Purification and Characterization of Vitamin B₆-Phosphate Phosphatase from Human Erythrocytes*

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Human erythrocytes rapidly convert vitamin B₆ to pyridoxal-P and contain soluble phosphatase activity which dephosphorylates pyridoxal-P at a pH optimum of 6-6.5. This phosphatase was purified 51,000-fold with a yield of 39% by ammonium sulfate precipitation and chromatography on DEAE-Sepharose, Sephacryl S-200, hydroxylapatite, and reactive yellow 86-agarose. Sephacryl S-200 chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the enzyme was a dimer with a molecular mass of approximately 64 kDa. The phosphatase required Mg²⁺ for activity. It specifically catalyzed the removal of phosphate from pyridoxic-P, pyridoxine-P, pyridoxamine-P, 4-pyridoxic acid-P, and 4-deoxy-pyridoxine-P at pH 7.4. Nucleotide phosphates, phosphoamino acids, and other phosphorylated compounds were not hydrolyzed significantly nor were they effective inhibitors of the enzyme. The phosphatase showed Michaelis-Menten kinetics with its substrates. It had a \( K_m \) of 1.5 \( \mu M \) and a \( V_{max} \) of 3.2 \( \mu mol/min/mg \) with pyridoxal-P. The \( V_{max}/K_m \) was greatest with pyridoxic-P > 4-pyridoxic acid-P > pyridoxine-P > pyridoxamine-P. The phosphatase was competitively inhibited by the product, inorganic phosphate, with a \( K_i \) of 0.8 \( \mu M \), and weakly inhibited by pyridoxal. It was also inhibited by \( Zn^{2+} \), fluoride, molybdate, and EDTA, but was not inhibited by levamisole, \( \alpha \)-phenylalanine, or 1,2-tartrate. These properties of the purified enzyme suggest that it is a unique acid phosphatase that specifically dephosphorylates vitamin B₆-phosphates.

Pyridoxine and pyridoxamine are phosphorylated by pyridoxal kinase (EC 2.7.1.35) and then converted to pyridoxic-P by pyridoxamine (pyridoxine) 5'-phosphate oxidase (EC 1.4.3.5). Most PLP is bound to proteins or hydrolyzed to pyridoxal by phosphatases. Although the kinase activity occurs in all mammalian tissues, the oxidase activity is very low or absent in many tissues. Human erythrocytes have the kinase, the oxidase, and phosphatase activities; therefore, they readily synthesize PLP and pyridoxal from dietary pyridoxine (1).

Some phosphatases rapidly hydrolyze PLP when free, but not when protein-bound (2-5). Subjects with alcoholism or liver disease often have increased degradation of PLP and low concentrations of plasma PLP which are inversely related to phosphatase activity (6-10). The alkaline phosphatase activities are high and PLP concentrations are low in plasma of children with hypophosphatemic rickets (11). Conversely, the alkaline phosphatase activities are low and PLP levels are high in plasma of patients with hypophosphatemia (9). Therefore, it has been suggested that tissue and plasma phosphatases play important roles in regulating tissue and plasma PLP concentrations (9, 12, 13).

PLP and pyridoxamine 5'-phosphate (PMP) are hydrolyzed by intestinal and tissue-nonspecific alkaline phosphatases which have broad substrate specificity for phosphomonoesters (8, 14). Acid phosphatases with broad substrate specificity have been purified from liver and kidney and shown to hydrolyze PLP (15, 16). Histochemical methods have demonstrated the presence of acid phosphatases in bone, neutrophils, and neuros which hydrolyze PLP (17-19). An acid phosphatase specific for PLP was partially purified from mouse liver nuclei (20); however this enzyme was not characterized. We describe herein the purification from human erythrocyte of a phosphatase which we had previously demonstrated to have high affinity for PLP (21). The purified phosphatase shows specificity toward phosphorylated vitamin B₆ compounds. It is hoped that characterization of this enzyme will help elucidate the role of catabolism in the regulation of PLP concentration.

