1978

Purification, characterization and initial rate kinetics of acyl-phosphate-hexose phosphotransferase from Aerobacter aerogenes

Joseph Paul Casazza
Iowa State University

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PHOSPHOTRANSFERASE FROM AEROBACTER AEROGENES.

IOWA STATE UNIVERSITY, PH.D., 1978
Purification, characterization and initial rate kinetics of
acyl-phosphate-hexose phosphotransferase
from Aerobacter aerogenes

by

Joseph Paul Casazza

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For the Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa
1978
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ADP</td>
<td>Adenosine-5'-diphosphate</td>
</tr>
<tr>
<td>APHP</td>
<td>Acyl-phosphate-hexose phosphotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>DEAE</td>
<td>Dietylaminoethyl cellulose</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetate</td>
</tr>
<tr>
<td>Glucose 6-P</td>
<td>Glucose 6-phosphate</td>
</tr>
<tr>
<td>Glucose 6-P dehydrogenase</td>
<td>Glucose 6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Mannose 6-P</td>
<td>Mannose 6-phosphate</td>
</tr>
<tr>
<td>Ribose 5-P</td>
<td>Ribose 5-phosphate</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PIPES</td>
<td>Piperazine-N,N'-bis (2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Inorganic orthophosphate</td>
</tr>
<tr>
<td>TRIS</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
</tbody>
</table>
INTRODUCTION

Acyl-phosphate-hexose phosphotransferase (acyl-phosphate: D-hexose phosphotransferase, E.C. 2.7.1.61) was first observed by Kamel and Anderson (1964). At first, the multifunctionality of this enzyme was not realized. It was of note only because it offered an alternate energy source, acetyl phosphate, instead of ATP, for the phosphorylation of glucose. Bergmeyer and Moellering (1965) established that not only did the enzyme possess a phosphotransferase activity, but it also showed a very potent phosphohydrolase activity. This activity was observed in the presence and absence of glucose as shown below with acetyl phosphate serving as the donor and glucose as the acceptor:

Phosphotransferase Activity

\[
\text{Acetyl phosphate} + \text{glucose} \rightarrow \text{glucose 6-phosphate} + \text{acetate}
\]

Phosphohydrolase Activity

\[
\text{Acetyl phosphate} + \text{H}_2\text{O} \rightarrow \text{acetate} + \text{phosphate}
\]

The copurification of these two activities in a constant ratio under standardized conditions, the similarity of the Michaelis constants for both the phosphotransferase and phosphohydrolase reactions, and the inhibition of the phosphatase activity by glucose provide strong evidence that both activities reside on the same enzyme (Kamel and Anderson, 1967).

Soon after the discovery of this enzyme it was reported that acyl-phosphate-hexose phosphotransferase was responsible for a wide
variety of phosphotransferase and phosphohydrolase reactions (Kamel and Anderson, 1967; Bergmeyer and Moellering, 1965). Table 1 lists some representative substrates for both the phosphotransferase and the phosphohydrolase reactions. It can be seen that when sugar phosphates are donors and sugars are acceptors, a novel situation in which the product is a substrate and the substrate is a product can occur (consider the glucose 6-phosphate, mannose substrate pair). Kamel and Anderson suggested that this hexose-phosphate-hexose phosphotransferase reaction might be the physiologically important reaction (Kamel and Anderson, 1966a; 1966b). They suggested that glucose 6-phosphate might supply the energy to phosphorylate other sugars for which no specific kinase might exist (i.e. mannose). No strong evidence for this point of view has been presented. No other reports concerning the physiological role of this enzyme are in the literature.

The dual functionality of APHP is not without precedent. At present, there are at least four other enzymes which are known to display both a transferase and a hydrolase activity. They are glucose 6-phosphatase (Arion and Nordlie, 1964), transglutaminase (Gross and Folk, 1973) and γ-glutamyltranspeptidase (Schuber et al., 1976). All of these enzymes are believed to have an obligatory covalent intermediate. The experimental evidence for these covalent intermediates vary from enzyme to enzyme. It has been proposed that catalysis involves an obligatory covalent intermediate on the basis of kinetic data and an observed nicotinamide, NAD exchange reaction.
Table 1. Representative Substrates of acyl-phosphate-hexose phosphotransferase

<table>
<thead>
<tr>
<th>Donors</th>
<th>Acceptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoyl phosphate$^a$</td>
<td>Glucose</td>
</tr>
<tr>
<td>Nictoyl phosphate</td>
<td>Mannose</td>
</tr>
<tr>
<td>Acetyl phosphate</td>
<td>Fructose</td>
</tr>
<tr>
<td>Propionyl phosphate</td>
<td></td>
</tr>
<tr>
<td>N-Benzoyl-glycol-phosphate</td>
<td></td>
</tr>
<tr>
<td>Carbamoyl phosphate</td>
<td></td>
</tr>
<tr>
<td>Glutaryl phosphate</td>
<td></td>
</tr>
<tr>
<td>Succinyl phosphate</td>
<td></td>
</tr>
<tr>
<td>1-3-diphosphoglycerate</td>
<td></td>
</tr>
<tr>
<td>p-Nitrophenol phosphate</td>
<td></td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td></td>
</tr>
<tr>
<td>Mannose 6-phosphate</td>
<td></td>
</tr>
<tr>
<td>Fructose 1,6-diphosphate</td>
<td></td>
</tr>
<tr>
<td>Ribose 5-phosphate</td>
<td></td>
</tr>
<tr>
<td>Glycerol 1-phosphate</td>
<td></td>
</tr>
<tr>
<td>Flurophosphate</td>
<td></td>
</tr>
<tr>
<td>Phosphoramidate</td>
<td></td>
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</table>

