1969

A physical-chemical study of pyridoxal phosphate in glycogen phosphorylase

George Franklin Johnson
Iowa State University

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PHOSPHORYLASE.

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Biochemistry

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A PHYSICAL-CHEMICAL STUDY OF PYRIDOXAL
PHOSPHATE IN GLYCOGEN PHOSPHORYLASE

by

George Franklin Johnson

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Graduate Faculty in Partial Fulfillment of
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Head of Major Department

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Dean of Graduate College

Iowa State University
Ames, Iowa

1969
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<td>Abbreviation</td>
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</tr>
<tr>
<td>-------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine-5'-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>CMDPL</td>
<td>Carboxymethyl-deoxypyridoxal</td>
</tr>
<tr>
<td>DPL</td>
<td>Deoxypyridoxal</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetate</td>
</tr>
<tr>
<td>G-HCl</td>
<td>Guanidine hydrochloride</td>
</tr>
<tr>
<td>glycerol-P</td>
<td>Glycerophosphate</td>
</tr>
<tr>
<td>G-1-P</td>
<td>α-D-Glucose-1-phosphate</td>
</tr>
<tr>
<td>G-6-P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-Mercaptoethanol</td>
</tr>
<tr>
<td>PL</td>
<td>Pyridoxal</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal-5'-phosphate</td>
</tr>
<tr>
<td>PMB</td>
<td>Para-Hydroxymercuribenzoate</td>
</tr>
<tr>
<td>PMP</td>
<td>Pyridoxamine-5'-phosphate</td>
</tr>
<tr>
<td>UDPG</td>
<td>Uridine-5'-diphosphoglucose</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

Glycogen in animals is synthesized and degraded by two separate enzyme systems. The degradative enzyme is glycogen phosphorylase (E.C. 2.4.1.1., α-1,4-glucan:orthophosphate glucosyl transferase). The following scheme illustrates the reaction catalyzed by the enzyme.

\[
\text{phosphorylase} \quad \text{orthophosphate} \quad \xrightarrow{\text{Mg}^{2+}, \text{ATP}} \quad \alpha-D\text{-glucose-1-phosphate} + n(\alpha-1,4\text{ linked glucosyl residues}) + n-1(\alpha-1,4\text{ linked glucosyl residues})
\]

This reaction is freely reversible, and can easily be studied in vitro in either direction.

Phosphorylase is present in animal tissue in two forms designated a and b. The two forms are interconvertible, and the process by which the interconversion takes place is shown in the scheme.

\[
\text{phosphorylase kinase} \quad \text{Mg}^{2+}, \text{ATP} \quad \text{phosphorylase b} \quad \xrightarrow{\text{phosphorylase phosphatase}} \quad \text{phosphorylase a}
\]

In the interconversion reaction two specific seryl residues are phosphorylated per mole of phosphorylase b by a specific kinase to produce phosphorylase a. The a form of the enzyme can be converted back to the b form by a specific phosphatase.
Phosphorylase a and b differ in their expression of enzymatic activity. Phosphorylase b requires the presence of the nucleotide adenosine-5'-phosphate for enzymatic activity. Phosphorylase a is active enzymatically without this activator being present. The currently accepted molecular weights for the two forms of phosphorylase in rabbit skeletal muscle are 370,000 for a and 185,000 for b (Seery et al., 1967).

The most enigmatic aspect of the phosphorylase system is presence of covalently bound pyridoxal-5'-phosphate. There are two moles of PLP per mole of phosphorylase b. Removal of the PLP under specific conditions causes the loss of enzymatic activity, and reconstitution of the apoenzyme with PLP restores the enzymatic activity. This thesis presents new data on PLP in phosphorylase. PLP analogues have been used to gain more information about the mode of binding of the coenzyme and the functional groups of the coenzyme itself that are necessary for expression of enzymatic activity. Spectral data are presented that give further information about the binding site for PLP in phosphorylase.
LITERATURE REVIEW

Introduction

It has now been just over a decade ago that pyridoxal-5'-phosphate was first discovered in glycogen phosphorylase from rabbit skeletal muscle (Baranowski et al., 1957). Since that time, much data has accumulated concerning PLP in phosphorylase. This review will attempt to examine all published data on the subject including the author's own published data. This has been done in order to make the literature review a complete entity that will stand by itself. The experimental section of the thesis will therefore be concerned primarily with the unpublished data of the author. It is hoped that this review will prove useful to those who will follow.

Occurrence of PLP in Glucosyl Transferases

Baranowski et al. (1957) reported that two moles of PLP were bound per mole of crystalline phosphorylase b. This value would have to be considered somewhat less than stoichiometric, since the old molecular weight of 242,000, determined by Keller and Cori (1953) was used. However, Kent et al. (1958) reported 2.5 ± 0.2 moles of PLP per 250,000 g of phosphorylase b. This observation along with the redetermined molecular

---

1 Unless specifically noted, the designations phosphorylase a and b used in this review will refer to the enzyme from rabbit skeletal muscle only.
weight for phosphorylase b of 185,000 reported by Seery et al. (1967) would give 1.9 moles of PLP per mole of phosphorylase b.

Since the time PLP was first reported in the glycogen phosphorylase of rabbit muscle, it has been subsequently found in the polyglucose phosphorylases of many diverse organisms as well as in phosphorylases of different tissues of the same organism. For example, PLP has been found in the polyglucose phosphorylases of E. coli (Schwartz and Hofnung, 1967; Chen and Segel, 1968), lobster (Cowgill, 1959), and potato (Lee, 1960). The glycogen phosphorylases of rabbit heart (Yunis et al., 1962), rabbit liver, (Appleman et al., 1966), and bovine kidney (Villar-Palasi and Gazquez-Martinez, 1968) all contain PLP. In all the cases cited, the presence of PLP was determined spectrophotometrically by either observing the spectrum of the native enzyme directly, or the spectrum of an extract of the enzyme under acidic or basic conditions.

An alternative method of detecting the presence of PLP in a phosphorylase is the pyridoxine deficiency test. By using animals which require pyridoxine for growth, and then depriving them of this substance, a deficiency can be induced. This deficiency can then be correlated with a decrease in the measured activity of an enzyme that requires PLP. Usually the activity of an enzyme which is known not to require PLP is also measured as a control. Using pyridoxine deficient rats Illingworth et al. (1960) showed that total muscle phosphorylase
activity \((a + b)\) undergoes a marked drop (35%) over the control group of rats which were fed pyridoxine. The phosphorylase \(a\) activity, however, remained relatively constant, and if only the \(a\) activity had been measured, an erroneous conclusion might have been reached. In this same study, the enzymatic activity of glycogen synthetase \((\text{UDPG}: \alpha-1,4\text{-glucan } \alpha\text{-glucosyltransferase})\) was also measured, and because no change was found in the activity of this enzyme, it was tentatively concluded that PLP was not required for this enzyme. This conclusion is equivocal, however, because it is now known that the synthetase exists in two forms, one of which is inactive without the presence of glucose-6-phosphate. The assay of Illingworth et al. did not include this activator. If the pyridoxine deficiency test is negative, the result must be accepted with some reservation. Lyon and Porter (1962) presented data that indicated that pyridoxine deficiency did not decrease the activity of liver phosphorylase. This is in contrast to the study of Eisenstein (1962) whose data show that liver phosphorylase activity does indeed decrease in deficient rats, but the decrease is not as marked as is found with the muscle enzyme. This was interpreted to mean that the liver enzyme turned over slower than the muscle enzyme. The pyridoxine deficiency test has been used to implicate the presence of PLP in the glycogen phosphorylases of the heart, brain (Valadares, 1967) and adrenal glands (Tiglao and Eisenstein, 1964) of the rat. Obviously, direct assays for PLP on highly
purified enzyme preparations is the most preferable method, but the pyridoxine deficiency test does provide useful preliminary data which can help direct more concentrated effort.

The only types of glucosyl transferases that have been shown to require PLP are those that catalyze the phosphorolysis of an α-1,4 linked polyglucose chain to produce α-D-glucose-1-phosphate. It would be very significant if an enzyme of this type were found that did not require PLP for activity. This would rule out any obligatory requirement for PLP in the mechanism of action of this kind of glucosyl transferase. There has not as yet been any polyglucose phosphorylase with this stereochemical, retention type of mechanism isolated that did not contain PLP. There are also polyglucose phosphorylases with mechanisms that involve inversion of configuration during catalysis. The β-1,3-oligoglucan:orthophosphate glucosyl transferase from Euglena gracilis (Marechal, 1967b) and the β-1,4-oligoglucan:orthophosphate glucosyl transferase from Clostridium thermocellum (Sheth and Alexander, 1969) are of this type. Whether these inversion polyglucose phosphorylases are related in an evolutionary sense to the retention type is not known, but if they are related, they might also require PLP for activity. These enzymes have not yet been investigated to determine whether PLP is bound as a prosthetic group.

The only other glucosyl transferases that utilize orthophosphate, and that might contain PLP are the disaccharide
phosphorylases, which can also be classified according to retention or inversion of configuration during catalysis. Sucrose phosphorylase is the only one that gives retention, and its mechanism has been shown to be ping-pong (Silverstein et al., 1967). This is in direct contrast to all other phosphorylases which appear to show ternary complex mechanisms. The inversion disaccharide phosphorylases include maltose phosphorylase, which is specific for δ-D-glucose-1-phosphate, cellobiose phosphorylase (Doudoroff, 1961), and laminaribose phosphorylase (Marechal, 1967a). Again, none of these enzymes have been critically examined in purified form for PLP.

A glucosyl transferase that might be related to glycogen phosphorylase is glycogen synthetase which is a UDPG:α-1,4-glucan α-4-glucosyl transferase. In analogy to the mammalian glycogen phosphorylase a, the glycogen synthetase also exists in a phosphorylated form. Larner and Sanger (1965) have isolated a hexapeptide containing the phosphoseryl residue from rabbit and rat muscle glycogen synthetase that is identical to the one isolated from phosphorylase a (Krebs and Fischer, 1962). This homology implies that the two proteins might be closely related in more than just this short sequence. As was previously indicated, the pyridoxine deficiency test proved negative for PLP involvement in this enzyme, but because of the inadequacy of this test, it is imperative that a purified preparation of this enzyme be tested directly for PLP.
No evidence has been presented that would indicate that phosphorylase has been derived from a more normal PLP containing enzyme. Hedrick and Fischer (1965) examined phosphorylase b and a closely for residual PLP enzyme activities. They found no transaminase, decarboxylase, or β- and γ-elimination activities characteristic of PLP enzymes. Their attempts to construct a nonenzymic model system were also negative.

Preparation and Properties of the Apoenzyme

The first preparation of apophosphorylase was reported by Cori and Illingworth (1957). The procedure, which could be used with either phosphorylase a or b, involved precipitation of holoenzyme with cold HCl in the presence of ammonium sulfate, centrifuging, and then washing the yellow precipitate a few times with alkaline ammonium sulfate to remove the PLP. The apoenzyme that was prepared could be reactivated to a substantial extent by excess PLP. If a lower pH were used in the acid precipitation, a greater specific activity of the reconstituted apoenzyme was obtained, but the loss of total enzyme to denaturation was greater. Illingworth et al. (1958) subsequently developed a new procedure for apo-b that resulted in better yield and reactivation by PLP. Phosphorylase b was dialyzed for 12 hours at 0° against pH 6.0 glycerophosphate buffer in the presence of L-cysteine to give apo-b. If phosphorylase a was treated in a similar manner, no resolution took place.
A better method was also found for the preparation of apo-α. To phosphorylase α in the presence of cysteine was added concentrated citrate buffer at pH 3.5; after a few minutes of reaction time at 0°, the apo-enzyme was precipitated with alkaline ammonium sulfate, centrifuged, and washed. Dialysis against pH 7.4 thioglycolate buffer in the cold gave crystalline apo-α (Illingworth et al.). The apo-α gave two principal peaks in the ultracentrifuge with sedimentation constants characteristic of monomeric and tetrameric forms of phosphorylase. Reconstitution with PLP gave an ultracentrifuge pattern characteristic of holo-α, showing only the tetrameric form. Apo-β, however, showed only one broad peak in the ultracentrifuge which sharpened on reconstitution with PLP with little change in sedimentation constant.