EXPERIMENTAL PROCEDURES

Materials—PLP, PMP, 4-deoxy-PNP, most of the other biochemicals, and reactive yellow 86-agarose were obtained from Sigma. We purchased AG 1-X2 (200-400 mesh) from Bio-Rad, DEAE-Sepharose CL-6B and Sephacryl S-200 High Resolution from Pharmacia LKB Biotechnology Inc., and hydroxylapatite high resolution from Behring Diagnostics. 4-Pyridoxic acid phosphate (4-PAP) was synthesized according to the procedure of Wada and Snell (22). Sodium \([^{3}H]borohydrde\) (specific activity 348 Ci/mol) was purchased from Du Pont-New England Nuclear. 4-\(^{[3H]}\)PNP was synthesized by reduction of PLP with sodium \([^{3}H]borohydrde\) and was then oxidized to 4-\(^{[3H]}\)PLP with manganese dioxide (23). \(^{[3H]}\)PNP and \(^{[3H]}\)PLP were purified by chromatography on AG 1-X2 acetate (24). The purity of the \(^{[3H]}\)PNP and \(^{[3H]}\)PLP was determined by high performance liquid chromatography (HPLC) as described previously (25). The specific radioactivity of the \(^{[3H]}\)PLP was 15.6 Ci/mol and that of the \(^{[3H]}\)PNP was 49 Ci/mol.

Solutions were prepared with distilled and deionized water. Solutions of vitamin B₆ and other light-sensitive compounds were protected from light, and all experiments involving light-sensitive compounds were performed in a room illuminated only with yellow fluorescent lights (Sylvania F15/8-GO).

Enzyme Assays—Phosphatase activity was routinely assayed with \(^{[3H]}\)PNP or \(^{[3H]}\)PLP as substrate (10). The assays were conducted...
in 1.5-ml microfuge tubes containing 0.2 ml of 36 μM [3H]PNP, 4 mM MgCl₂, and 37 mM triethanolamine-HCl, pH 7.4, unless indicated otherwise. At zero min and after 60 min of shaking at 37 °C, 50-μl aliquots were removed and applied to DE81 discs (Whatman). The [3H]pyridoxine was removed from the disc by agitation with 1.4 ml of 100 mM sodium citrate at 4 °C for 4 min, and the radioactivity in the wash was determined by liquid scintillation spectrometry. PNP phosphatase activity was routinely measured during purification because [3H]PNP is easier to synthesize than [3H]PLP, and [3H]PLP binds to protein present in fractions containing high protein concentration.

Hydrolysis of all other potential substrates was measured in 37 mM triethanolamine-HCl, pH 7.4, containing 4 mM MgCl₂, and 37 mM triethanolamine-HCl, pH 7.4, unless indicated in 1.5-ml microfuge tubes containing 0.2 ml of 36 pM [3H]PNP, 4 mM triethanolamine-HCl, pH 7.4, containing 4 mM MgCl₂, and 37 mM triethanolamine-HCl, pH 7.4. Other aromatic compounds tested as putative substrates were incubated with the enzyme and subsequently applied to an HPLC ion-exchange column. Absorbance was monitored with a Kratos Spectroflow 357 spectrometer at 260 nm for ATP, ADP, AMP, and CAMP, 270 nm for phenylphosphate, 312 nm for p-nitrophenylphosphate, and 375 nm for FMN. ADP and AMP were separated on a Vydac 401TP cation-exchange column isocratically with 0.05 M KH₂PO₄, pH 2.2, from 0 to 3 min, from 0.05 M and 0.5 M KH₂PO₄, pH 3.5, at 4 min, from 0.05 M A and 0.5 M B at 4 min to 90% B and 10% C (0.5 M NaH₂PO₄, pH 5.9) at 13 min, and from 90% B and 10% C at 13 min to 100% C at 23 min, and maintaining 100% C from 23 to 25 min. The effluent was reacted with sodium bisulfite. The fluorescence was detected with a McPherson FL-750 fluorometer equipped with a 24-μl flow cell and with a CF-360 filter at the entrance to the emission monochrometer, excitation wavelength set at 392 nm and emission wavelength at 460 nm.