$^a$From data of Kamel and Anderson (1967) and Bergmeyer and Moeller (1965).
for nicotinamide adenine dinucleotide glycohydrolase (Schuber, et al., 1976). There is both kinetic and chemical evidence for a Ping Pong mechanism for glucose 6-phosphatase (Nordlie, 1971) and transglutaminase (Gross and Folk, 1973). A stable thiotrimethylacetyl enzyme intermediate has been isolated from transglutaminase (Folk et al., 1967). Feldman and Butler have reported isolating a phosphohistidine intermediate from glucose 6-phosphatase (Feldman and Butler, 1969; 1972). The experimental evidence for proposing an obligatory covalent intermediate for γ-glutamyl transpeptidase is solely from kinetic data (Karkowsky et al., 1976).

Acyl-phosphate-hexose phosphotransferase and glucose 6-phosphatase show a striking similarity. Besides catalyzing a phosphotransferase and a phosphohydrolase activity, both enzymes share a number of common substrates (Nordlie, 1971). Sugar phosphates, acyl phosphates, phosphoramidates and carbamoyl phosphate are some of the common donors. Fructose, glucose and mannose are also common acceptors. Glucose 6-phosphatase also catalyzes a hexose phosphate-hexose phosphotransferase reaction (Hass and Byrne, 1960; Segal, 1959). Notably, nucleoside polyphosphates and pyrophosphate are substrates for glucose 6-phosphatase (Nordlie, 1971), but not for acyl-phosphate-hexose phosphotransferase (Bergmeyer and Moellering, 1965; Kamel and Anderson, 1967).

Glucose 6-phosphatase plays an important part in glucose metabolism. Although the physiological significance of the hydrolase activity is thought to be well-understood (Cahill et al., 1959; Ashmore and Weber, 1959), the significance of the phosphotransferase reaction is
a source of controversy (Nordlie, 1971; Reeves, 1976). Part of this confusion is undoubtedly due to the fact that glucose 6-phosphatase is tightly associated with the endoplasmic reticulum. Liver and kidney glucose 6-phosphatase have not been solubilized successfully (Nordlie, 1971). Consequently there are some experimental problems in working with glucose 6-phosphatase. Glucose 6-phosphatase preparations are very sensitive to a variety of detergents used for purification. Some detergents activate at low concentration and inhibit at high concentrations, while others only inhibit (Beaufay and de Duve 1954a; Carruthers and Baumler, 1962; Snoke and Nordlie, 1967). Detergents can effect the $V_{\text{max}}$, $K_m$ and even shift the pH optima to either a higher or lower pH level (Nordlie, 1971). In addition, naturally occurring metabolites have profound effects on catalysis. Certain long chain fatty acids exert a discriminating action on phosphohydrolase and phosphotransferase activities at different pH's. (Nordlie et al., 1967; Nordlie, 1968; 1969). Glucose 6-phosphatase preparations are even effected by the level of phospholipids in the membrane (Beaufry and de Duve, 1954b).

The sensitivity of glucose 6-phosphatase to various effectors and the impurity of glucose 6-phosphatase preparations make experimental interpretation difficult. Not only does this limit the interpretation of kinetic data, it also effects the interpretation of the chemical findings. Because of glucose 6-phosphatase's tight association with the microsomal fraction, Feldman and Butler's proposal of a phosphoryl intermediate is open to some doubt (Feldman and Butler, 1969; 1972).
Since other enzymes are present in the microsomal fraction, Feldman and Butler's work, although suggestive of a covalent intermediate, cannot be considered conclusive.

Because of the similarity in both activity and specificity of glucose 6-phosphatase and acyl-phosphate-hexose phosphotransferase, we studied the kinetics of the bacterial enzyme as a model for the particulate enzyme. We also investigated the mechanism of control of acyl-phosphate-hexose phosphotransferase. Because of its wide range of substrates, its ability to use products and substrates interchangeably, and its phosphotransferase and phosphohydrolase activities, acyl-phosphate-hexose phosphotransferase has the capabilities of being a one enzyme futile cycle. Uncontrolled, this enzyme would drain the bacterium of its energy sources. It seems improbable that this enzyme would not be under rigorous metabolic control.
EXPERIMENTAL PROCEDURE