The most useful procedure for preparation of apo-β has been described by Shaltiel et al. (1966). Phosphorylase b is reacted with L-cysteine in the presence of an imidazole-citrate buffer at pH 6.2 and 0°. The resolution is complete with the conditions employed in about one-half hour. The resolved enzyme is then precipitated with ammonium sulfate and desalted on a Sephadex column. The apo-β made by this procedure can be crystallized, but only in the presence of Mg$^{2+}$, AMP and β-mercaptopethanol. The apoenzyme usually shows a specific activity of 0.1-1.0% of the original holoenzyme. This residual activity correlates well with residual bound PLP as
determined by spectrophotometric means. The correlation suggests that within experimental error, the apoenzyme is enzymatically completely inactive.

The resolution procedures that have been outlined utilize the same general steps. The enzyme is deformed by appropriate conditions in the presence of a PLP trapping reagent, cysteine, which removes the PLP from the enzyme as a thiazolidine derivative. This adduct will not recombine with the apoenzyme in the presence of the deforming agents.

Shaltiel et al. have extensively studied the mechanism by which their resolution procedure takes place. In the presence of the imidazole buffer, $[^{32}P]$ PLP free in solution was found to be readily exchangeable with PLP on the enzyme, a condition that did not occur in the absence of the buffer. Ultracentrifuge studies showed a $S_{20,w}$ of 5.5 in the presence of the imidazole-citrate buffer at pH 6.0, in contrast to a $S_{20,w}$ of 8.4 in the presence of glycerophosphate buffer. Dialysis reversed the change in sedimentation constant. The $S_{20,w}$ of 5.5 is very close to that of PMB induced monomer reported by Madsen and Cori (1956), and it was concluded by Shaltiel et al. that imidazole-citrate buffer induces monomerization of dimer phosphorylase $b$. AMP which blocks monomerization completely blocked the resolution of $b$, and G-6-P, which inhibits monomerization, inhibited the resolution of $b$ (Fischer et al., 1968). In further support of the importance of monomer in
resolution, Fischer and Krebs (1966) have indicated that PMB induced monomer can be resolved with hydroxylamine. The only negative evidence for the hypothesis that monomerization is a prerequisite to resolution is the observation that NaCl promotes dissociation but blocks resolution (Shaltiel et al., 1966).

Phosphorylase a was not resolved with the conditions employed for resolution of b. However, if glycogen were present, resolution took place (Fischer et al., 1968). Glycogen has been shown to promote the dissociation of tetramer a to dimer a (Wang and Graves, 1964; Metzger et al., 1967). Fischer et al. (1968) have found conditions for phosphorylase a resolution without glycogen: hydroxylamine in the presence of a maleate-imidazole-citrate buffer at pH 5.5 and 22° is required. It has not been reported whether these conditions favor monomerization of a.

Shaltiel et al. (1966) have shown that the resolution reaction is first order in L-cysteine, indicating that the protein is attacked directly by cysteine. The reaction shows considerable sterospecificity: D-cysteine is completely inactive in the resolution reaction. Hydroxylamine was the only aldehyde reagent that was as effective as L-cysteine. The following compounds were ineffective in bringing about resolution: cysteamine, homocysteine, β-mercaptpropionic acid, ethylene diamine, sodium bisulfite, and S-methyl cysteine. Compounds that were poorer resolving agents than L-cysteine included
penicillamine, 2,3-dimercaptopropanol, semicarbazide and isonicotinyl hydrazide.

The apo-b made by the procedure of Shaltiel et al. (1966) was most stable at pH 6.0 in the presence of AMP. While the apo-b is relatively stable below 30°, it rapidly denatures at elevated temperatures, showing much more heat instability than the native enzyme. Apo-b appears as a rapidly associating-dissociating system in the ultracentrifuge (Hedrick et al., 1966). From the observed sedimentation constants, the apo-enzyme sediments as a monomer at 35°, a dimer at 23°, and as a tetramer or higher aggregates near 0°. Holophosphorylase b remains a dimer throughout this temperature range. Hedrick et al. observed that AMP favors aggregation of the apo-b, proving that AMP will still bind to the apoenzyme. High concentrations of NaCl (1-3M) also favored aggregation of apo-b.

Kastenschmidt et al. (1968) have done direct binding studies, using a gel filtration technique, on the interaction of AMP with apo-b, and have determined a dissociation constant of $5.3 \times 10^{-4}$; under the same conditions, a dissociation constant of $3.7 \times 10^{-4}$ was determined for the holoenzyme. The cooperative binding of AMP characteristic of holo-b, however, is absent with apo-b. Apo-b was found by these same workers to bind the substrate glycogen as tightly as the holoenzyme.

Illingworth et al. (1958) had previously demonstrated that apo-b could be converted to apo-a with phosphorylase kinase. This result was confirmed by Hedrick et al. (1966), and it was
further shown that apo- and holoenzymes were phosphorylated at similar rates by phosphorylase kinase. Removal of PLP from the enzyme does not therefore destroy the conformation at the seryl site required by phosphorylase kinase.

Much of the experimental data available on the properties of apo-β is consistent with conformational changes taking place on removal of PLP from holoenzyme. These conformational changes are all reversible upon addition of PLP to the apo-enzyme. Optical rotatory dispersion studies of Johnson and Graves (1966) indicate no significant changes in secondary structure between apo- and holoenzymes which is consistent with a limited conformational change involving secondary structure. It cannot be concluded from the observed properties of the apoenzyme that PLP has only a structural role in phosphorylase. The apoenzymes of other PLP enzymes also have properties distinct from their holoenzymes. Apotryptophanase, for example, exists as a tetramer which dissociates reversibly upon dilution or low temperatures to a dimer, whereas the holoenzyme stays as a tetramer under the same conditions (Morino and Snell, 1967). PLP may, in fact, have a structural role as well as a catalytic role in many of the normal PLP requiring enzymes.
Effect of Coenzyme Structure on Binding and Activity

Reconstitution of apoenzyme

The reconstitution of apo-b with PLP has been studied by Hedrick et al. (1966). The reconstitution process was found to be quite temperature dependent. An Arrhenius activation energy of 22.3 Kcal/mole was obtained by following the return of enzymatic activity at different temperatures. Reconstitution seemed to be only slightly dependent on pH: in a series of experiments where the pH was varied from 5.0 to 8.0, 80-100% reactivation was obtained in all cases within three minutes at 37°, and full reconstitution was obtained within nine minutes. The extent and rate of reconstitution was not effected by the following compounds: AMP, G-1-P, glycogen and glucose-6-phosphate (Fischer et al., 1968). As was discussed earlier, both AMP and glucose-6-phosphate blocked the resolution process. Reconstitution was found to be inhibited in the presence of a large excess of PLP. This inhibition of reconstitution was not specific for PLP, since compounds such as formaldehyde, glyceraldehyde and pyridine-4-aldehyde also inhibited. The holoenzyme was much less sensitive to aldehyde inhibition than the apoenzyme.

Surprisingly, L-cysteine even though it forms a stable complex with PLP does not block reconstitution, whereas its enantiomer, D-cysteine, is quite effective in blocking reconstitution. Fischer et al. (1968) reported that 95% reactivation of
apo-b could be obtained with L-cysteine after 30 min at 30°, while only 3% reactivation was attainable with D-cysteine under the same conditions. Reconstitution in the presence of a large excess of L-cysteine was investigated using both 14C- and 35S-labeled cysteine (Fischer and Krebs, 1966). Ammonium sulfate precipitation followed by Sephadex chromatography gave a reconstituted enzyme with one mole of cysteine incorporated per mole of monomer. Apoenzyme treated in the same manner showed no incorporation of cysteine. The bound cysteine in the reconstituted enzyme could be exchanged with unlabeled cysteine, however. Further investigation of cysteine binding to holoenzyme and enzyme reconstituted in the presence of cysteine is needed to establish the nature of the cysteine-enzyme complex.

Illingworth et al. (1958) have reported a second order rate constant of 185 M\(^{-1}\) sec\(^{-1}\) for the reaction of PLP with apo-b in 0.04 M sodium glycerophosphate, 0.005 M EDTA buffer at pH 7.0 and 25°. The reaction was followed by the decrease in absorbance at 390 nm with time. The value reported for the rate constant is probably in some error, since the old molecular weight for phosphorylase b was used and the experimental procedure involved adding a stiociometrical amount of PLP to the apoenzyme. In contrast to apo b, the reconstitution of apo-a with PLP has been reported to be instantaneous at 15°C by Fischer et al. (1968). It is not known whether the difference in rate for PLP binding to apo-a and apo-b has much physiological importance. PLP could bind to either apo-a or b
in vivo. Lyon and Porter (1963) have reported a strain of mice that exhibit no phosphorylase kinase activity in their skeletal muscles. These mice, which appear normal, therefore have no apo-a, and only apo-b would be available for combination with PLP.

**Analogue studies**

A variety of PLP analogues have been tested for their ability to bind to apo-b and -a and to restore the enzymic activity once bound. The names and structural formulas of these analogues, along with their ability to restore enzymic activity can be found in Table 1. Illingworth et al. (1958) demonstrated that incubation of apo-a with excesses of pyridoxal and 5-deoxypyridoxal gave reconstituted enzymes that were indistinguishable from the holoenzyme in the ultracentrifuge. A compound that lacks a 4-formyl group, pyridoxamine phosphate, had no effect on the sedimentation of apo-a. None of these derivatives gave any reactivation of the apoenzyme. Further evidence for the binding of pyridoxal and 5-deoxypyridoxal was obtained in reactivation experiments with PLP in the presence of varying amounts of these derivatives. 5-deoxypyridoxal was more effective than pyridoxal in preventing the reconstitution of the apoenzyme with PLP. Since pyridoxal exists mainly as a hemiacetal in solution (Martel, 1963; Metzler and Snell, 1955), it is not surprising that it is not as effective as 5-deoxy- pyridoxal. Pyridoxamine phosphate was completely ineffective
Table 1. Structure and activity of coenzyme analogues tested with apophosphorylase

<table>
<thead>
<tr>
<th>Structure</th>
<th>Name</th>
<th>R</th>
<th>R'</th>
<th>R''</th>
<th>R'''</th>
<th>Activates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Pyridoxal</td>
<td></td>
<td>CH₃</td>
<td>OH</td>
<td>CHO</td>
<td>CH₂OH</td>
<td>No</td>
</tr>
<tr>
<td>B. 5-Deoxypyridoxal</td>
<td></td>
<td>CH₃</td>
<td>OH</td>
<td>CHO</td>
<td>CH₃</td>
<td>No</td>
</tr>
<tr>
<td>C. Pyridoxal sulfate</td>
<td></td>
<td>CH₃</td>
<td>OH</td>
<td>CHO</td>
<td>CH₂OSO₃H</td>
<td>No</td>
</tr>
<tr>
<td>D. Pyridoxine phosphate</td>
<td></td>
<td>CH₃</td>
<td>OH</td>
<td>CH₂OH</td>
<td>CH₂OPO₃H₂</td>
<td>No</td>
</tr>
<tr>
<td>E. Pyridoxic acid phosphate</td>
<td></td>
<td>CH₃</td>
<td>OH</td>
<td>CO₂H</td>
<td>CH₂OPO₃H₂</td>
<td>No</td>
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<tr>
<td>F. Pyridoxamine phosphate</td>
<td></td>
<td>CH₃</td>
<td>OH</td>
<td>CH₂NH₂</td>
<td>CH₂OPO₃H₂</td>
<td>No</td>
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<tr>
<td>G. 4-Deoxypyridoxine phosphate</td>
<td></td>
<td>CH₃</td>
<td>OH</td>
<td>CH₃</td>
<td>CH₂OPO₃H₂</td>
<td>No</td>
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<tr>
<td>H. 3-0-Methylpyridoxal phosphate</td>
<td></td>
<td>CH₃</td>
<td>OCH₃</td>
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<td>I. ω-Methylpyridoxal phosphate</td>
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<td>CH₃CH₂</td>
<td>OH</td>
<td>CHO</td>
<td>CH₂OPO₃H₂</td>
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</tr>
<tr>
<td>J. N-Methylpyridoxal phosphate</td>
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<td>(See column I)</td>
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<td></td>
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in preventing reconstitution of apo-a by PLP. These results of Illingworth et al. (1958) clearly indicate the role of the 4-formyl group in coenzyme binding and the requirement of the 5'-phosphate group for return of enzymatic activity after coenzyme binding.