Other aromatic compounds tested as putative substrates were incubated with the enzyme and subsequently applied to an HPLC ion-exchange column. Absorbance was monitored with a Kratos Spectroflow 357 spectrometer at 260 nm for ATP, ADP, AMP, and CAMP, 270 nm for phenylphosphate, 312 nm for p-nitrophenylphosphate, and 375 nm for FMN. ADP and AMP were separated on a Vydac 401TP cation-exchange column isocratically with 0.02 N HCl. AMP and CAMP were separated from adenosine on the cation-exchange column isocratically with 0.1 M ammonium acetate, pH 5.0. ATP and ADP were separated on a 4.1 × 250-mm SynChropak Ax300 anion-exchange column isocratically with 0.05 M KH₂PO₄, and 0.5 M NaCl. FMN and riboflavin, p-nitrophenolphosphate and p-nitrophenol, and phenylphosphate and phenol were separated on the anion-exchange column isocratically with 0.1 M KH₂PO₄. The phenolphomino acids tested as putative substrates were incubated with the enzyme and subsequently were derivatized with orthohthalaldehyde and chromatographed on an Ultrasphere-ODS reversed-phase column (Beckman) as described by Hill et al. (27). The dephosphorylated products were detected fluorometrically with excitation at 394 nm and emission at 440 nm.

Phosphate release from putative substrates was measured colorimetrically as the molybdate complex with malachite green (28). Hydrolysis of p-nitrophenolphosphate was also assessed by measuring the production of p-nitrophenol; the reaction was stopped by the addition of the absorbance was measured at 405 nm and was measured at 1000 rpm. The cells were washed three times with 0.9% NaCl, frozen for 30-60 min at −70 °C, and then resuspended in 2 volumes each of D (2 mM 2-mercaptoethanol, 2 mM MgCl₂, 1 mM EGTA, 50 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin, 0.7 mg/ml pepstatin, 0.62% sodium deoxycholate, 0.1% Triton X-100, and 10 mM triethanolamine-HCl, pH 7.1) containing 40 mM KCl at 10 °C. Two-mL fractions were collected. Fractions containing PNP phosphatase activity were concentrated to 2 ml, diluted 3-fold with buffer E, and applied to a hydroxyapatite column (1 × 5-cm) equilibrated with buffer E. The flow rate was 10 ml/h, and 1.8-ml fractions were collected. The column was washed with 5 ml of buffer B followed by a gradient of 30 ml of buffer B versus 30 ml of buffer E with 0.1 M phosphate, pH 7.1. Active fractions were concentrated to approximately 2 ml, diluted 3-fold with buffer E, and applied to a 1 × 10-cm column of reactive yellow 86-agarose equilibrated with buffer E. The column was eluted at a flow rate of approximately 20 ml/h with 20 ml of buffer E, 20 ml of buffer E containing 0.1 N NaCl, and 20 ml of buffer E containing 0.2 M NaCl. Fraction size was 3 ml. The active fractions were concentrated to approximately 1 ml and were stored in the presence of 20% glycerol at −20 °C.

Protein concentrations were determined by the Coomassie Brilliant Blue assay using the reagent and micro procedure from Pierce Chemical Co. Bovine serum albumin was used as a standard. SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (29) with 12% acrylamide gels. Protein bands on the gels were visualized by using either Coomassie Brilliant Blue R-250 or silver staining (Bio-Rad kit). Apparent molecular weight was estimated using a commercial mixture of molecular weight standards (Bio-Rad).

RESULTS

Human erythrocytes had at least two forms of PNP (PLP) phosphatase activity when assayed with 36 μM PNP or PLP and 4 mM MgCl₂ from pH 5 to 10. The hemolysate supernatant contained phosphatase activity with a pH optimum between 6 and 6.5. Alkaline PNP (PLP) phosphatase activity with an optimum at pH 9 was associated with the stromal fraction. Since the soluble enzyme had considerable activity at physiological pH and low Kₘ values with PNP and PLP, this enzyme activity was purified.