Materials

ATP, acetyl phosphate, NADP, NAD, NADH, mannose 6-P, ribose 5-P, PIPES, phosphoglucoisomerase and acetate kinase were obtained from Sigma. Glucose was obtained from Pfanstiehl and recrystallized from hot ethanol. Glycylglycine, N-acetyl-glucosamine, hexokinase, pyruvate kinase, egg albumin, cytochrome c, liver alcohol dehydrogenase and lactate dehydrogenase were purchased from Calbiochem. Glucose 6-P dehydrogenase and phosphomannoisomerase were purchased from Boehringer-Mannheim. All reagents for gel electrophoresis, AG-1X-2 anion exchanger and Cellex D were supplied by Bio-Rad Laboratories. DE 52 was obtained from H. Reeve Angel Inc. Sephadex G-200 and S-200 gels were purchased from Pharmacia. Gold label p-nitrophenol phosphate was from Aldrich. Low ion water was obtained by passing distilled water through a Barnstead high capacity ion-exchanger and then a Continental water deionizer. All other reagents were of the highest possible purity.

Methods

Standard assay

Enzyme activity was routinely assayed by measuring NADP reduction using a glucose 6-P dehydrogenase couple. Changes in optical density were observed using a Cary 15 or Cary 118 recording spectrophotometer thermostated at 28 °C. Standard assay conditions were 8 mM glycyl-glycine, pH 7.5, 4 mM acetyl phosphate, 2 mM glucose, 180 μM NADP, and 1.7 IU of glucose 6-P dehydrogenase. Reaction volumes of 3 mL were used.
A unit of APHP was defined as the amount of enzyme that catalyzed the phosphorylation of 1 μMole of glucose per minute at 28 °C under standard assay conditions.

**Protein determinations**

Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Specific activity is expressed as units of enzyme activity per mg of protein.

**Reagent concentrations**

Glucose concentrations were determined by enzymatic assay in a reaction mixture containing 50 mM TRIS-Cl buffer, pH 7.6, 5 mM ATP, 10 mM MgCl₂, 1 mM NADP, 1.67 IU yeast hexokinase, 1.7 IU glucose 6-P dehydrogenase and approximately 60 μM glucose. Reactions were initiated by addition of hexokinase, in a volume of 1 μL, after the spectrophotometer had been zeroed to the reaction mixture in the absence of hexokinase. The reaction was followed to conclusion at 340 nm. Glucose concentration was calculated on the basis of the amount of NADPH formed, using its molar extinction coefficient of 6.22 × 10³ M⁻¹ cm⁻¹.

Acetyl phosphate concentrations were determined by enzymatic assay in a reaction mixture containing 50 mM TRIS-Cl buffer, pH 7.6, 3.3 mM MgCl₂, 1.66 mM glucose, 0.33 mM ADP, 0.33 mM NADP, 1.7 IU glucose 6-P dehydrogenase, 1.67 IU hexokinase, 0.85 IU acetate kinase and approximately 60 μM acetyl phosphate. Reactions were initiated by addition of 1 μL of acetate kinase. The concentration of acetyl phosphate was calculated on the basis of the amount of NADPH formed as in the glucose determinations.
Glucose 6-P, fructose 6-P and mannose 6-P concentrations were determined by enzymatic assay. The assay procedure for all three sugar phosphates was identical except for the enzyme added to the reaction mixtures. Assays were 50 mM TRIS-Cl, pH 7.6, 1 mM MgCl₂, 0.33 mM NADP and approximately 60 μM in the sugar phosphate being assayed. Assay volume was 3 mL. Glucose 6-P assays contained 1.7 IU glucose 6-P dehydrogenase. Fructose 6-P assays contained 1.7 IU glucose 6-P dehydrogenase and 1.0 IU phosphoglucoisomerase. Mannose 6-P assays contained 1.7 IU glucose 6-P dehydrogenase, 1.0 IU phosphoglucoisomerase and 0.24 IU phosphomannoisomerase. Glucose 6-P assays were initiated by addition of 1 μL glucose 6-P dehydrogenase. Fructose 6-P assays were initiated by addition of 1 μL phosphoglucoisomerase. Mannose 6-P assays were initiated by addition of 2 μL of phosphomannoisomerase. Concentrations were calculated on the basis of NADP reduction as in the glucose determinations.

ATP concentrations were determined by an enzymatic assay in a reaction mixture 50 mM in TRIS-Cl buffer, pH 7.6, 5 mM glucose, 1 mM MgCl₂, 0.33 mM NADP, 1.7 IU glucose 6-P dehydrogenase, 1.6 IU hexokinase and approximately 60 μM in ATP. Assay volume was 3 mL. Reactions were initiated by addition of hexokinase in a volume of 1 μL. ATP concentrations were determined on the basis of NADP reduction as in the glucose determinations.