The most comprehensive recent investigations of PLP analogues and apophosphorylase have been reported by Fischer and Krebs (1966), and Fischer et al. (1968). In agreement with Illingworth et al. (1958), it was found that any replacement of the 4-formyl group resulted in a derivative with no capacity for reactivation. Pyridoxine phosphate, pyridoxamine phosphate, and pyridoxic acid phosphate, and 4-deoxypyridoxine phosphate all failed to reactivate the apoenzyme. All derivatives that lacked the 5'-phosphate group were also inactive. 5-carboxymethylpyridoxal and pyridoxal sulfate were in this category. Other substituents on the pyridine ring that could be involved in the ability of PLP to reactivate the apoenzyme include the 3-hydroxy group, the 2-methyl group and the pyridine nitrogen itself. The role of the 3-hydroxy group was tested with 3-0-methylpyridoxal phosphate. This derivative was found to re-activate apo-b to 25% of that obtained with PLP, and apo-a to 40-45% of the activity obtained with PLP. The 3-hydroxy group of PLP cannot therefore be involved in an obligatory protonation-deprotonation step during enzymic catalysis.
Illingworth et al. (1958) reported that $\omega$-methylpyridoxal phosphate, the 2-ethyl analogue of PLP, gave 50% of the re-activation that could be obtained with PLP and apo-$b$. Fischer et al. (1968) repeated this experiment with another sample of analogue and found no reactivation. It would be doubtful that the 2-methyl group of PLP could be involved in catalysis, but it is reasonable that replacement of the methyl with a larger alkyl group might produce steric problems that would result in loss of enzymic activity.

The involvement of the pyridine nitrogen was tested by Fischer et al. with N-methylpyridoxal phosphate. This derivative gave no reactivation of apophosphorylase; however, ultracentrifuge experiments indicated that this derivative did not bind to the apoenzyme. A mechanism requiring protonation-deprotonation of the pyridine nitrogen during catalysis is therefore not ruled out by the analogue work.

Kastenschmidt et al. (1968) have studied the properties of apo-$b$ reconstituted with pyridoxal and 5-deoxypyridoxal. Reconstitution with these analogues, as will be recalled from an earlier section, returns the ultracentrifugal properties characteristic of dimer phosphorylase $b$. While the enzyme activity is not restored, the allosteric properties observed in AMP binding to the holoenzyme are restored. The binding of AMP to the 5-deoxypyridoxal reconstituted enzyme showed typical homotropic interactions with $n = 2$ for AMP between 1.0 and $3 \times 10^{-5}\text{M}$. These interactions, as discussed earlier, are not
observed with the apoenzyme. In $5 \times 10^{-2}$ M glycyglycine buffer holophosphorylase b exhibits a dissociation constant for AMP binding greater than $50 \times 10^{-4}$ M; the presence of $2 \times 10^{-2}$ M G-1-P lowers the dissociation constant to $0.8 \times 10^{-4}$ M. This heterotropic interaction between AMP and G-1-P in glycyglycine buffer could also be observed with the 5-deoxypyridoxal reconstituted enzyme. In this instance, the presence of $2 \times 10^{-2}$ M G-1-P lowers the AMP dissociation constant from greater than $50 \times 10^{-4}$ to $4.6 \times 10^{-4}$ M. Combining these results with the observation that apophosphorylase b binds glycogen as tightly as the holoenzyme would indicate that the 5-deoxypyridoxal reconstituted enzyme is not inactive because it cannot bind substrates and activator. However, nonproductive complexes cannot be ruled out with the data presented.

**NaBH₄ reduced enzyme**

A unique modification of the PLP in phosphorylase can be brought about by reduction of the enzyme under appropriate conditions with sodium borohydride (Fischer et al., 1958). The species that is reduced is the imine between PLP and an ε-amino group of a protein lysyl residue. The reduction causes the PLP to be fixed on the enzyme irreversibly as a substituted pyridoxamine derivative. Direct proof of the structure of the reduced species was obtained by extensive chymotryptic digestion of the reduced enzyme which gave the dipeptide ε-N-pyridoxal-phosphatelysylphenylalanine (Nolan et al., 1964). Normally, the reduction does not take place between pH 5 and
9.5, but at the extremes of this pH range, a yellow PLP-enzyme imine is formed that is readily reducible. The PLP is not thought to be bound to the enzyme as an imine between pH 5 and 9.5, but rather, as an imine addition product with some side chain nucleophile. The basis for this assignment will be thoroughly discussed in a later section of the review. The most important aspect of the enzyme is the retention of catalytic activity. At pH 6.8, 50-60% of the specific activity of the native enzyme is retained. The reduced phosphorylase is therefore unique among PLP enzymes in that it does not require an imine linkage between the PLP and the enzyme as a prerequisite for catalytic activity.

The properties of reduced phosphorylase b have been reported to be very similar to those of the native enzyme (Fischer et al., 1963; Strausbauch et al., 1967). The native and reduced enzymes have similar sedimentation constants, pH dependence of enzyme activity, activation energies, Michaelis constants for AMP and G-1-P, urea and heat stabilities, and degree of inhibition by phlorizin. The reduced b is converted to the a form by phosphorylase kinase and back to the b form by phosphorylase phosphatase at essentially the same rates found with the native enzyme. Sealock and Graves (1967) found the same sigmoidal effect of AMP on the kinetics of reduced b as was observed with native b. This has been verified by the direct binding experiments of Kastenschmidt et al. (1968); the
heterotropic interactions between G-l-P and AMP characteristic of the native enzyme were also observed for the reduced enzyme by these workers.

Spectral Properties of PLP in Phosphorylase

Absorption spectra

Phosphorylase b and a exhibit a well defined UV absorbance maximum at 333 nm which has essentially constant absorbancy in the pH range between 5.0 and 9.5 (Kent, 1959; Kent et al., 1958). At pH 6.8 and at an enzyme concentration of 10 mg/ml, the absorbance at 333 nm is 0.54. A small subsidiary maximum is also seen around 415 nm in phosphorylase preparations. This shoulder increases with age of the enzyme and the increase roughly parallels loss of enzymic activity. Kent has observed that the 415 nm absorbance can be as low as 8% of the 333 nm absorbance in fresh preparations, and as much as 30% in aged preparations. No method of enzyme preparation was found that would reduce the 415 nm absorbance below the 8% level.

If the pH of a solution of phosphorylase is lowered below pH 5.0, a new maximum at 415 nm is seen with a concomitant decrease at 333 nm, and a reasonably sharp isosbestic at 357 nm. The observation of the isosbestic absorbance is consistent with the 333 nm species being transformed directly to a new species absorbing at 415 nm. At extreme acid pH, the 415 nm species faded. This phenomena could be seen in 0.3 N perchloric acid where the fading could be correlated with the appearance of
295 nm absorbing material (the absorbance maximum for PLP in acid solution) in the perchloric acid supernatant. Kent interpreted the data to mean that the bound PLP passes through a form absorbing at 415 nm before it is removed from the enzyme in acid solution.

A similar phenomenon could be observed at alkaline pH. In this instance a species is formed absorbing in the 400 nm region which continually shifts its absorption to 388 nm, the absorbance maximum of free PLP in alkaline solution. A sample of enzyme dialyzed at pH 10.8 gave an absorbance maximum at 408 nm. In accordance with titration data at acid pH, Kent felt that the data at alkaline pH indicated that the 333 nm species of bound PLP passed through a form absorbing above 400 nm when the PLP was split from the enzyme. The 333 nm to 415 nm transformation observed at acid pH is not a process that can be assigned a definite pK. Kent observed that with aged enzyme the 415 nm species develops at appreciably higher pH values.

There is good evidence that the 333 nm to 415 nm transformation depends strongly on protein conformation. A transient species that absorbs at 415 nm was observed by Kent when the enzyme was diluted in 7 M urea at 0°. A fairly stable 415 nm species could also be generated with various aqueous detergents at neutral pH. Ford and Mason (1968) reported that incubation of phosphorylase b with various anionic steroids resulted in the formation of a species absorbing in the 400 nm region with a resulting decrease of the 333 nm species and enzymic activity.
Estradiol disulfate formed a species absorbing at 420 nm, and dialysis of the enzyme to remove the steroid returned the original spectrum and 70% of the enzymic activity. A reversible 333 nm to 415 nm transformation at neutral pH has also been observed by Graves.\(^1\) Phosphorylase in the absence of AMP and sulfhydryl reagents was made 1.5 M in NaCl and cooled to -5°. A slow transformation to a 415 nm absorbing species was observed; subsequent rewarming of the enzyme solution to room temperature reversed the spectral change.

Kent et al. (1958) postulated the following structures for the bound PLP to explain the 333 nm and 415 nm spectral forms.

\[
\begin{align*}
\text{NH} & \quad \text{X} \\
\text{CH} & \\
\text{O} & \quad \text{PO}_3\text{H}^- \\
\text{333 nm} & \\
\end{align*}
\]

\[
\begin{align*}
\text{+HN} & \quad \text{XH} \\
\text{CH} & \\
\text{O} & \quad \text{PO}_3\text{H}^- \\
\text{415 nm} & \\
\end{align*}
\]

The 415 nm species was interpreted to be a hydrogen-bonded, protonated Shiff base and the 330 nm form was considered to be a zwitterionic addition product of some nucleophilic group (X)

\(^1\)Graves, Donald J., Ames, Iowa. Data from cold inactivation studies. Private communication. 1963.
on the protein with the Schiff base. The 333 nm to 415 nm conversion was visualized to be the result of a protein conformational change. The schiff base formed would be relatively stable or would hydrolyze, depending on the extent of the conformational change.

Kent observed that the yellow, 415 nm absorbing imine could be reduced with NaBH$_4$, allowing the PLP to be irreversibly fixed to the enzyme. This is illustrated in the scheme that follows:

![Scheme](image)

The 333 nm form in the native enzyme maximally present between pH 5.0 and 9.0 could not be fixed to the enzyme by NaBH$_4$ reduction. There was no appreciable spectral change even in the 415 nm region after reduction at neutral pH. It would appear, then, that the 415 nm species characteristic of the native enzyme is not identical with the 415 nm form in the conformationally perturbed enzyme. Incubation of the enzyme with NaBH$_4$ at pH 10, where a definite increase in 415 nm
absorbance is visible, results in 60% fixation of PLP in two hours which is consistent with the postulated mechanism of alkaline hydrolysis of PLP from the enzyme. As indicated earlier, chymotryptic cleavage of reduced phosphorylase $a$ yields the dipeptide $\epsilon$-N-pyridoxalphosphatelysylphenylalanine (Nolan et al., 1964). Forrey (1963) observed that the insoluble core which is obtained by precipitation at pH 4 after trypptic attack constitutes only 25-35% of the protein but contained 60-70% of the bound PLP. This result intimates that the PLP might be bound in a more hydrophobic portion of the protein.

Kent considered the possibility that the PLP in the reduced enzyme did not occupy the site at which it was initially bound in the native enzyme. Migration of the PLP was checked for by adding enzyme, $(\text{NH}_4)_2\text{SO}_4$ and $\text{NaBH}_4$ together at alkaline pH and then dropping the pH quickly to pH 4.5 with acetic acid. Any free aldehyde formed would be reduced to pyridoxine phosphate and would show up in the supernatant, and in fact, none was found. This experiment did not rule out the possibility of transimination, in which the PLP could be transferred from one $\epsilon$-amino group of lysine to another without involving a free aldehyde intermediate. However, such a transformation would have to be quite specific and complete, since a good yield of the chymotryptic dipeptide was isolated.

The reduced enzyme, while having many catalytic properties similar to the native enzyme, had spectral properties that were
quite distinct from the native enzyme (Kent, 1959). In contrast to the native enzyme which shows essentially constant absorbance between pH 5 and 9, the reduced enzyme shows a pH dependent spectral transition centered around pH 6. Above pH 6.5, the reduced enzyme shows no well defined peak at 330 nm, and at pH 9.0 the absorption for 10 mg/ml of phosphorylase b was reported to be only 0.105 at 332 nm, while at pH 4.5 a value of 0.78 and a well defined peak was obtained. Kent speculated that the spectral change might be the result of a change in the pK of the pyridinium nitrogen possibly caused by the proximity of some positively charged residue.