Nearly all the soluble PNP phosphatase activity was precipitated from human hemolyzate supernatant with 55% ammonium sulfate, and none was precipitated with 32.5% ammonium sulfate. The activity bound to the DEAE-Sepharose column in 10 mM triethanolamine buffer, pH 7.1, and eluted as a single peak between 70 and 140 mM KCl (Fig. 1A). The PNP phosphatase activity was then chromatographed on a Sephacryl S-200 column (Fig. 1B) and a hydroxyapatite column (Fig. 1C) from which it eluted as a single peak between 30 and 44 mM phosphate. Finally, chromatography on a reactive yellow 86-agarose column separated the PNP phosphatase activity from other phosphatases (Fig. 1D). Of the total activity recovered from the dye column, approximately 30% came off in the buffer wash along with phosphoglycocolate phosphatase, other phosphatase activities, and most of the protein, and 70% of the PNP phosphatase activity eluted with 0.1 N NaCl. Some of the PNP phosphatase activity that eluted in the buffer wash may have been due to nonspecific phosphatases. A typical purification of the phosphatase starting with 130 ml of human blood is shown in Table I. Overall, the PNP phosphatase activity was purified approximately 51,000-fold with a yield of 39% based on the total PNP phosphatase activity in the hemolysate.

Other chromatography resins were tested for effectiveness
FIG. 1. Chromatography of PNP phosphatase activity on consecutive columns. Panel A, DEAE-Sepharose chromatography. Panel B, Sephacryl S-200 chromatography. The elution positions of molecular mass standards obtained from separate runs are shown: 1) blue dextran 2000; 2) yeast alcohol dehydrogenase, 150 kDa; 3) bovine serum albumin, 66 kDa; 4) ovalbumin, 45 kDa; and 5) carbonic anhydrase, 29 kDa. Panel C, chromatography on hydroxylapatite. Panel D, chromatography on reactive yellow-86 agarose. Phosphatase activity assayed with [3H]PNP (○) or phosphoglycolate (△) and protein concentration (△) were measured.

TABLE I
Purification of PNP phosphatase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>nmol/min</td>
<td>nmol/min/mg protein</td>
<td>-fold</td>
<td>%</td>
</tr>
<tr>
<td>Hemolysate</td>
<td>12,900</td>
<td>147</td>
<td>0.0113</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate (25–53%)</td>
<td>758</td>
<td>147</td>
<td>0.193</td>
<td>17.2</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>25.0</td>
<td>104</td>
<td>4.16</td>
<td>368</td>
<td>71</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>9.12</td>
<td>83.8</td>
<td>9.08</td>
<td>803</td>
<td>57</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>0.96</td>
<td>64.3</td>
<td>67.0</td>
<td>5,950</td>
<td>44</td>
</tr>
<tr>
<td>Reactive yellow 86-agarose</td>
<td>0.10</td>
<td>57.5</td>
<td>575</td>
<td>51,000</td>
<td>39</td>
</tr>
</tbody>
</table>

in purifying the enzyme. PNP phosphatase activity bound less tightly to reactive yellow 86-agarose in 10 mM triethanolamine-HCl, pH 7.1, than to other dye agaroses (Blue 3-, Blue 4-, Red 120-, Brown 10-, or Green 19-agarose). PNP phosphatase activity did not bind to concanavalin A-Sepharose.

The purified PNP phosphatase was unstable at low protein concentration. The addition of 0.002% Triton X-100 stabilized the enzyme. The enzyme was unstable to freezing in the absence of glycerol. Currently, we routinely purify the enzyme from at least 1 liter of blood to obtain higher protein concentrations.