Orthophosphate assays

The method of Berenblum and Chain (1938) was utilized to assay phosphate with slight modification. One mL of a stopped reaction
mixture or $P_i$ standard was transferred to a test tube containing 4 mL of water-saturated 1:1 isobutanol-benzene mixture. The mixture was mixed vigorously on a vortex mixer and cooled on ice for 10 minutes. One-half mL of an ammonium molybdate solution, that was prepared by adding 5 grams of ammonium molybdate to 100 mL of 4 M $H_2SO_4$, was added to the isobutanol-benzene mixture and mixed vigorously for 1 minute. The two phases were allowed to separate after which 3 mL of the nonaqueous phase was transferred to a test tube containing 1 mL of 95% ethanol 0.36 M $H_2SO_4$. The mixture was mixed rapidly using a vortex mixer and color developed by addition of 0.2 mL of a 17.7 mM $SnCl_2$, 1 M $H_2SO_4$ solution. After 10 minutes, absorbance was read at 660 nm. All assays were done in duplicate. This assay procedure allowed the determination of $P_i$ in the presence of labile materials, such as acetyl phosphate.

**Initial velocity studies**

Initial velocity studies were carried out by one of four methods: a direct spectral assay, a coupled spectral assay, a discontinuous $P_i$ release assay using spectral detection of $P_i$, and a discontinuous assay in which mannose release was determined radioactively. All assays were made at $28^\circ C$.

**p-Nitrophenol release assays**  
$p$-Nitrophenol release assays were made using a direct spectral assay. The increase in the $p$-nitrophenolate anion was measured continuously at 410 nm as a function of time on a Cary 118 or Cary 15 recording spectrophotometer. The 0-0.1 slide wire was used. All reaction mixtures were incubated at $28^\circ C$ for 5 minutes before initiation with APHP. Rates were observed at 410 nm with the
spectrophotometer cell housing maintained at 28° C by water circulating from a temperature controlled bath through thermospacers. Change in the concentration of p-nitrophenolate anion was calculated using an extinction coefficient of 18.1 x 10^3 M^{-1} cm^{-1}.

**Coupled spectral assays** The formation of glucose 6-P was followed using the coupling enzyme glucose 6-P dehydrogenase. The NADPH production of this enzyme was measured continuously as a function of time on a Cary 118 or Cary 15 recording spectrophotometer on the 0-0.1 slide wire. Addition of 1.7 IU of coupling enzyme in the presence of 180 μM NADP was sufficient to assure the initial velocity was linear with respect to the amount of enzyme added. All reaction mixtures were incubated at 28° C for 5 minutes before initiation with APHP. Rates were observed at 340 nm with the spectrophotometer cell housing maintained at 28° C by water circulating from a temperature controlled bath through thermospacers. Changes in absorbance over a given time were converted to velocity by using an extinction coefficient for NADPH of 6.22 x 10^3 M^{-1} cm^{-1}. Michealis constant determinations for phosphotransferase experiments were made under conditions identical to the **standard assay**. All other spectral assays were made in 20 mM glycylglycine with the exception of P_i inhibition studies. P_i inhibition experiments were carried out in 40 mM glycylglycine with KCl added to maintain constant ionic strength. Care was always taken to insure that the phosphohydrolase and phosphotransferase activities never used more than 10% of total phosphoryl donor present. p-Nitrophenyl phosphate transferase assays were made at 342 nm, the isobestic point for the
conversion of $p$-nitrophenol phosphate to $p$-nitrophenol.

Phosphatase assays Phosphatase activity was measured over a 10 minute period using a modification of the Berenblum and Chain procedure (1938), as previously described, in orthophosphate assays. Assay volumes were 1 mL. Reactions were buffered at pH 7.5 with 8 mM glycyl-glycine. Assay mixtures were stopped by the addition of 0.2 mL of 21% HClO$_4$ and immediately placed on ice. Reaction blanks were made at each concentration of phosphoryl donor. All assays were carried out in duplicate.

Mannose release assays Enzymatic release of mannose from mannose 6-P was assayed by the method of Fromm et al. (1964). After 10 minute incubations, the reactions were terminated by adding 1 mL of a 1.1 mL reaction volume to a 1 x 1.5 cm AG-1X-2 column. Columns were washed with 6 mL of water. The pass-through and wash volumes were collected. A 3 mL aliquot was counted in 10 mL of Bray's solution (Bray, 1960).

Molecular exclusion chromatography

The molecular weight of APHP was determined by ascending gel chromatography on a G-200 Sephadex column with various proteins of known molecular weight as described by Andrews (1964). The column (2.5 x 100 cm) was equilibrated with 100 mM KCl and 20 mM PIPES, pH 6.5, at 5°C, prior to sample application. A uniform flow rate of 12 mL per hour was maintained with 3 mL fractions collected. Blue dextran was read at 620 nm, egg albumin at 280 nm, and cytochrome c at 415 nm. APHP was assayed using the standard assay; lactate dehydrogenase activity was
measured by following the oxidation of NADH in the presence of pyruvate; alcohol dehydrogenase activity was measured by following the reduction of NAD in the presence of ethanol; pyruvate kinase activity was assayed by following pyruvate formation with a lactate dehydrogenase couple, in the presence of phosphoenol pyruvate, ADP, and NADH.