The interpretation of the spectra of reduced and native phosphorylase by Kent was based on model compound data available at the time. This data indicated that a zwitterionic PLP analogue with a saturated carbon at the 4' position would be expected to absorb in the 330 nm region (Metzler and Snell, 1955). Because of the 330 nm to 415 nm conversion, Kent proposed an imine addition product as the most likely species to explain the 333 nm absorbance. For instance, the reaction of cysteine and cysteinylglycine with PLP results in thiazolidine ring formation yielding a stable adduct that absorbs at 330 nm (Matsuo, 1957; Buell and Hanson, 1960). More recent model compound studies have indicated that there are other species that could also explain the 333 nm absorbance in phosphorylase. Heinert and Martell (1963) have shown that a tautomeric imine of PLP and PLP analogues can absorb in the 330 nm region. This
species is a neutral form that is favored in solvents less polar than water such as methanol and dioxane. If such a species were present in phosphorylase, the environment around the bound PLP would have to favor this neutral species. The species under discussion is depicted below.

\[ \text{Structure} \]

An enzyme conformational change could then bring this species into contact with a more polar environment which would result in the formation of a protonated, dipolar imine absorbing at 415 nm identical to that discussed earlier.

An analogous scheme can be proposed to explain the spectral properties of the reduced enzyme. In this case the bound PLP can be regarded as a substituted pyridoxamine derivative. Matsushima and Martell (1967) have shown that pyridoxamine has a form that absorbs at 289 nm in neutral methanol solution. This species is interpreted to be a neutral, nonpolar form. The presence of such a species in the reduced enzyme would explain the absence of absorption in the 330 nm region at neutral pH. The following structure indicates the species under
To explain the appearance of absorption in the 330 nm region in the reduced enzyme as the pH is lowered, a conformational change would have to be postulated that would expose the bound PLP to a more polar environment. The original proposal of Kent involved changing the pK of the pyridinium nitrogen in a drastic way. Model compound studies (Metzler and Snell, 1955) would predict a shift of the absorption spectrum to lower wavelengths by about 15 nm. The reappearance of 330 nm absorption would then be a result of protonating the pyridine nitrogen to reform the dipolar anion.

There are a number of other protein systems in which bound PLP shows a principal absorption maximum at 330-340 nm. An equimolar mixture of bovine plasma albumin and PLP forms an adduct which absorbs at 332 nm. An imine addition product has also been proposed in this system to explain the observed absorbancy maximum (Dempsey and Christensen, 1962). Glutamate
decarboxylase from *E. Coli* exhibits a single maximum at 335 nm when the pH is 6.5 (Shukuya and Schwert, 1960). Anderson and Chang (1965) demonstrated that reaction of this form of the enzyme with \( \text{NaBH}_4 \) would not fix the PLP irreversibly to the enzyme or destroy the enzymic activity. Reduction of the enzyme at a lower pH where a 420 nm absorbing form predominates does cause loss of activity and PLP fixation. The \( \gamma \)-form of supernatant glutamate-asparatate transaminase from pig heart exhibits a single maximum at 340 nm which shows no change in absorbance between pH 5.4 and 8.5. The enzyme is not affected by treatment with \( \text{NaBH}_4 \) or the usual procedures for resolution (Martinez-Carrion et al., 1965). Finally, Soda and Misono (1968) have purified an L-lysine-\( \alpha \)-ketoglutarate amino transferase from *Achromobacter liquidum*. This enzyme has two absorption maxima, one at 340 nm and one at 415 nm, and contains two moles of PLP for each mole of enzyme. Resolution of the enzyme by a common procedure results in an inactive "semiapo-enzyme" which still has the 340 nm band and one mole of PLP. As in the case of phosphorylase, the 330-340 nm absorbance in the PLP systems that have been discussed could be interpreted in terms of either an imine adduct with a protein nucleophile or a tautomeric, neutral PLP imine. A protein environment that would stabilize the tautomeric imine might also be expected to protect it from reduction with \( \text{NaBH}_4 \).
Fluorescence

PLP enzymes have distinctive fluorescence properties in addition to UV absorption properties. By comparing the fluorescence properties of PLP bound to an enzyme to the properties of PLP model compounds, information can be obtained about how the PLP is bound. The fluorescence spectrum can therefore serve as a useful adjunct to the absorption spectrum in interpretive work. Fluorescence also offers the advantages of usually requiring lower protein concentrations and being more sensitive to the protein environment around the bound chromophore. The first attempt at observing PLP fluorescence in phosphorylase was negative. It was reported that neither native nor reduced phosphorylase exhibited any fluorescence due to PLP at pH 6.8 (Fischer et al., 1963; Forrey, 1963). However, reduced phosphorylase did show PLP fluorescence below pH 6.0. Inspection of the data of Forrey (1963) indicates the emission maximum for this fluorescence is about 390 nm and the excitation maximum about 330 nm. The observed fluorescence correlates well with the appearance of the 330 nm band observed by Kent below pH 6.0 in the reduced enzyme. More recent work indicates that the PLP in phosphorylase is indeed fluorescent although the intensity is low. Shaltiel and Fischer (1967) observed an emission maximum at 530 nm and an excitation maximum at 330 nm. In good agreement with this data was the report of Ford and Mason (1968), who found an emission maximum at 525 nm and an
excitation maximum at 340 nm. These workers found that in the presence of estradiol disulfate, which mediates a 333 nm to 420 nm conversion in the absorption spectrum, the emission maximum changed to 510 nm and the excitation maximum shifted to 425 nm.

Other PLP enzymes show a long wavelength fluorescence around 500 nm. Glutamate-aspartate transaminase from pig heart at acid pH has an excitation maximum at 430 nm and an emission maximum at 500 nm (Fasella et al., 1961). Shukuya and Schwert (1960) have reported that glutamate decarboxylase has a low pH form which emits at 490 nm maximally when 420 nm light is used for excitation. In both of these enzymes, the absorption data indicates that the PLP is bound as a protonated imine which would be expected to absorb in the 420 nm region (Metzler, 1957; Matsushima and Martell, 1967). The long wavelength fluorescence around 500 nm therefore indicates the presence of the protonated imine. With phosphorylase such a facile interpretation cannot be made. Although phosphorylase does show some absorption around 415 nm in the native enzyme, the observed excitation maximum indicates that only the species at 333 nm contributes appreciably to the observed fluorescence.

Bridges et al. (1966) have compiled fluorescence data on the various ionic species of PLP analogues that are present in aqueous solution. In particular, the dipolar ionic species that have saturated 4' carbons and show absorption in the 330 nm region all have Stokes shifts of 5-6,000 cm\(^{-1}\). The dipolar
imine addition product proposed by Kent to explain the 333 nm form would be expected to have a similar shift. The experimentally reported shift of \(10-11,000 \text{ cm}^{-1}\) is therefore clearly anomalous. Anomalous Stokes shifts have also been observed under certain conditions in compounds analogous to PLP and PLP imines. These systems will be discussed in some detail in order to provide a basis for interpreting phosphorylase fluorescence. An interpretation will be presented here since none has yet appeared in the literature.

Weller (1955, 1956) has studied the fluorescence of salicylic acid and its derivatives. Salicylic acid exhibits fluorescence in methanol with a long Stokes shift (10,900 cm\(^{-1}\)). Weller demonstrated that the ortho hydroxyl group of salicylic acid was necessary for this long Stokes shift. Replacement of the hydroxyl hydrogen with a methyl group to give \(0\)-methoxy benzoic acid caused the Stokes shift to become normal (5,900 cm\(^{-1}\)). Weller interpreted the long Stokes shift to be the result of a proton transfer in the excited state. The process for salicylic acid is shown in the following scheme.

\[
\begin{align*}
\text{COOH} & \quad \text{h} \nu \\
\text{COOH}^+ & \text{COOH}_2^-
\end{align*}
\]
The hydrogen from the hydroxyl group is transferred to the protonated carboxyl group in the excited state to form a positively charged, doubly protonated carboxyl group. The methyl ester of salicylic acid behaved the same as salicylic acid, giving a Stokes shift of 10,500 cm\(^{-1}\). The 0-methoxy benzoic acid methyl ester exhibited a normal Stokes shift of 4,800 cm\(^{-1}\). Further support for the excited state, intramolecular proton transfer was obtained from fluorescence studies of salicylic acid in 6 N potassium hydroxide and concentrated sulfuric acid. The experimental conditions should prevent a proton transfer in the excited state, and the observed Stokes shifts of 5,600 cm\(^{-1}\) in potassium hydroxide, and 5,700 cm\(^{-1}\) in sulfuric acid are in agreement with Weller's interpretation. The formation of the zwitterionic excited state results from ionization constant changes in going from the ground state to the excited state. Weller estimates the acidity of the aromatic OH is 5 to 8 pH units higher in the excited state, and the acidity of the aromatic CO\(_2\)H\(^+\) is 6 to 8 pH units lower in the excited state; these conditions would combine to make the zwitterionic excited state the predominant species.

Cohen and Schmidt (1962) have studied the spectral properties of the thermochromic anil 5'-chlorosalicylidine. This compound in the crystalline state undergoes a tautomerization when warmed from -153° to -49° with a resulting shift of an absorption band to longer wavelengths. The process is inter-
interpreted as a benzoid to quinoid tautomerization and is illustrated in the following scheme.

\[
\begin{array}{c}
\text{Cl} \quad \text{H} \quad \text{N} \quad \text{O} \\
\text{H} \quad \text{N} \quad \text{O} \quad \text{Cl}
\end{array}
\]

Cohen and Schmidt have observed a large Stokes shift in fluorescence for the species at \(-153^\circ\) and have indicated that this fluorescence emission band approximately mirrors the absorption band observed at \(-49^\circ\). An explanation similar to that Weller used for the salicylic acid system was used to explain the observed long Stokes shift i.e., excitation of the benzoid ground state present at \(-153^\circ\) leads to an intermolecular proton transfer in the excited state yielding an excited species which gives the long wavelength fluorescence upon emission.

In analogy with the two systems discussed, we can interpret the long wavelength fluorescence that is derived from the 333 nm absorbing species in phosphorylase, if we first assume that the 333 nm form is a tautomeric imine. This tautomeric imine could also undergo intramolecular proton transfer in the excited state to give an excited species fluorescing at 525 nm. This is illustrated in the following scheme.
Unfortunately, model PLP imines which exhibit a 330 nm band have not as yet been tested for the ability to show a long Stokes shift when excited in the 330 nm band. At this time it can only be said that the fluorescence characteristics of phosphorylase would seem to be best explained if we postulate the tautomeric imine as the species absorbing at 333 nm.

Of the four PLP-proteins previously discussed that also have absorption bands at 333 nm, fluorescence data is available only on two: the adduct of PLP and bovine plasma albumin and the 340 nm form of glutamate decarboxylase. Fasella et al. (1961) reported that the adduct of PLP and bovine plasma albumin exhibits an excitation maximum at 330 nm and an emission maximum at 390 nm. Shukuya and Schwert (1960) indicated that glutamate decarboxylase in the 340 nm form emits at 380 nm when excited with 335 nm light. Both systems have the type of fluorescence that would be expected from dipolar imine addition products. Phosphorylase is therefore unique in its fluorescence at this time.
Difference spectra

Because of the lack of any detailed structural work on phosphorylase involving sequence analysis, active site-directed reagents, and ultimately x-ray crystallography, it cannot now be decided whether the PLP in phosphorylase is at the catalytic site or at a site distinct from it. However, data has been presented that show a spectral change can occur in the absorption band of the bound PLP when phosphorylase b binds its substrates. The spectral change was experimentally observed as a small difference spectrum with a dual beam spectrophotometer that had a concentrated phosphorylase b solution in the reference beam, and at equal concentration in the sample beam, a b solution that contained AMP and substrate (Bresler et al., 1966, 1968; Bresler and Firsov, 1968). The substrate could be either phosphate or G-1-P but not glycogen. The difference spectrum was observed only with AMP and anionic substrate together and not with either alone. The data of Bresler et al. (1966) show the difference spectrum with a maximum at about 360 nm, a crossover at about 340-345 nm and a minimum at 320 nm with G-1-P as the substrate. With phosphate as the substrate, the minimum appeared to be nearer 330 nm. In a later paper (Bresler and Firsov, 1968) somewhat different data were obtained: the difference spectrum with G-1-P (no data was reported for phosphate) was said to have a maximum near 360 nm, a crossover near 333 nm, and a minimum about 310 nm. Buc and Buc (1968) have used the difference spectrum to follow the
binding of phosphate to phosphorylase in the presence and absence of AMP. It was found that in the absence of AMP, the difference spectrum appeared only at high phosphate concentration. The binding curves and constants obtained from them were consistent with the allosteric model proposed by these authors. The difference spectrum observed by Buc and Buc in the presence of phosphate had a maximum at 365 nm, a crossover at 345 nm and a minimum at 320 nm. Bresler et al. (1966) observed that ATP and glucose which inhibit the rate of enzymic catalysis in an allosteric manner, also cause a decrease in the difference spectrum. The observed decrease in the intensity of the spectrum would presumably reflect a decreased capacity for phosphate and G-1-P to bind to the enzyme in the presence of these allosteric inhibitors.