The soluble PNP phosphatase activity eluted as a single peak just after bovine serum albumin on a calibrated Sephacryl S-200 column (Fig. 1B). The elution of the phosphatase corresponded to a molecular mass of approximately 64,000 daltons when the data were plotted as $K_{AV}$ versus log (molecular mass). The elution data plotted as $(-\log K_{AV})^3$ versus Stokes radius indicated an apparent Stokes radius of approximately 3.5 nm (30). The highly purified phosphatase migrated as a single 33-kDa protein band on SDS-polyacrylamide gels with (Fig. 2) or without mercaptoethanol. The phosphatase is apparently a dimer.

Many phosphatases require a divalent metal ion for activity. The effect of EDTA on the PNP phosphatase activity eluted from the Sephacryl S-200 column was examined. The activity was completely inhibited by 0.2 mM EDTA in the absence of added Mg$^{2+}$. In the presence of 1 mM Mg$^{2+}$, the activity was inhibited 50% by 2 mM EDTA. The activation by MgCl$_2$ of

FIG. 2. SDS-polyacrylamide gel electrophoresis of purified phosphatase. Denatured and reduced samples were subjected to electrophoresis on 12% SDS-polyacrylamide gels. The gel was stained with Coomassie Blue. Lane 1, molecular mass markers: phosphorylase b (97 kDa); bovine serum albumin (66 kDa); ovalbumin (45 kDa); bovine carbonic anhydrase (31 kDa); soybean trypsin inhibitor (21.5 kDa); and lysozyme (14.4 kDa). Lane 2, 1.7 μg of fully purified phosphatase.
the enzyme pretreated with EDTA followed saturation kinetics (Fig. 3). Under these conditions, the concentration of Mg\(^{2+}\) giving 50% activation (K<sub>s</sub>) was approximately 130 μM. The enzyme had 17% as much activity with 1 mM CaCl<sub>2</sub> as with MgCl<sub>2</sub> and had only 4, 2, 2%, and zero activity with 1 mM Cu\(^{2+}\), Mn\(^{2+}\), Ca\(^{2+}\), and Zn\(^{2+}\), respectively. In fact, Zn\(^{2+}\) was a very potent inhibitor since the PNP phosphatase activity in the presence of MgCl<sub>2</sub> was inhibited 50% by 10 μM ZnCl<sub>2</sub>.

PNP phosphatase activity from the Sephadryl S-200 column was not affected significantly by 100 mM NaCl, KCl, NaBr, NaI, NaNO<sub>3</sub>, or sodium acetate when assayed with 1 mM MgCl<sub>2</sub>, 36 μM PNP, and buffer which contained approximately 28 mM chloride. Therefore, in the presence of MgCl<sub>2</sub>, the partially purified enzyme was not activated by these monovalent cations or anions.

The highly purified phosphatase specifically catalyzed the removal of the phosphate from PLP, PNP, PMP, 4-PAP, and 4-deoxo-PNP (Table II). The velocities with PLP, PMP, PNP, and 4-PAP followed Michaelis-Menten kinetics (Fig. 4). The enzyme had the lowest K<sub>m</sub> and highest V<sub>max</sub> values for PLP and 4-PAP (Table II). It had intermediate K<sub>m</sub> and low V<sub>max</sub> values with PMP. The specificity constant (V<sub>max</sub>/K<sub>m</sub>) was highest with PLP followed by 4-PAP > PNP > PMP. Kinetic constants obtained for PLP with the HPLC assay were similar to those obtained with the radiometric assay.

The purified phosphatase catalyzed the hydrolysis of p-nitrophenylphosphate slowly (Table II). Under the conditions used, the purified enzyme did not catalyze the dephosphorylation of other physiologically important phosphorylated compounds, such as nucleotides, phosphoamino acids, or phosphoglycerate. The active fractions eluted from the Sephadryl S-200 column contained a mixture of phosphatases that hydrolyzed many phosphorylated compounds. This fraction had a specific activity in nmol/min/mg of 8.3 with PNP, 86 with phosphoglycerate, 43 with p-nitrophenylphosphate, 3.7 with phosphoserine, 0.2 with phosphothreonine, and 0.5 with phosphotyrosine.