**Gel electrophoresis**

Native gels were electrophoresed at $3^\circ$ C using the procedure of Davis (1964). Sodium dodecyl sulfate gel electrophoresis was done at room temperature using the method of Laemmli (1970). Sodium dodecyl sulfate gels were 10% in acrylamide. Gels were stained for protein overnight in a 0.05% Coomassie brilliant blue R-250, 25% isopropyl alcohol, 10% acetic acid solution. Gels were electrophoretically destained in a 10% acetic acid solution. Activity staining was done in 2 mM acetyl phosphate, 8 mM glycylglycine, pH 7.5 and 200 mM CaCl$_2$. After a 30 minute incubation at $27^\circ$ C, a band of white calcium phosphate could be seen, indicating the presence of phosphatase activity.

**Determination of molecular weight by short column equilibrium sedimentation**

Equilibrium sedimentation experiments were carried out using the short column sedimentation equilibrium method described by Van Holde and Baldwin (1958). This method has the advantage that equilibrium conditions are reached quickly. Molecular weight determinations were made in a Spinco Model E analytical ultracentrifuge at $3^\circ$ C. Protein concentrations were determined using the photoelectric scanning system of
the ultracentrifuge. All optical density readings were corrected using an internal standard.

**Statistical analysis**

Statistical analysis was done according to the method of Siano et al. (1975). Data was fit to a particular model using the method of weighted least squares. Weighting was assigned using the formula

$$W(1/V_i) = \frac{1}{\sum V_i^{4-a}}$$

where $N$ is the number of points, $V$ is the velocity and $a$ is an experimentally determined constant. $a$ was determined for each different kinetic assay. This weighting procedure has received support from investigators in other laboratories (Storer et al., 1975; Askelof et al., 1976). The variance was assumed to follow the equation

$$\sigma_i^2 = CV_i^\alpha$$

A log plot of $\sigma_i^2$, standard deviation, vs. log velocity allows evaluation of $C$ and $\alpha$. The $\alpha$'s were 0.67, 0.41, and 0.95 for the glucose 6-P dehydrogenase couple, phosphatase assays and the $p$-nitrophenol release assays, respectively.

All velocity measurements represent initial reaction velocity. This portion of the product-time progress curve was determined either from continuous spectrophotometric tracings or by a series of stop-time


determinations when the former procedure could not be used, i.e., for the isotope experiments. Velocity is reported in \( \text{M}^{-1} \text{ min}^{-1} \).
RESULTS AND DISCUSSION

Experimental Data

Purification of APHP

APHP was first purified by Kamel and Anderson (1967). Although the enzyme was purified to the point where it was free of contaminating activities, the enzyme was not homogeneous. Bergmeyer and Moellering (1965) reported crystallizing the enzyme. Unfortunately they gave no details of their purification scheme. The first four steps of the purification reported here are almost identical to Kamel and Anderson's purification procedure. All operations were performed at 3⁰ C unless otherwise indicated.

Bacterial growth A. aerogenes were grown on a minimal media as described by Lieberman (1956). Bacterial growth from lyophilized cultures and slants were started on media 0.5% in ribotol to insure culture purity. After growth was well-established, cultures were transferred to media 0.5% in glucose and 0.1% in yeast extract. Final bacterial growth occurred in 2 liter flasks containing 400 mL of media. These flasks were inoculated with 40 mL of freshly grown culture. The bacteria were allowed to grow overnight with vigorous shaking. All growth occurred at 37⁰ C. Once growth was established from slants, cultures were always started by addition of a 5-10% volume of inoculum.

Bacteria were harvested in the morning. Cells were spun down at 5,000 x g for 30 minutes. The bacteria were resuspended in 10 mM P₁, at 3⁰ C, pH 7.0 and centrifuged at 5,000 x g again.
**Cell lysis**  
Cells were suspended in approximately half their volume of 10 mM \( P_1 \), pH 7.0, and worked into an even paste. This paste was transferred to a French pressure cell which had been precooled to 3° C. The bacteria were passed through the cell twice. Pressure in the cell was maintained at 20,000 psi during cell lysis. The ruptured cells were diluted to approximately four times their wet weight with 20 mM \( P_1 \), pH 7.0, and then centrifuged at 16,000 \( \times \) g for 15 minutes. The cellular debris was discarded and the supernatant cell free extract treated immediately.

**Protamine sulfate treatment**  
The cell free extract was made 0.1 M in ammonium sulfate. This was followed with slow addition of 0.2 volumes of 2% protamine sulfate. The mixture was stirred for 10 minutes. The resultant milky white precipitate was removed by centrifugation and discarded.

**First ammonium sulfate fractionation**  
Finely ground solid ammonium sulfate was slowly added to the supernatant fluid from the protamine sulfate step until the solution was 50% of saturation and allowed to stir for 30 minutes. The pH of the solution was carefully monitored during addition of ammonium sulfate to insure that the solution remained approximately at pH 7.0. After centrifugation at 16,000 \( \times \) g, the precipitate was discarded. The supernatant fluid was then brought to 90% of saturation with finely ground ammonium sulfate and again allowed to stir for 30 minutes. The precipitate was collected by centrifugation. The supernatant solution was discarded and the precipitate was dissolved in 20 mM PIPES, pH 6.7.
Heat treatment  The solution was immersed in a 50° C water bath with continual stirring and quickly brought to temperature. After 5 minutes, the solution was cooled to 3° C. Denatured protein was removed by a 20 minute centrifugation at 16,000 x g. The supernatant was dialyzed overnight against 2 liters of 20 mM PIPES, pH 6.7.