The difference spectrum itself could originate in two main ways. In one model, the enzyme saturated with AMP and anionic substrate could have a PLP absorption band that is slightly red shifted with respect to the free enzyme. The alternative model would require that a small amount of a new species in equilibrium with the normal 333 nm species be formed when the enzyme bound AMP and substrate. The first model predicts that the crossover in the difference spectrum will occur very near 333 nm, while the second model would assign the 360 nm maximum in the difference spectrum to a new species, and would predict that the crossover would not be at 330 nm. Unfortunately, there are discrepancies in the reported results about where
the crossover in the difference spectrum is located. Until further experimental results are available, a profitable discussion cannot be made about the origin of the difference spectrum. Bresler and Firsov (1968) did indicate that the magnitude of the difference spectrum was a linear function of enzyme concentration (5-20 mg/ml) and that the magnitude of the spectrum did not change between 20° and 40°. The results were interpreted to mean that dimerization of phosphorylase b was not responsible for the difference spectrum.

It should be made clear that the spectral change in the bound PLP does not necessarily mean that PLP in phosphorylase is or is not at the catalytic site. The binding of AMP and substrate to phosphorylase b could cause a conformational change in the enzyme that might produce changes in environment at the PLP site, even if it were far removed from the catalytic site. In fact an enzyme species might be involved that has an altered PLP spectrum, but which might not even be on the main catalytic sequence; all that is required is that the species be in equilibrium with an active complex.

Circular dichroism

The bound PLP in phosphorylase exhibits optical activity in its absorption band. This has been found to be the case for all PLP enzymes that have been investigated for optical activity. The circular dichroism band in phosphorylase b appears as a symmetrical band centered at 333 nm where the absorption
maximum also occurs (Torchinsky et al., 1965, 1968; Johnson and Graves, 1966). The 415 nm absorbing form produced at acid pH was observed to be optically inactive, and it was further observed by Johnson and Graves that the 415 nm form produced by detergent was also optically inactive. Reduced phosphorylase was found to have lost at least 60% of the CD characteristic of the native enzyme (Johnson and Graves). This observation is consistent with the loss of absorption at 330 nm observed by Kent (1959) in the reduced enzyme.

The presence of PLP CD in phosphorylase provided another spectral probe into the protein environment around the bound cofactor. Both Torchinsky et al. (1968) and Johnson and Graves (1966), have reported that neither G-1-P, AMP or an equilibrium system containing glycogen perturbs the observed CD of the enzyme. As in the case of absorption spectroscopy, however, small absolute changes would have gone undetected. In contrast to the CD results, changes in the optical rotatory dispersion of the enzyme has been reported when AMP is present (Hedrick, 1966). These changes were reflected in the parameters of a Drude plot that was obtained between 400 nm and 600 nm. Because of the CD results, it is doubtful that the observations of Hedrick would indicate a change in PLP optical activity. It is more probable that the environments of other protein chromophores were affected by AMP binding, giving rise to the optical activity change.
The state of enzyme aggregation also had little effect on the observed CD. Phosphorylase \( b \) (a dimer) and \( a \) (a tetramer) gave the same CD within experimental error (Johnson and Graves). Dissociation of phosphorylase \( b \) into monomers could be induced at alkaline pH. Under conditions where 50-90% of the \( b \) existed in the monomeric form, very little change in CD was found (Torchinsky et al., 1968). This was interpreted to mean that PLP was not involved in contact interactions between monomers. Another means of monomerization involves the use of the sulfhydryl reagent PMB, whose action can be reversed by addition of mercaptan to the enzyme. In this instance, CD changes were observed. Torchinsky et al. (1968) report about a 40% loss of CD, and Johnson et al. (1968) report a reversible loss of 15-20% of the CD. The CD results would seem to indicate that monomerization by PMB results in a larger conformational change than monomerization by alkaline pH.

Direct comparison of the CD results discussed in this section is most easily accomplished by use of the anisotropy factor of Kuhn (1958). This factor is equal to \( \Delta \varepsilon / \varepsilon \), in which \( \varepsilon \) is the usual molar absorption coefficient, and \( \Delta \varepsilon \) is the observed CD expressed as the difference between the molar absorption coefficients of left and right circularly polarized light. The most useful aspect of the factor is its concentration independence. For instance, it allows comparison between PLP enzymes where the purity of the enzyme is uncertain.
Obviously for the comparison to be valid, the absorption band for the bound PLP must be far enough removed from the protein absorption band. The value of $\Delta \varepsilon / \varepsilon$ at 333 nm in phosphorylase $b$ at neutral pH has been reported to be $1.1 \times 10^{-3}$ by Johnson and Graves (1966), and $0.86 \times 10^{-3}$ by Torchinsky et al. (1968). The two results agree well considering the possible errors in machine calibration and variability of enzyme preparations. Values of $\Delta \varepsilon / \varepsilon$ at 333 nm are also available for lobster muscle $b$ and potato phosphorylase. Johnson and Graves report a value of $0.92 \times 10^{-3}$ for the lobster enzyme, and Kamogawa et al. (1968) give a value of $0.89 \times 10^{-3}$ for the potato enzyme. This similarity of values for the different phosphorylases indicates a similarity in the binding sites for PLP.
EXPERIMENTAL

Materials

Pyridoxal phosphate analogues

The analogues of PLP used in this work were kind gifts from the laboratory of Dr. David E. Metzler at Iowa State University, Ames. Some of the analogues were synthesized in Dr. Metzler's laboratory and some were synthesized in other laboratories. Data on the origin of each compound can be found in the Ph.D. thesis of Furbish (1969). Pyridoxal Phosphate itself was obtained from the Sigma Chemical Company. The names and structures of the analogues used in this work that were not described previously in Table 1 can be found in Figure 1.

Chemicals

All chemicals were obtained from commercial sources and were reagent grade. Imidazole was obtained from the Sigma Chemical Company as an off-white material. It was recrystal-lized from ethyl acetate and dried under reduced pressure overnight in a vacuum desiccator.

Native phosphorylase b

The enzyme was prepared from frozen rabbit muscle essentially as described by Fischer and Krebs (1962). β-Mercapto-ethanol replaced L-cysteine·HCL in the preparation. The enzyme
Figure 1. Names and structures of analogues

I Carboxymethyl-deoxypyridoxal
II Deoxypyridoxalyl phosphonic acid
III Pyridoxal phosphate cyanoethyl ester
IV Pyridoxal methylphosphonic acid
V N-Oxide pyridoxal phosphate
was routinely recrystallized at least three times at 0° from a pH 6.8 buffer consisting of 0.05 M glycer-P, 0.05 M β-ME, 0.001 M AMP and 0.01 M Mg$^{2+}$. The last recrystallization was usually done without added AMP and Mg$^{2+}$. Residual AMP was removed by treating the enzyme with acid washed Norit (charcoal). A ratio of absorbancies ($A_{260\text{ nm}}/A_{280\text{ nm}}$) of 0.54-0.56 was considered a satisfactory indication of AMP removal.

Reduced phosphorylase b

The method for preparation of reduced enzyme was essentially that of Graves et al. (1965). A typical reduction involved the following steps. The third crystals of the native b obtained from 2 lbs. of frozen rabbit muscle were dissolved in 0.1 M glycer-P at pH 6.8 to give 30 ml of enzyme solution (20-30 mg/ml), and residual AMP was removed. Enough solid NaCl was added to the enzyme solution to give a final concentration of 1.5 M, and the solution was subsequently cooled in a glass-stoppered flask to -5° in a refrigerated bath of water-ethylene glycol. After the enzyme solution had turned a bright yellow, small crystals of NaBH$_4$ (1-3 mg) were added with gentle stirring. This immediately bleached the yellow color which slowly returned after about 15 min.; at this time another addition of NaBH$_4$ was made. Usually a total of 3-4 additions were required at 15 min. intervals to give a colorless final solution. After reaction was complete 70 ml of 0.05 M glycer-P, 0.05 M β-ME at pH 6.8 were added; this was followed by
100 ml of neutral, saturated \((\text{NH}_4)_2\text{SO}_4\) to precipitate the enzyme. The precipitate was centrifuged in the cold, dissolved in the glycero-P, \(\beta\)-ME buffer, and dialyzed against this buffer overnight. The dialyzed, reduced enzyme was crystallized in the usual manner \((\text{AMP} + \text{Mg}^{2+})\). Crystallization was slower than with native enzyme and gave crystals that appeared much whiter. The reduced enzyme was stored as a crystalline suspension at 0°.

**Apophosphorylase b**

Apophosphorylase b was prepared according to the procedure of Shaltiel et al. (1966). Usually 250 mg of native b were used in the procedure. For desalting after resolution, a 1.5 cm x 22 cm Sephadex G-25 column was used (8.8 g of G-25); the apoenzyme came off the column at 15-16 mg/ml. Apoenzyme was usually stored in pH 6.0 buffer at 0°.

**Methods**

**Spectrophotometric measurements**

UV spectra were recorded in 1 cm silica, semimicro cells (1.5 ml) on a Cary 15 recording spectrophotometer. CD spectra were taken in the same cells on a modified Jouan dichrograph that has been previously described (Johnson and Graves, 1966; Johnson et al., 1968). Sensitivity of the instrument was 1-2 \(\times 10^{-5}\) \(\Delta A\) as determined from the noise on the trace with the experimental conditions employed. The CD spectra that are
presented in this thesis were obtained by drawing smooth curves though the traces and baseline, and then replotting the data every 5 nm and drawing a smooth curve through the points.

**Protein concentration**

Protein concentration was determined spectrophotometrically using an absorbency index of $A_{278}^{1\%}$ of 11.9 for phosphorylase $b$ (Appleman et al., 1963). The same index was used in calculating concentrations of apo-$b$ and reduced $b$. To determine concentration, AMP was removed as previously described and the concentrated enzyme diluted in 0.1 M glyceo-P buffer at pH 6.8.

**Molecular weight**

A molecular weight of 185,000 for dimer phosphorylase $b$ (Seery et al., 1967) was used when it was necessary to calculate enzyme molarity.

**Assay of phosphorylase**

Phosphorylase was assayed in the direction of glycogen synthesis by the method of Illingworth and Cori (1953). Initial rates of reaction were determined by stopping the reaction at intervals and measuring the phosphate released from G-1-P. A fixed time assay with the substrate concentrations of Hedrick and Fischer (1965) was used in some of the analogue work.
RESULTS AND DISCUSSION

Native Enzyme

Spectral properties

Figure 2 illustrates the type of absorption and CD data characteristic of native phosphorylase b. This data is presented here to allow comparison with data that will be presented in later sections on reduced b, and apo-b reconstituted with analogues. The bound PLP exhibits a principle absorption maximum at 333 nm, and for this particular preparation an $\Delta A / A$ of 0.55 was observed. There is also a small shoulder visible in the absorption spectrum near 420 nm. The absorption in this region is only 0.1 of that of the 333 nm absorbancy maximum.

The CD spectrum shown at the right of the UV spectrum in Figure 2 was taken on the same sample used for the absorption spectrum. The CD spectrum is also centered at 333 nm. A $\Delta A / A$ value calculated at 333 nm equals $1.3 \times 10^{-3}$; at 360 nm the $\Delta A / A$ value equals $0.92 \times 10^{-3}$. The half-widths for the CD and UV data on the long wavelength side of the maxima are approximately equal with values of 29 nm for the absorption data and 25-27 nm for the CD data.

Figure 3 shows CD data obtained at very high enzyme concentration in the long wavelength region (400-500 nm). The CD goes off scale as the 333 nm maximum is approached. The data indicate that the bound PLP species responsible for the
Figure 2. Absorption and CD spectra of phosphorylase b
Phosphorylase b, 16.6 mg/ml, in 0.02 M glycerol-P, 0.015 M L-cysteine at pH 6.8. Noise level in the CD baseline was $+2 \times 10^{-5}$ ΔA.
Figure 3. CD of phosphorylase b in the long wavelength region

Phosphorylase b, 46.2 mg/ml in 0.05 M glycerol-P, pH 6.8. Noise level in the CD baseline was $+3 \times 10^{-5}$ $\Delta$A.
absorption shoulder at 420 nm is also optically active. A value of $\Delta A/A$ of $1.7 \times 10^{-3}$ was obtained for this band at 425 nm. The presence of the enzyme substrate phosphate (.01 M) and the activator AMP (.001 M) had no effect on the intensity of this CD band. This is in agreement with data obtained previously by Johnson and Graves (1966) and Torchinsky et al. (1968) where it was shown that AMP and substrates did not perturb the 333 nm CD band.

