mol wt 200,000 of phosphorylase dimer was recovered (Table V). The substance released from the protein was identified by paper electrophoresis as the original 3′-*O*-methylpyridoxal-P *N*-oxide (see Methods). No 3′-*O*-methylpyridoxal-P was found. Upon removal of 3′-*O*-methylpyridoxal-P *N*-oxide and subsequent reconstitution of the apoprotein with pyridoxal-P, a preparation was obtained with 96% of the specific activity of a phosphorylase which likewise went through two cycles of reconstitution with pyridoxal-P. The 3′-*O*-methylpyridoxal-P phosphorylase *b* is about 25% as active as pyridoxal-P phosphorylase *b* (*cf.* Shaltiel *et al.*, 1969). Therefore, if apophosphorylase would

[3] In the following paper (Pfeuffer *et al.*, 1972), data are presented indicating that pyridoxal-P *N*-oxide does not properly fit into the phosphorylase protein. The incorrect fit also induces a different aggregation state.

have deoxygenated 3'-*O*-methylpyridoxal-P *N*-oxide to the same extent as pyridoxal-P *N*-oxide, the 3'-*O*-methylpyridoxal-P *N*-oxide phosphorylase should have been about 5% active. However, reconstitution of apoenzyme with 3'-*O*-methylpyridoxal-P *N*-oxide yielded an inactive enzyme. No activity could be detected in three different assay systems.

Modification of the 5'-Phosphate Group of Pyridoxal Phosphate. PYRIDOXAL-P MONOMETHYL ESTER. This monomethyl ester was prepared to obtain a pyridoxal-P analog with the phosphate group, $pK_2 = 6.2$ blocked but with the phosphate group, $pK_1 = 2.5$ fully ionized at physiological pH. The latter charged group was expected to be sufficient for properly positioning the pyridoxal-P in the apoprotein by ionic interaction with a positively charged amino acid side chain. Stoichiometric reconstitution of apophosphorylase *b* with pyridoxal-P monomethyl ester yielded completely inactive phosphorylase (*cf.* Table V). In the analytical ultracentrifuge, pyridoxal-P monomethyl ester phosphorylase *b* sedimented like active pyridoxal-P phosphorylase *b*. The uv and visible spectra, as well as the fluorescence spectra, of pyridoxal-P and pyridoxal-P monomethyl ester phosphorylase are also indistinguishable (*cf.* Ehrlich *et al.*, 1971; K. Feldmann *et al.*, 1972).[4]

Results obtained with these two new analogs of pyridoxal-P which are pertinent to the function of pyridoxal-P in glycogen phosphorylase are reported in the following paper (Pfeuffer *et al.*, 1972).

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Role of Pyridoxal 5′-Phosphate in Glycogen Phosphorylase. II. Mode of Binding of Pyridoxal 5′-Phosphate and Analogs of Pyridoxal 5′-Phosphate to Apophosphorylase *b* and the Aggregation State of the Reconstituted Phosphorylase Proteins†

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ABSTRACT: The inactive pyridoxal phosphate monomethyl ester is, like pyridoxal-P, nearly irreversibly bound to apophosphorylase *b*. Inactive pyridoxal phosphate monomethyl ester and active 3′-*O*-methylpyridoxal phosphate (and pyridoxal phosphate) bound to apophosphorylase *b* do not exchange with pyridoxal phosphate (or [4′-^{3}H]pyridoxal phosphate). Enzyme-bound 3′-*O*-methylpyridoxal phosphate *N*-oxide and pyridoxal are slowly displaced by pyridoxal phosphate. Differences in the reactivity of the C_4–azomethine bond which links pyridoxal phosphate to a lysyl residue in the phosphorylase protein were found in the case of pyridoxal phosphate analogs. At neutral pH and in the absence of deforming agents such as imidazole citrate the inactive pyridoxal phosphate monomethyl ester and pyridoxal phosphate bound to phosphorylase *b* were least attacked by $NaBH_4$ and L-cysteine. Pyridoxal, 3′-*O*-methylpyridoxal phosphate, pyridoxal phosphate *N*-oxide, and 3′-*O*-methylpyridoxal phosphate *N*-oxide were more reactive. The apparent p*K* values of the protonatable groups of pyridoxal and pyridoxal phosphate respectively were determined in a milieu approximating that of the hydrophobic pyridoxal phosphate binding site in phosphorylase. The apparent pK_2 value of the 5′-phosphate group of pyridoxal phosphate increased from 6.2 in water to 9.3 in 80% dioxane–water. Therefore, the 5′-phosphate group (pK_2) of pyridoxal phosphate in phosphorylase must project to a more polar environment, if it is to function as proton donor–acceptor group in phosphorylase catalysis. The aggregation state of holophosphorylase reconstituted from apophosphorylase *b* with pyridoxal phosphate and both active and inactive analogs of pyridoxal phosphate was compared by polyacrylamide gel electrophoresis either with or without prior reaction with dimethyl suberimidate. The pyridoxal phosphate monomethyl ester maintained (up to 30°) a quaternary structure which was indistinguishable by these criteria from that of active holophosphorylase *b*. At still higher temperatures (35°), the pyridoxal phosphate monomethyl ester phosphorylase *b* was less stable than pyridoxal phosphate phosphorylase *b*. The amount of cross-linked oligomers formed with dimethyl suberimidate indicates a high degree of structural homology and complementarity of the contact surfaces of the subunits of phosphorylase *b*.

Nature may have found it advantageous to utilize protonatable groups of a vitamin rather than those of amino acid side chains to facilitate catalysis. If pyridoxal-P[1] should have such a function in glycogen phosphorylase one would expect its position in the catalytic site to be as strictly determined as that of a particular amino acid residue in the covalent structure of the protein. The optimum topography of the catalytic region in an enzyme is preserved by a genetic translation mechanism which operates with a high degree of fidelity. X-Ray crystallography of lysozyme, and of other enzymes, has taught us that the exact arrangement of protonatable groups at hy-

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‡ Doctoral fellow. This work is part of the Ph.D. thesis of J. E. which will be submitted to the Natural Science Faculty of the University of Würzburg.

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[1] Abbreviations of vitamin B_6 analogs follow IUPAC–IUB rules (1966, 1970) (see Pfeuffer *et al.*, 1972); FDNB, fluoro-2,4-dinitrobenzene; glycogen phosphorylase (EC 2.4.1.1), α-1,4-glucan:orthophosphate glucosyltransferase (EC 2.4.1.1).