High capacity DEAE chromatography  High capacity DEAE (Cellex D) was prepared by washing with 0.5 N HCl followed by 0.5 N NaOH. The Cellex D was then washed to neutrality, with copious amounts of water, on a Buchler funnel. The DEAE was then equilibrated with 100 mM PIPES, pH 6.7. After 1 hour, the mixture of Cellex D and buffer was adjusted to pH 6.7. The resin was allowed to settle and the excess buffer and fine particles poured off. The resin was then equilibrated with 20 mM PIPES, pH 6.7. After 1 hour, the mixture of Cellex D and buffer was again brought to pH 6.7. The DEAE was allowed to settle once more and the buffer and fine particles were again poured off. The remaining slurry was brought to 2.5 times the volume of the resin with 20 mM PIPES and a 2.5 x 30 cm column prepared. The column was washed with several volumes of 20 mM PIPES pH 6.7 until equilibration occurred. Before any protein was applied to any column, the pH and ionic strength of the eluate were checked to insure that it was the same as the wash buffer.

Dialyzed, heat treated, protein was applied to the column. The APHP activity was washed off with 20 mM PIPES, pH 6.7. Fractions were collected in a refrigerated fraction collector using a drop counter to measure volume. Fractions of 10 mL were collected and those fractions with activity were pooled. APHP activity was precipitated by bringing
the pooled fractions to 90% saturation with ammonium sulfate. After 1 hour, the precipitate was collected by a 20 minute centrifugation at 16,000 x g. The supernatant solution was discarded and the precipitate dissolved in a minimal volume of 20 mM glycylglycine and dialyzed against 2 liters of glycylglycine, pH 7.5, overnight. Denatured protein was removed by centrifugation before addition to a S-200 column.

**S-200 gel chromatography** A 2.5 x 70 cm S-200 column was prepared and equilibrated with several volumes of 20 mM glycylglycine, pH 7.5. The concentrated protein from the Cellex D step was applied to the column. Fractions of 4 mL were collected in a refrigerated fraction collector. Fractions with activity were pooled and concentrated on an Amicon concentrator. Either a PM-10 or UM-10 membrane was used.

**DE 52 chromatography** DE 52 was prepared in the same manner as Cellex D. A 2.5 x 25 cm DE 52 column was prepared and equilibrated with 20 mM glycylglycine, pH 7.5. The concentrated protein solution from the S-200 column was applied to the column. After washing the protein onto the column with 40 mL of buffer, the protein was eluted with a 1 liter 0-100 mM KCl gradient. A refrigerated fraction collector was used to take 10 mL fractions. Fractions with activity were pooled.

**Hydroxylapatite chromatography** Bio-Rad HTP hydroxylapatite was prepared by swelling the dry HTP powder in a 1 part hydroxylapatite to 6 parts buffer, 10 mM P₄, pH 7.5. The slurry was mixed with gentle swirling and then allowed to settle for 10 minutes. Excess buffer and fine particles were poured off. Enough buffer was added to make a slurry approximately 3 parts buffer to 1 part hydroxylapatite. A 2 x
10 cm column was prepared and equilibrated with several volumes of buffer.

The pooled fractions from the DE 52 chromatography were added directly to the hydroxylapatite column. The column was washed with 10 mM P$_4$, pH 6.7, until the 280 nm absorbance was less than 0.02. APHP was washed from the column with a 300 mL, 0-100 mM P$_4$ gradient, pH 6.7. Pure APHP was eluted in a single peak. The specific activity was constant across the entire peak. APHP activity was pooled and concentrated on an Amicon concentrator as before.

Concentrated enzyme was dialyzed against 1 liter of 20 mM PIPES pH 6.5. APHP could be frozen without loss of activity. APHP prepared in this manner gave a single band on native gels stained with Coomassie brilliant blue R-250. This band coincided with the phosphatase activity seen on native gels which had been run in parallel with those stained with Coomassie blue. Sodium dodecyl sulfate gel electrophoresis also gave a single band. Under standardized conditions the ratio of transferase to phosphatase activity remained constant from preparation to preparation.

**Molecular weight determinations**

The molecular weight of APHP was determined by two methods, gel chromatography and short column equilibrium sedimentations. Both methods gave molecular weights that were in good agreement.

**Gel chromatography**

A 2.5 x 100 cm G-200 column was calibrated with samples of known molecular weight as described in experimental methods. Standards and APHP were added together in a 2 mL solution.
containing 5% sucrose. Fractions were collected in a refrigerated fraction collector.

APH eluted between lactate dehydrogenase and pyruvate kinase as shown in figure 1. The molecular weight determined by this method was approximately 150,000.