Potential substrates were tested as inhibitors of the purified enzyme with PNP as substrate (Table III). PLP and 4-PAP were very effective inhibitors, and PMP was less inhibitory than PLP and 4-PAP. p-Nitrophenylphosphate was a poor inhibitor, and phenylphosphate was even poorer. No significant inhibition was observed with 5 mM ATP or phosphoglycerate, or with 0.5 mM α- or β-glycerophosphate, 3-phosphoglycerate, or 2,3-bisphosphoglycerate.

The products of the reaction were tested as inhibitors. Phosphate was a competitive inhibitor with respect to PNP with a K<sub>s</sub> value of 0.8 mM (Fig. 5). Approximately 11 mM pyridoxal was required to give 50% inhibition with either PNP or PLP as substrate (Table III). No significant inhibition was obtained with 5 mM of the other products, pyridoxine, pyridoxamine, or 4-pyridoxic acid or with 5 mM of the product analogs, 4-pyridine-carboxaldehyde, isonicotinate, 3-hydroxypryridine, salicylaldehyde, or benzaldehyde. The effect of common acid or alkaline phosphatase inhibitors was also studied. Fluoride appeared to give complex noncompetitive

### Table II

Kinetic parameters of phosphatase with various potential substrates

<table>
<thead>
<tr>
<th>Compound</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (μM)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; or activity (μmol/min/mg)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; (min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxal-P</td>
<td>1.47 ± 0.23</td>
<td>3.18 ± 0.122</td>
<td>10,800</td>
</tr>
<tr>
<td>Pyridoxine-P</td>
<td>5.19 ± 0.65</td>
<td>0.68 ± 0.026</td>
<td>656</td>
</tr>
<tr>
<td>Pyridoxime-P</td>
<td>54 ± 4.7</td>
<td>0.38 ± 0.030</td>
<td>56</td>
</tr>
<tr>
<td>4-Pyridoxic acid-P</td>
<td>2.55 ± 0.38</td>
<td>2.20 ± 0.032</td>
<td>4,510</td>
</tr>
<tr>
<td>4-Deoxypyridoxine-P</td>
<td>0.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Nitrophenylphosphate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatase</td>
<td>&lt;0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>&lt;0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>&lt;0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>&lt;0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-AMP</td>
<td>&lt;0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FMN</td>
<td>&lt;0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoserine</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphothreonine</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphotyrosine</td>
<td>&lt;0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoglycerate</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Activity with 40 μM compound.
The PNP phosphatase activity in the stromal fraction had an alkaline pH optimum and was probably due to a tissue-specific alkaline phosphatase (EC 3.1.3.1), which is present in the plasma membrane of virtually all tissues (33). The soluble PNP phosphatase which we have purified appears to be a unique acid phosphatase. It is not an alkaline phosphatase, since these enzymes are zinc metalloenzymes with molecular masses greater than 100 kDa (34). Tissue-specific alkaline phosphatases are inhibited potently by levamisole and are not inhibited by fluoride, and other mammalian alkaline phosphatases are inhibited by t-phenylalanine (33). The soluble PLP phosphatase apparently is not any of the ectrotycic acid phosphatases which have been characterized. Low molecular weight acid phosphatase (EC 3.1.3.2) in human erythrocytes has a molecular mass of 17-18 kDa (35), is not inhibited by fluoride or EDTA, is not activated by Mg**, and has broad substrate specificity including phosphorysorntase activity (36). Phosphoglycocyte phosphatase (EC 3.1.3.18) activity copurified with the PNP phosphatase through the Sephacryl S-200 chromatography step; however, the specific activity of the phosphoglycocyte phosphatase increased much less than that of the PNP phosphatase. The two activities separated on the reactive yellow 86-agarose column. Erythrocyte phosphoglycocyte phosphatase is specific for phosphoglycocyte, which is our substrate for the phosphatase we isolated, is a homodimer of 72 kDa (37), is activated as effectively by Co** and Mn** as by Mg**, and is also activated by chloride, bromide, and iodide (28). Other specific phosphatases in human erythrocytes have different substrate specificities than our enzyme.