The presence of a CD band for the 420 nm species indicates that it is bound in an asymmetric environment. The random association of a small excess of PLP with the many $\varepsilon$-amino side chains of lysine that are available in phosphorylase to form an imine absorbing at 420 nm would not be expected to produce observable optical activity. The argument against excess PLP, randomly bound, is strengthened by the new molecular weight of Seery et al. (1967) for phosphorylase b which, combined with the observation of Kent (1959) of 2.5 moles of PLP for each 250,000 g of enzyme, would indicate only a stoichiometric amount of PLP is bound to the enzyme. Moreover, Johnson et al. (1968) have indicated that loss of CD caused by resolution of phosphorylase b with 0.1 M NH$_2$OH in an imidazole-citrate buffer occurs at about the same rate at 333 nm and at 420 nm. Randomly bound PLP imines would be expected to be immediately removed by 0.1 M NH$_2$OH even without the deforming buffer present. Finally, Kent (1959) has reported that incubation of phosphorylase b with NaBH$_4$ at neutral pH caused no change in
absorbance at 420 nm. The species absorbing at 333 nm and near 420 nm at neutral pH would therefore seem to be bound to the enzyme in a similar fashion, and, in fact, could represent PLP species bound at the same enzyme site. There is as yet no data that would unequivocally demonstrate that the two PLP species are in direct equilibrium with each other.

Reduced Enzyme

Spectral properties

Figure 4 shows the CD of a reduced b preparation at pH 6.75 and pH 4.6. At pH 6.75, the absorbance at 332 nm was 0.190 while at pH 4.6, the absorbance at 332 nm was 0.715. The appearance of a CD band at low pH therefore coincides with the appearance of an absorption band in the reduced enzyme. The CD seen in this particular preparation at pH 6.75 is undoubtedly due to incomplete reduction. In well reduced preparations, values of $A_{332}^{1\%}$ (pH 6.8) of 0.076 have been obtained; this is to be contrasted with the value of $A_{332}^{1\%}$ of 0.210 shown by this particular preparation. Using the value of $\Delta\lambda/\Delta$ at 333 nm calculated earlier for the native enzyme, it can be estimated from the CD results that the preparation of Figure 4 was about 83% reduced. The CD band at pH 4.6 shows a $\Delta\lambda/\Delta$ of $0.7 \times 10^{-3}$ at 332 nm. In terms of absolute CD values, $\Delta\lambda_{332}^{1\%}$ for the reduced enzyme at pH 4.6 is about $55 \times 10^{-5}$, whereas native b at pH 6.8 gives a $\Delta\lambda_{333}^{1\%}$ of $72 \times 10^{-5}$. 
Figure 4. CD of reduced b at pH 4.6 and pH 6.75

The top curve is reduced b (8.9 mg/ml) in 0.05 M potassium acetate pH 4.6. The bottom curve is reduced b (8.9 mg/ml) in 0.05 M glycero-P, pH 6.75.
Because of the intense protein band centered at 280 nm, it is difficult to detect where the PLP in the reduced enzyme is absorbing at pH 6.8. Kent (1959) speculated that the pK of the pyridinium nitrogen in the substituted pyridoxamine phosphate residue of the reduced enzyme was drastically lowered by the proximity of a positively charged residue. From model compound studies (Metzler and Snell, 1955) it can be estimated that the monoanion should absorb about 15 nm lower than the dipolar form. The appearance of an absorption band at 332 nm in the reduced enzyme at low pH would predict that the high pH form proposed by Kent should absorb about 317 nm. No absorption peak or shoulder can be observed above 300 nm in the reduced enzyme at neutral pH. The CD results of Figure 4 also indicate that a new CD band near 317 nm is not formed. However, a new band above 300 nm might be formed that is optically inactive or shows little optical activity, but absorption data appears to already have eliminated this possibility. Thus both CD and absorption results would appear to place the hidden PMP band below 300 nm.

Since a substituted PMP derivative in contact with an aqueous environment at neutral pH would be expected to absorb near 330 nm from model compound studies, it should be possible to generate a 330 nm form from the hidden PMP band at neutral pH by deforming the enzyme. Figure 5 shows such an experiment: the lower curve shows reduced enzyme at pH 6.8, and the upper
Figure 5. Spectrum of reduced b in the presence of guanidine·HCl

The bottom curve is reduced b (9.25 mg/ml) in 0.05 M glycero-P at pH 6.8. The top curve is reduced b (9.25 mg/ml) in 4.25 M guanidine·HCl, 0.025 M glycero-P at pH 6.8.
curve shows the reduced enzyme at the same concentration and pH, but in the presence of 4.25 M guanidine-HCl. The absorption maximum appears at 330 nm and exhibits a value of $A_{330}^{\alpha}$ of 0.76. Sodium dodecyl sulfate (0.3%) in an experiment similar to the one in Figure 5 also deformed the enzyme, and resulted in a band near 330 nm. In this case the maximum was at 327 nm and gave a value of $A_{327}^{\alpha}$ of 0.75. In good preparations of reduced enzyme, the 332 nm band formed at pH 4.6 gave an $A_{332}^{\alpha}$ value of 0.77; Kent (1959) reported a similar value of 0.78.

An attempt to find the hidden band was made by difference spectroscopy. A solution containing reduced b in the 330 nm band form was placed in the sample beam of the Cary 15, and in the reference beam a solution was placed containing reduced b, at equal concentration, in the hidden band form. The reference solution simply contained reduced b at neutral pH, and the sample solution had either reduced b at neutral pH in the presence of SDS (0.3%), or reduced b at pH 5.0. The results of these experiments can be seen in Figure 6. Since reduced b with the hidden band is in the reference beam, it can only show up as a negative contribution to the difference spectrum. The first band that will be observed as $\lambda$ decreases should be a positive 330 nm band. In Figure 6 this is what is observed in both the SDS and pH 5.0 experiments. The absorbance at 330 nm in the SDS experiment is .055 and in the pH 5.0
Figure 6. Difference spectra of reduced b

Top curve. Sample cell contains reduced b (0.80 mg/ml) in 0.1 M sodium acetate at pH 5.0. Reference cell contains reduced b (0.80 mg/ml) in 0.1 M glycero-P at pH 7.0. Bottom curve. Sample cell contains reduced b (0.72 mg/ml) in 0.05 M glycero-P and 0.3% SDS at pH 6.8. Reference cell contains reduced b (0.72 mg/ml) in 0.05 M glycero-P at pH 6.8.
experiment is .056; these values agree to within 2% of the expected absorbance calculated from dilution factors in each case. The absorbance curve crosses the zero line at 302 nm in the SDS experiment and at 300 nm in the pH 5.0 experiment. The SDS experiment shows two negative maxima at 287 nm and 293 nm; similar negative maxima are observed in the pH 5.0 experiment, but are more diminished, and in this case are located at 285 nm and 293 nm. These negative maxima are probably the result of enzyme conformational changes that expose tyrosine and tryptophan residues resulting in a typical denaturation blue shift. A positive maximum is observed near 254 nm in the SDS experiment and at 257 nm in the pH 5.0 experiment.

If we assume that the enzyme is not too greatly perturbed by the conditions used to generate the 330 nm band, the crossover point in the difference spectrum would be determined by the isosbestic point between the hidden band and the 330 nm band. Since the crossover in both cases is near 300 nm, this would indicate that the maximum of the hidden band must be located below 300 nm. Because of the observed perturbation of enzyme aromatic residues, the negative contribution of the hidden band might be covered up, and it cannot be said with certainty where this band is located.

In the case of the pH 5 experiment, it seems logical to assign the 257 nm positive band to the dipolar substituted PMP
residue. With the parent compound pyridoxamine, there is a band at 326 nm ($\epsilon = 7.9 \times 10^3$), and a band at 252 nm ($\epsilon = 4.5 \times 10^3$) in neutral, aqueous solution (Metzler and Snell, 1955). Heinert and Martell (1963) have shown that the electronic absorption spectra of vitamin B$_6$ analogues are always characterized by two absorption bands designated by them as $\pi_1$ and $\pi_2$ bands. If we assume that the ratio of extinction coefficients of the $\pi_1$ and $\pi_2$ bands present in reduced $\beta$ at pH 5.0 is equal to the ratio exhibited by the same bands in the parent compound pyridoxamine, we can calculate that an absorbance of .032 should be associated with the band near 260 nm. The observed absorbance at 257 nm in the pH 5.0 experiment of Figure 6 is .037. The SDS experiment shows a very large absorbance near 260 nm, and it must be assumed that the substituted PMP residue in the reduced enzyme is not responsible for more than a fraction of this absorbance.

The only likely species from model compound studies that could be assigned to the hidden band is the neutral tautomer of the dipolar PMP species. Matsushima and Martell (1967) have shown that pyridoxamine in slightly acidic methanol shows bands at 287 nm, 333 nm, 260 nm, and 220 nm. The bands at 333 nm and 260 nm have been assigned as the $\pi_1$ and $\pi_2$ bands of dipolar pyridoxamine; the bands at 287 nm and 220 nm have been assigned as the $\pi_1$ and $\pi_2$ band of the neutral pyridoxamine tautomer. The pH 5.0 difference spectrum in Figure 6 is
clearly consistent with the hidden band being near 290 nm. The 220 nm $\pi_2$ band is outside the range of the spectrum.

Because of the observed CD at pH 4.6 in the reduced enzyme, a large conformational change around the PLP probably has not taken place at pH 5.0. A larger conformational change probably takes place when SDS is added to the reduced enzyme. It will be recalled that Johnson and Graves (1966) showed that incubation of native enzyme with SDS gave a 420 nm imine that was optically inactive. The presence of larger aromatic residue contributions in the SDS difference spectrum of Figure 6 would also implicate a larger conformational change taking place.

The effect of substrates and activator on reduced phosphorylase b in the hidden band form were tested in order to see if they might affect a conversion to the spectral form absorbing at 330 nm. At pH 7.5, AMP ($1.3 \times 10^{-3}$ M) and phosphate (.036 M) had no effect on the spectrum of the reduced enzyme, and at pH 6.8 the presence of an equilibrium system with initial concentrations of 0.1 M phosphate, .001 M AMP and .04% glycogen produced some turbidity but no appearance of the 330 nm band. The results indicate that when reduced enzyme is substituted PMP must remain in the hidden band form; the possibility that a very small fraction of the hidden band is converted to the 330 nm species is not ruled out by these results.
Figure 7 indicates that there are conditions under which AMP will affect the spectrum of the reduced enzyme. At pH 6.0, the reduced enzyme at 330 nm exhibits about 55% of the maximal absorbance reached at pH 4.6. Addition of .01 M AMP to the reduced enzyme at pH 6.0 causes a 59% reduction in intensity of the 330 nm band. Reduced enzyme at pH 6.0 in the presence of both .01 M AMP and .02 M phosphate did not show any greater diminution of the spectrum than AMP produced by itself. ATP (7.5 x 10^{-3} M) also caused the decrease in the pH 6.0 spectrum of reduced enzyme. The spectrum of the reduced enzyme at pH 6.8 is also shown in Figure 7. It is apparent that AMP will not cause complete reversion to the hidden band form.

The experiment shown in Figure 7 obviously requires a more complicated interpretation than a simple protonation of the pyridine nitrogen which was earlier postulated by Kent (1959) to explain the hidden band to 330 nm conversion. A more likely interpretation is that the hidden band form and the 330 nm form of reduced b represent different protein conformations. To explain the data, AMP would bind more tightly to the conformation represented by the hidden band form. The inability of saturating AMP to completely reverse the spectrum would indicate that the 330 nm form of the enzyme also has some capacity to bind AMP. The idea of an enzyme conformational change is also more compatible with the previous assignment of...
Figure 7. Effect of AMP on the spectrum of reduced b

The pH 6.0 curve is reduced b (8.0 mg/ml) in 0.05 M glycero-P and 0.05 M β-ME at pH 6.0. The (pH 6.0 + AMP) curve is reduced b (8.0 mg/ml) in 0.05 M glycero-P and 0.05 M β-ME at pH 6.0 with 0.01 M AMP present. The pH 6.8 curve is reduced b (7.8 mg/ml) in 0.05 M glycero-P, 0.05 M β-ME at pH 6.8.
the substituted PMP species responsible for the hidden band. The neutral, nonpolar PMP residue characteristic of the reduced enzyme at neutral pH would be brought into a more polar environment by a conformational change as the pH was lowered, resulting in the dipolar 330 nm form.