Short column equilibrium sedimentation  Short column equilibrium centrifugation as described by Van Holde and Baldwin (1958), at 3° C, gave a molecular weight of 153,000. This value is in very good agreement with the molecular weight determined by gel chromatography.

Sodium dodecyl sulfate gel electrophoresis

Three proteins of known molecular weight were used to standardize sodium dodecyl sulfate gels, cytochrome c, 12,500 molecular weight; α-chymotrypsinogen, 25,000 molecular weight; and egg albumin, 45,000 molecular weight. Egg albumin monomers associate to some extent under the denaturing conditions which were employed. As a result, egg albumin migrates as both a monomer of 45,000 molecular weight and as a dimer of 90,000 molecular weight. α-Chymotrypsinogen was denatured separately from APHP samples and the other standards. Gels were electrophoresed with standards only, APHP alone, and with both APHP and standards. Standard and APHP bands were identified from the first two sets of gels. Molecular weight was assigned using the gels which were loaded with both the standards and APHP. The results of a sample electrophoretogram are shown in figure 2. A monomer weight of 24,000 was indicated by these experiments. On the basis of sodium dodecyl sulfate gel electrophoresis,
Figure 1. Plot of tube number (fraction collector) versus molecular weight for 6 different marker proteins and APHP. The proteins are (○) myoglobin, molecular weight 16,500 (□) egg albumin, molecular weight 45,000; (△) malate dehydrogenase, molecular weight 70,000; (△) horse liver alcohol dehydrogenase, molecular weight 80,000; (□) rabbit muscle lactate dehydrogenase, molecular weight 142,000; (⊗) rabbit muscle pyruvate kinase, molecular weight 212,000; APHP (X).
Figure 2. Plot of the Molecular weight of 4 different sodium dodecyl sulfate gel electrophoresis protein standards and APHP versus relative migration with respect to a bromphenol blue marker dye. Proteins are (Δ) cytochrome c, molecular weight 12,500; (□) α-chymotrypsinogen, molecular weight 25,000; (○) egg albumin monomer, molecular weight 45,000; (▽) egg albumin dimer, molecular weight 90,000; (X) APHP.
G-200 column chromatography, and equilibrium sedimentation determinations, it appears that APHP is a hexamer.

**Metal ion studies**

Notably absent from the APHP purification scheme outlined here, and from the standard assay is the presence of any metal ions. This is also the case with studies done by Bergmeyer and Moellering (1965) and Kamel and Anderson (1967). Our studies have shown that magnesium has no effect on the phosphatase or phosphotransferase activities. We have also checked a wide variety of metals and found that they have no effect on the phosphatase activity of the enzyme.

In an attempt to remove any metal ions bound to the enzyme, 0.5 mL of APHP was dialyzed against 100 mL of 50 mM TRIS, pH 6.5, 100 mL of 50 mM glycylglycine, pH 8.5 and 100 mL of 20 mM P$_4$, pH 6.7, at 3° C, for 24 hours. All dialysis buffers were 5 mM in 8-hydroxy-5-quinolinesulfonic acid. Some 8-hydroxy-5-quinolinesulfonic acid precipitated out of solution, at 3° C, in the buffers at the lower pH's. In this case solutions were saturated with respect to 8-hydroxy-5-quinolinesulfonic acid. Dialysis solutions without 8-hydroxy-5-quinolinesulfonic acid, were used as controls for each buffer. After 24 hours no difference in activity could be observed between the APHP dialyzed against buffers with, and without, 8-hydroxy-5-quinolinesulfonic acid.

The data suggest that if a metal ion is associated with APHP catalytic activity, it is very tightly bound to the enzyme. Preliminary atomic absorption studies were done in an attempt to learn if a metal ion was bound to the enzyme and if so, what it was.
Approximately 0.6 mL of enzyme was dialyzed against 8 liters of doubly distilled deionized water in a rocking dialyzer. The enzyme solution was then removed and dialyzed against 500 mL of water in a beaker that had previously been once boiled in 1 mM EDTA and twice boiled with doubly distilled deionized water. Approximately 35% of the enzyme activity was lost as a result of this treatment. Atomic absorption studies were then carried out at the Ames Laboratory. The results are shown in table 2. These data indicate that there is no metal ion associated with APHP. These results are only preliminary studies and are not meant to be conclusive.

**Kₘ determinations**

A number of graphical procedures have been employed for the evaluation of kinetic parameters for two substrate systems (Frieden, 1957; Dalziel, 1957; Florini and Vestling, 1957). These procedures usually allow the investigator to segregate kinetic mechanism, by inspection, into two classes, either Ping Pong or sequential. A Ping Pong mechanism is one in which the first product is released by the enzyme before the second substrate is bound. As a direct result of this, the enzyme must be covalently modified during catalysis. This is compared to a sequential mechanism in which all substrates must be bound to the enzyme before catalysis can occur.