Although the erythrocytes we used were washed and centrifuged at low speed several times to remove other blood cells, it is possible that there were some leukocytes present. Leukocyte phosphohosomes contains tissue-nonspecific alkaline phosphatase which hydrolyzes many phosho-compounds, including PLP, and is inhibited by levamisole (38). Leukocytes also have an acid phosphatase which hydrolyzes PLP but differs from the enzyme that we isolated in that it is not inhibited by N-ethylmaleimide (38, 39). The phosphatase we isolated is distinct from leukocyte phosphatases.

The only other phosphatase that we are aware of which may be specific for phosphorylated vitamin B_6 compounds is a phosphatase partially purified from the nuclear fraction of mouse liver (20). It has high activity with PLP, 6% as much activity with PMP, and no activity with four other phosphorylated compounds tested. However, no other properties were reported for this enzyme. Other nonspecific phosphatases have been shown to have high activity with PLP. An alkaline phosphatase partially purified from human brain cleaved PLP better than any of the nine other substrates tested (40). Rat pineal glands have PLP phosphatase activity (41) which appears to be due to a nonspecific phosphatase. Intermediate molecular weight acid phosphatases purified from bovine kidney (15) and rat liver (16) have approximately the same relative activity with PLP as with several other substrates tested. These intermediate molecular weight phosphatases differ from the phosphatase we isolated in that they are monomers with a molecular weight of 44,000 and are not inhibited by sulfhydryl reagents (15, 16).

The concentration of PLP in human erythrocytes is 50-100 nM (10, 42), which is well below the $K_{m}$ value of the PLP phosphatase for PLP. Thus, the in vivo hydrolysis of PLP would be first order with respect to PLP. Most of the PLP in the erythrocyte is bound to hemoglobin (1) and is more resistant to hydrolysis by the phosphatase than is free PLP (5). Phosphatases appear to be important in regulating PLP

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**TABLE III**

*Inhibition of PNP phosphatase activity by various compounds*

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{i}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxal-P</td>
<td>0.03</td>
</tr>
<tr>
<td>Pyridoxamine-P</td>
<td>0.5</td>
</tr>
<tr>
<td>4-Pyridoxic acid-P</td>
<td>0.02</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>11</td>
</tr>
<tr>
<td>L-P,Nitrophenylphosphate</td>
<td>4.0</td>
</tr>
<tr>
<td>o-Phosphate</td>
<td>0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>o-Aminobenzylphosphonate</td>
<td>7</td>
</tr>
<tr>
<td>Fluoride</td>
<td>2</td>
</tr>
<tr>
<td>EDTA (with 1 mM Mg&lt;sup&gt;2+&lt;/sup&gt;)</td>
<td>2</td>
</tr>
<tr>
<td>Molybdate</td>
<td>0.0029</td>
</tr>
</tbody>
</table>

<sup>a</sup> $K_{i}$ value.

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**DISCUSSION**

The results reported here describe the first purification and partial characterization of a phosphatase specific for phosphorylated vitamin B<sub>6</sub> compounds. The 51,000-fold purification of the PNP phosphatase from human erythrocytes resulted in a preparation that was nearly homogeneous based on SDS-polyacrylamide gel electrophoresis. MgCl<sub>2</sub> was an essential activator of this 64-kDa dimer. The $K_{m}$ and $V_{max}$ values of the enzyme differed for PLP, PNP, 4-PAP, and PMP. Apparently, the enzyme active site has greatest affinity for substrates with a positive charge in the 4 position. Since other phosphorylated compounds were poor inhibitors, these compounds did not bind to the phosphatase substrate-binding site when tested as substrates at 40 µM.

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<sup>1</sup>G.-J. Gao and M. L. Fonda, unpublished results.
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concentration in erythrocytes, since there is an increase in PLP concentration when inorganic phosphate is added to erythrocytes at pH 7.4 to inhibit phosphatases (43). On the basis of various metabolic studies, tissue phosphatases have been suggested to be involved in intracellular regulation of vitamin B₆ metabolism (9, 12).

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