**Catalytic properties**

Figure 8 shows the pH profiles of the enzymatic activity of native and reduced phosphorylase b measured under the same conditions. The most obvious difference between reduced b and native b is seen at pH 5.4: here, native enzyme has 41% of the activity observed at its pH optimum while the reduced enzyme has fallen to 0%. If the pH optimum curves had been identical, then the ratio of reduced b activity to native b activity at all pH values would have been invariant. This is not the case; it can be seen that the activity ratio decreases smoothly in going from high pH to low pH. The ratios that are actually observed at various pH values are given as follows: pH 7.8 (.79), pH 7.4 (.72), pH 7.0 (.67), pH 6.4 (.61), and pH 6.0 (.57). The results clearly show a skewness in the reduced b pH profile.

The results of Figure 8 give further support to the idea that a conformational change is taking place in the reduced enzyme as the pH is lowered that is not found in the native enzyme. The results reported here are in contradiction to the studies reported by Fischer et al. (1963) in which it was indicated
Figure 8. Effect of pI on the enzymic activity of native and reduced $b$

Native and reduced $b$ were diluted in 0.04 M glycero-P, 0.04 M $\beta$-ME at the indicated pH values, and assayed using the substrate of Illingworth and Cori (1953) which also had been adjusted to the correct pH. Initial rates of phosphate release were determined.
MICROMOLES/min /mg

pH
6.5
7.5

NATIVE

REDUCED

6  12  18

74
that reduced and native enzyme have essentially identical pH-dependence activity curves. Fischer et al. (1963) and Strausbauch et al. (1967) have stressed the similarities between native and reduced enzyme. The results shown in Figures 4, 5, 6, 7, and 8 stress the differences between native and reduced enzyme.

Analogue Reconstituted Enzymes

Spectral properties

Figure 9 shows the CD spectra that were obtained when apo-b was reconstituted with pyridoxal, deoxypyridoxal, and carboxymethyl-deoxypyridoxal (I). In these experiments the apoenzyme was incubated with an excess of the analogues, and then dialyzed in the cold to remove free analogue. The resulting UV spectra of the dialyzed enzymes indicated that the analogues were not bound in equivalent amounts. If the absorption in the 330 nm region is taken as an index of binding, then the order of binding was CMDPL > DPL > PL. The CD spectra of Figure 9 are also in this order. The CMDPL enzyme gave a peak at 333 nm with an observed $\lambda_{333}$ of 0.55; the DPL enzyme had a peak at 330 nm with an $\lambda_{330}$ of 0.41; the PL enzyme showed no peak and only a weak shoulder near 320 nm with an $\lambda_{320}$ of 0.36. These absorbances should all be taken to be somewhat high, because some turbidity was noticeable in all the samples.
Metzler and Snell (1955) have indicated that DPL has absorption characteristics almost identical to PLP. With this observation in mind, it was assumed that absorbances at the peak positions in the analogue enzymes above that observed in the apoenzyme alone would be the most useful index of binding. Using this criterion, the ratio of \( \frac{A_{\text{DPL}} - A_{\text{apo}}}{A_{\text{CMDPL}} - A_{\text{apo}}} \) was found to be 0.57 while the ratio of \( \frac{\Delta A_{\text{DPL}}}{\Delta A_{\text{CMDPL}}} \) at the CD peak positions in Figure 9 was found to be 0.56. The PL enzyme showed only 14% of the CD exhibited by the CMDPL enzyme and because of its absorption characteristics, it is difficult to estimate the amount of PL bound. In another experiment under the same conditions used in Figure 9 apo-\( \beta \) was reconstituted with CMDPL and PLP. The ratios of the corrected absorbances at 333 nm, \( \frac{A_{\text{CMDPL}} - A_{\text{apo}}}{A_{\text{PLP}} - A_{\text{apo}}} \), was found to be 0.75; \( \frac{\Delta A_{\text{CMDPL}}}{\Delta A_{\text{PLP}}} \) from CD measurements at 333 nm was found to be 0.82.

The results would appear to indicate that the PLP, CMDPL, and DPL enzymes will give very nearly the same CD spectra when compared with equivalent amounts of analogue bound. This would imply that all these analogues bind at the same site in nearly the same way. Other workers have presented data that would support this conclusion. Illingworth et al. (1958) and Fischer et al. (1968) have shown that reconstitution of apo-\( \beta \) with DPL results in an inactive enzyme that resembles dimer native \( \beta \) in its ultracentrifugal properties and not apo-\( \beta \). Also, Kastenschmidt et al. (1968) have shown that reconstitution
Figure 9. CD of PL, DPL and CMDPL reconstituted apo-b

Apo-b (14.9 mg/ml) in 0.05 M glycerol-P, 0.05 M β-ME at pH 6.0 was reacted 2 hr. at 30° with a 3 fold molar excess of analogue over PLP binding sites. Enzyme solutions were dialyzed against the above buffer at 4° to remove excess analogue.
of apo-b with DPL returns the allosteric heterotropic and homotropic interactions characteristic of native dimer b which are absent in the apoenzyme. Finally, the results of Figure 9 demonstrate that CD can be used in itself as a criterion of specific binding for PLP analogues with the apoenzyme.

A number of new PLP analogues were tested in this thesis for their ability to bind to apo-b and cause reactivation. Table 2 shows spectral properties of the analogue reconstituted enzymes. To affect reconstitution, apo-b (7.9 mg/ml) in 0.1 M glycero-P, .005 M EDTA at pH 6.8 was incubated with a molarity of analogue equivalent to the molarity of monomer apo-b for at least one hour at 25°. β-Mercaptoethanol was not included in these studies to prevent hemimercaptal formation with the free aldehyde forms of the analogues. Such forms would be expected to absorb in the 330 nm region and would not allow use of the spectrum as a means to show binding. All the analogue enzymes exhibited peaks in the 330 nm region. The N-methyl PLP was used as a control. Fischer et al. (1968) have reported on the basis of ultracentrifuge data that this analogue does not bind to the enzyme. The N-methyl PLP in the presence of apo-b gave a spectrum with a small, broad band near 330 nm and a more distinct peak at 400 nm which is characteristic of the absorption of the free compound. Although the spectral results cannot rule out some binding of the N-methyl PLP, it is apparent for all other analogues tested that a substantial 330 nm band is formed. The small shoulders near 400 nm probably represent
Table 2. Spectral properties of analogue reconstituted enzymes

<table>
<thead>
<tr>
<th>Analogue</th>
<th>$\lambda_{max}$</th>
<th>$A^1_{max}$</th>
<th>$\lambda^a_{S}$</th>
<th>$A^1_{S}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Deoxypyridoxal phosphonic acid (II)</td>
<td>335 nm</td>
<td>0.505</td>
<td>420 nm</td>
<td>0.099</td>
</tr>
<tr>
<td>2. Pyridoxal phosphate cyanoethyl ester (III)</td>
<td>326 nm</td>
<td>0.357</td>
<td>400 nm</td>
<td>0.097</td>
</tr>
<tr>
<td>3. Pyridoxal methylphosphonic acid (IV)</td>
<td>330 nm</td>
<td>0.418</td>
<td>400 nm</td>
<td>0.158</td>
</tr>
<tr>
<td>4. N-Methylypyridoxal phosphate</td>
<td>333 nm, 400 nm</td>
<td>0.214, 0.219</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>5. Pyridoxal phosphate</td>
<td>333 nm</td>
<td>0.534</td>
<td>395 nm</td>
<td>0.127</td>
</tr>
</tbody>
</table>

$^a_{\lambda_S}$ and $^1_{A_S}$ refer to small shoulders in the 400 nm region.
either imine formation with the enzyme or unreacted analogue. It is tentatively concluded that all the derivatives with the exception of N-methyl PLP show some capacity to bind to enzyme and produce the characteristic band near 330 nm.

Apo-b reconstituted with PLP N-oxide (V) gave a spectrum that was distinctly different than the spectra of the reconstituted enzymes shown in Table 2. This spectrum is shown in Figure 10; a peak at 418 nm was observed ($A_{418}^{1%} = 0.52$), and a distinct shoulder at 322 nm was seen ($A_{322}^{1%} = 0.85$). When spectra were taken at different times after addition of PLP N-oxide to apo-b, a decrease in the absorption of the free compound at 398 nm was observed with a parallel increase at 322 nm and 420 nm. It would appear that both bands are formed at very nearly the same rate during the reaction of V with apo-b.

**Catalytic properties**

The ability of the various analogues to reactivate apo-b is shown in Table 3. While it was tentatively concluded that all the analogues in Table 2 bind to the apoenzyme, it can be seen that only two of the analogues show any measurable ability to reactivate: the deoxypyridoxalyl phosphonic acid (II) showed 4% reactivation, and the PLP N-oxide (V) gave 26% reactivation.

From examination of space-filling models, it was found that the carboxymethyl side-chain of I would not extend quite
Figure 10. Spectra of apo-b reconstituted with PLP N-oxide (V)

Apo-b (8 mg/ml) in 0.1 M glycerophosphate, 0.005 M EDTA at pH 6.8 was reacted with a stoichiometric amount of V for one hr. at 30°.
Table 3. Activity of reconstituted enzymes

<table>
<thead>
<tr>
<th>Analogue</th>
<th>% activity^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Carboxymethyl-deoxypyridoxal</td>
<td>0%</td>
</tr>
<tr>
<td>2. Pyridoxal phosphate cyanoethyl ester</td>
<td>0%</td>
</tr>
<tr>
<td>3. Pyridoxal methyl phosphonic acid</td>
<td>0%</td>
</tr>
<tr>
<td>4. Deoxypyridoxalyl phosphonic acid</td>
<td>4%</td>
</tr>
<tr>
<td>5. N-Oxidepyridoxal phosphate</td>
<td>26%</td>
</tr>
</tbody>
</table>

^aActivities were measured in 0.05 M glycerc-P, 0.05 M β-ME at pH 6.8, and are expressed as % reactivation compared to PLP under the same conditions.

as far as the phosphate of PLP. This might explain the inability of this analogue to reactivate. However analogues III and IV would not have this problem. The inability of III to reactivate might be explained by the presence of the bulky cyanoethyl group. In analogue IV the substitution of a methyl group for a phosphate oxygen of PLP should result in much smaller steric problems than found in III, but this analogue was also inactive. The phosphonic acid analogue (II) is the first analogue to be reported that gives reactivation without having an actual phosphate group at the 5' position. Although this analogue gave 4% reactivation with apo-β, it has been reported to give only about 0.2% reactivation to glutamic
oxalacetic transaminase (Furbish, 1969). This would rule out any possible contamination by PLP. The observation that the PLP N-oxide (V) reactivates apo-b would seem to make participation of the pyridine nitrogen less likely in whatever PLP does to bring about the enzymic activity of phosphorylase.

The data presented here and the previous data of other workers would suggest a unique role of the 5' phosphate in the function of PLP in phosphorylase. It would seem reasonable to use the normally ionized phosphate oxygen for binding purposes; the other phosphate oxygen could either be ionized or protonated to confer enzymic activity. Even if this were known, it would still not be possible to decide whether PLP is at the catalytic site or at another distinct site in phosphorylase. Such knowledge will hopefully be obtained in the near future from structural work.

**Reaction of apo-b-V with NH₂OH**

Although the PLP N-oxide reconstituted apo-b was enzymatically active, the spectrum in Figure 10 indicated that a large portion of the bound analogue was present as an imine absorbing at 418 nm. The reaction of NH₂OH was studied with apo-b-V in order to see if this imine could be removed without affecting the enzymic activity. Kinetics of the reaction of apo-b-V with NH₂OH is presented in Figure 11. The reaction was carried out at pH 6.8 and 28° and followed by the decrease in absorbance at 420 nm. An Aᵦ point was calculated by adding
Figure 11. Kinetics of reaction of apo-b-V with NH$_2$OH

Apo-b-V (6.2 mg/ml), prepared as in Figure 9, was reacted with 0.1 M NH$_2$OH·HCl in 0.08 M glycero-P, 0.004 M EDTA at pH 6.8 and 28°. Reaction was followed at 420 nm with a Cary 15.
\[ k = 3.1 \times 10^{-1} \text{ hr}^{-1} \]
\[ t_{1/2} = 2.2 \text{ hr.} \]
NH₂OH to V and then adding the apoenzyme. In the presence of 0.1 M NH₂OH pseudo-first order kinetics were observed with a half-life for removal of the imine of 2.2 hr. In a separate experiment, using the same conditions, samples of apo-b-V were incubated for approximately 6 hr. in the presence and absence of 0.1 M NH₂OH. After this time it was found that the sample containing NH₂OH had lost 76% of enzymic activity when compared to the control sample. Using the rate constant calculated from the experiment of Figure 11, we can calculate that 84% of the 418 nm imine should have reacted after 6 hr. This is in reasonable agreement with the observed loss of enzyme activity over the same time period.

From the available results, it seems probable that the PLP N-oxide species absorbing at 322 nm and 418 nm in apo-b-V are bound at the same site. It is not possible however, to say which species is enzymatically active. The 418 nm species in apo-b-V seems to behave like the small amount of 420 nm species observed in the native enzyme that was discussed in an earlier section i.e., both imines appear to be "buried".
GENERAL DISCUSSION

PLP Spectra-Structure Correlations in Phosphorylase

In the literature review section of this thesis, it was proposed that the assignment of the 333 nm absorbing, PLP species in phosphorylase to a dipolar imine addition product might be incorrect. The tautomeric, neutral imine described by Heinert and Martell (1963) was also considered a good model to explain the available data. In particular, the neutral imine model was considered to be more consistent with the fluorescence characteristics of bound PLP in phosphorylase, in which a long Stokes shift had been observed. The data presented in this thesis can also be more easily explained by invoking the neutral imine model.

The UV and the CD spectra (Figures 4, 5 and 6) of reduced phosphorylase b at neutral pH are all consistent with the absorption band of the substituted pyridoxamine phosphate being located below 300 nm. The most likely species to explain this observation is the tautomer of pyridoxamine in which the 3-oxygen of the pyridine ring is protonated and the pyridine nitrogen is unprotonated. By studying spectra of pyridoxamine in methanol solution, Matsushima and Martel (1967) concluded that this tautomer should absorb around 290 nm; this is clearly consistent with the results described in this thesis on reduced enzyme. This spectral assignment in reduced enzyme would mean that the microscopic environment around the bound
PMP would have to be less polar than water in order for the neutral PMP species to be favored over the dipolar species found in aqueous solution. Since the reduced enzyme has catalytic and physical properties very similar to the native enzyme at neutral pH, we would expect the environment around the bound coenzyme to also be similar in both enzyme forms. In this regard, if the imine addition product of Kent (1959) were assumed to be the predominant species in native phosphorylase, it would be difficult to explain why the absorption should be at 333 nm. In analogy with the reduced enzyme, the environment around the PLP should also favor the neutral tautomer which would be expected to absorb at 290 nm. The assignment of a neutral PLP imine to the 333 nm band in the native enzyme, however, would allow the native and reduced enzymes to have the same microscopic environment around the bound coenzyme.

Hedrick and Fischer (1965) have indicated that the bound PLP in native phosphorylase is "buried" by a number of criteria; chief among these being the inability of the bound coenzyme to react with aldehyde reagents. The same conclusion can be reached about the small amount of 420 nm absorbing species in native phosphorylase. The CD results of Figure 3 indicate optical activity in the 420 nm absorption band of native enzyme. Resolution of the enzyme with NH$_2$OH in the presence of a deforming buffer has previously been shown to cause a parallel loss in the CD of both the 333 nm and 420 nm species (Johnson...
et al. (1968). The presence of a substantial amount of 420 nm imine in the spectra of apophosphorylase reconstituted with PLP N-oxide (Figure 10), and the slow reactivity of this species with \( \text{NH}_2\text{OH} \) (Figure 11) again indicates an environment that would permit a buried imine. In terms of the proposed neutral imine model, the absorption in the 330 nm region and 420 nm region found in native phosphorylase and in apoenzyme reconstituted with PLP N-oxide could simply result from an equilibrium between two imine tautomers. For the 330 nm species, a proton would be on the 3-oxygen of the pyridine ring; in the 420 nm species this proton would reside on the imine nitrogen. Such a scheme would allow the tautomeric equilibrium to exist with no enzyme conformational change taking place; however, equilibria between different enzyme conformers that would either favor the 330 nm species or the 420 nm species cannot be ruled out.

The chemical evidence that PLP is buried in phosphorylase really only indicates that the 4' carbon cannot be attacked by aldehyde reagents, whereas the proposed neutral imine would require a less polar environment than water around the pyridine ring. Bartlett (1969) in this laboratory has shown by solvent perturbation difference spectroscopy that PLP in phosphorylase is not perturbed by ethylene glycol. This would support the idea that the pyridine ring of the bound PLP is "tucked away". However, the solvent perturbation studies did show that the
bound PLP could be perturbed by D$_2$O. It is doubtful, though, that a chromophore could be completely buried to ethylene glycol and completely exposed to D$_2$O at the same time.

Conformational Changes in Reduced Phosphorylase

The appearance of the 332 nm band in reduced b as the pH is lowered could reflect a conformational change in the reduced enzyme. This proposal was strengthened by the results of Figures 6 and 7. The activator, AMP, caused a partial reversal at pH 6.0 in the hidden band to 332 nm conversion which would imply different conformations for the two spectral forms of reduced enzyme. The pH-dependence of activity curve also fell very sharply as the pH was lowered for the reduced enzyme; this phenomena was not seen in pH studies of the native enzyme.

The origin of this conformational change in reduced enzyme is not known. It is possible that NaBH$_4$ could have done more to the enzyme than just reduce the PLP; for instance, a peptide bond might have been reduced, but the mild conditions employed in the reduction would make this possibility improbable. Another possibility is that the PLP was not reduced on the same ε-amino side-chain of lysine on which it was originally bound, but this change would probably cause greater observed differences in catalytic properties between native and reduced enzyme at neutral pH. Using the proposed neutral imine model for bound PLP in the native enzyme, reduction would cause a change in hybridization of the 4' carbon of PLP from sp$^2$ to
sp\(^3\). The different steric disposition that would result around the 4' carbon might contribute to the observed low pH conformational change.

Pyridoxal Phosphate Function in Phosphorylase

The main approach that has been used to gain knowledge about the function of PLP in phosphorylase has been the synthesis of analogues. If PLP has a catalytic function in phosphorylase, there are only certain functional groups of the coenzyme that could be involved. Either the 3-oxygen, the pyridine nitrogen, or the phosphate group could act as an acid-base catalyst at the active site. As mentioned before in the literature section, Fischer and Krebs (1966) reported that the 3,0-methyl derivative of PLP was an active analogue, ruling out participation of the 3-oxygen in catalysis.

The PLP N-oxide (Table 3) tested in this thesis was an active derivative (26%), and would likely rule out catalytic participation of the pyridine nitrogen. There are some pitfalls, however: the oxygen of the N-oxide might be able to replace the pyridine nitrogen to some extent in a catalytic manner, or conceivably the PLP N-oxide might be converted to PLP on the enzyme. The latter possibility was not checked for, but in view of the data it seems unlikely. PLP in the native enzyme is quite refractory to \(\text{NH}_2\text{OH}\), but apoenzyme reconstituted with PLP N-oxide lost 76% of its activity of its activity in 6 hr. when incubated with 0.1 M \(\text{NH}_2\text{OH}\). If this activity loss
were to represent attack on PLP that had arisen from PLP N-oxide, the enzyme structure would have to have been altered to permit such rapid attack by \( \text{NH}_2\text{OH} \).

The other analogues tested in this thesis involved changes in the 5' phosphate portion of PLP. Analogues I, III and IV allowed only one negative charge on the 5' side-chain and, while all seemed to be effective in binding, they did not return enzymic activity. The only active analogue was deoxy-pyridoxalyl phosphonic acid (II) which gave 4% reactivation. The activity shown by this analogue intimates that either a second negative charge is needed on the 5' side-chain of PLP for interaction with the enzyme to give the active conformation, or that one of the phosphate oxygens of PLP is involved in acid-base catalysis. Analogue II might give only the observed 4% reactivation either because of a shorter 5' side-chain than PLP, or because the pK of the phosphonic acid might not be close enough to the pK exhibited by the phosphate of PLP. A phosphonic acid analogue with a side-chain extension of one methylene residue might prove useful in resolving this question.

The data suggesting that PLP is buried in phosphorylase does not preclude either a catalytic or structural function for the coenzyme. A conformational change could alter the environment of the PLP during catalysis, or if only the 5'-phosphate portion were involved catalytically, the environment
around the bound coenzyme might change very little during catalysis.

From the study presented here and the cited work of many others, it is indeed apparent that there is still a long way to go in understanding the function of PLP in phosphorylase. Experimentally, ways must be found to locate the PLP binding site in relation to the catalytic binding sites in order to precisely determine the coenzyme function; this means that laborious structural work is ahead. The answer to how PLP ever got to be in phosphorylase in the first place, of course, lies in antiquity. The emerging field of biochemical evolution will undoubtedly find the study of phosphorylase to be a rewarding endeavor.
SUMMARY

Native Enzyme

Phosphorylase b was shown to have a small CD band at 425 nm in addition to the large band at 333 nm. The presence of phosphate (0.01 M) and AMP (0.001 M) did not perturb the observed long wavelength CD.

Reduced Enzyme

The observations of Kent (1959) that reduced b does not exhibit an absorption band for its substituted PMP residue at neutral pH, and that a 332 nm band appears at low pH were confirmed and extended. The 332 nm band at low pH in the reduced enzyme was found to be optically active, \((\Delta A/A)_{332\text{ nm}} = 0.7 \times 10^{-3}\). CD of the reduced b at pH 7.0 indicated that no new band was formed above 300 nm; the UV spectrum also provided no evidence for a PMP band above 300 nm.

Guanidine·HCl (4.25 M) and SDS (0.3\%) caused the appearance of a band near 330 nm at pH 6.8. Difference spectra of reduced b (+ SDS; pH 5 versus pH 7) indicated that the hidden band must be below 300 nm.

AMP (.01 M) was shown to cause partial reversal of the hidden band to 332 nm conversion at pH 6.0; ATP was also effective. Substrates and AMP had no effect on the spectrum of the reduced b at pH 6.8. Native b and reduced b showed different pH profiles of enzyme activity, with the reduced b activity falling off more sharply than the native b below pH
7.0. The data were interpreted to indicate different conformations for the 332 nm and hidden band forms.

**Analogue Studies**

Apo-b reconstituted with deoxypyridoxal, pyridoxal, and carboxymethyl-deoxypyridoxal exhibited CD bands centered around 330 nm. Comparison of the CD and UV spectra of apo-b reconstituted with either DPI, CMDPL, or PLP indicated that all gave approximately the same CD when corrected for the amount of analogue bound.

All the analogues tested gave a band near 330 nm when reacted with stoichiometric amounts of apo-b. Carboxymethyl-deoxypyridoxal (I), PLP cyanoethyl ester (III) and pyridoxal methylphosphonic acid (IV) all failed to reactivate apo-b. Deoxypyridoxalyl-phosphonic acid (II) gave 4% reactivation of apo-b, and PLP N-oxide (V) gave 26% reactivation when compared with PLP.

The PLP N-oxide enzyme showed a substantial imine maximum at 418 nm and a distinct shoulder at 322 nm. Apo-b-V was reacted with NH₂OH (0.1 M) at pH 6.8 and 28°. Disappearance of the imine at 420 nm showed pseudo-first order kinetics with an observed half life of 2.2 hr. Loss of enzyme activity paralleled the loss of the imine.
Spectra-Structure Correlation

From the results obtained with the reduced b, a neutral tautomer of the substituted PMP, absorbing near 290 nm, was assigned to the hidden band. The 332 nm species in reduced b was postulated to arise from a conformational change at low pH that exposed the PMP residue to a more polar environment.

In analogy with reduced b, the species absorbing at 333 nm in native b was assigned to be a neutral imine tautomer (imine-nitrogen unprotonated and 3-oxygen protonated). The neutral imine tautomer was postulated to be in an environment less polar than water. Tautomerization of the neutral imine was postulated to explain the unreactive 420 nm imines in native b and apo-b-V.
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