A double reciprocal plot for a Ping Pong mechanism can be described as follows:
Table 2. Metal ion content of APHP<sup>a</sup>

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Detection limit</th>
<th>Amount Found&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Amount Expected&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beryllium</td>
<td>.0001</td>
<td>-</td>
<td>.007</td>
</tr>
<tr>
<td>Calcium</td>
<td>.012</td>
<td>-</td>
<td>.034</td>
</tr>
<tr>
<td>Cadmium</td>
<td>.004</td>
<td>-</td>
<td>.078</td>
</tr>
<tr>
<td>Cobalt</td>
<td>.004</td>
<td>-</td>
<td>.051</td>
</tr>
<tr>
<td>Copper</td>
<td>.04</td>
<td>-</td>
<td>.054</td>
</tr>
<tr>
<td>Iron</td>
<td>.02</td>
<td>-</td>
<td>.047</td>
</tr>
<tr>
<td>Magnesium</td>
<td>.002</td>
<td>-</td>
<td>.02</td>
</tr>
<tr>
<td>Manganese</td>
<td>.0001</td>
<td>-</td>
<td>.047</td>
</tr>
<tr>
<td>Molybdate</td>
<td>.04</td>
<td>-</td>
<td>.082</td>
</tr>
<tr>
<td>Selenium</td>
<td>.03</td>
<td>-</td>
<td>.067</td>
</tr>
<tr>
<td>Vanadium</td>
<td>.0007</td>
<td>.003</td>
<td>.018</td>
</tr>
<tr>
<td>Zinc</td>
<td>.005</td>
<td>-</td>
<td>.05</td>
</tr>
</tbody>
</table>

<sup>a</sup> All values are given as micrograms/mL.

<sup>b</sup> As determined by atomic absorption spectroscopy

<sup>c</sup> Values given are based on protein present as determined by the method of Lowry et al. (1951) assuming identical subunits per molecule of APHP.
Since there is no (A)(B) term, when one substrate is varied and the other held constant, at different fixed levels, a family of parallel lines is generated. Often a choice can be made between a Ping Pong mechanism and a sequential mechanism, which does have an (A)(B) term, on the basis of kinetic experiments.

If an enzyme with an obligatory covalent intermediate catalyzes both a transferase and a hydrolase reaction, when the reciprocal of velocity is plotted as a function of the last product of the enzyme, the hydrolase activity introduces an (A)(B) term into the rate equation (Arion and Nordlie, 1964). The resulting kinetic plot has lines that converge, and the plots appear of the sequential type.

Assuming a modified Ping Pong mechanism, one would expect, for APHP, that the dephosphorylated donor would be the first off the enzyme and that $P_i$, or a phosphorylated acceptor, would be the second product. If APHP catalysis does involve an obligatory covalent intermediate following the release of glucose 6-P would lead to the situation described in the preceding paragraph. This postulated Ping Pong mechanism is the one suggested for glucose 6-phosphatase by Arion and Nordlie (1964). As reported for glucose 6-phosphatase by Arion and Nordlie (1964), the initial rate pattern observed for the $K_m$ determination—

\[
\frac{E_0}{c} = \phi_0 + \frac{\phi_A}{(A)} + \frac{\phi_B}{(B)} + \frac{\phi_{AB}}{(A)(B)}
\] (1)

\[1\text{Nomenclature is that of Dalziel (1957).} \]
tions of APHP, are sequential, as shown in figures 3 and 5. In this case, it would appear that nothing could be learned about the kinetic mechanism from the $K_m$ determinations, since both a modified Ping Pong mechanism and any modified sequential mechanism will give qualitatively identical plots. This is not the case.

The modified Ping Pong mechanism as proposed by Arion and Nordlie (1964) is shown below.

Scheme I

$$\begin{array}{cccccc}
A & P & B & Q \\ 
k_1 & k_2 & k_3 & k_4 & k_5 & k_6 & k_7 & k_8 \\ 
E & EA & F & FB & E \\ 
k_9 \\ 
E + R
\end{array}$$

$A, B, P, Q, R, E$ and $F$ are phosphoryl donor, phosphoryl acceptor, dephosphorylated donor, phosphorylated acceptor, $P_i$, enzyme and phosphoryl enzyme, respectively. The equation describing the release of $Q$ for the phosphotransferase reaction is:

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_A}{(A)} + \frac{\phi_B}{(B)} + \frac{\phi_{AB}}{(A)(B)} \quad (2)$$

In the above equation $\phi_0 = (1/k_3 + 1/k_9)$, $\phi_A = (k_2 + k_3)/k_1 k_3$, $\phi_B = (k_6 + k_7)(k_3 + k_9)/k_3 k_5 k_7$ and $\phi_{AB} = k_9 (k_2 + k_3)(k_6 + k_7)/k_1 k_3 k_5 k_7$. The equation describing the phosphatase activity in the absence of acceptor is

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_A}{(A)} \quad (3)$$
Figure 3A. Plot of the reciprocal of velocity \((v)\) versus the reciprocal molar concentration of glucose at 222 \(\mu\text{M} (\Theta)\), 285 \(\mu\text{M} (\nabla)\), 400 \(\mu\text{M} (\Box)\), 660 \(\mu\text{M} (\bigcirc)\) and 2 \(\text{mM} (\triangle)\) mannose 6-phosphate. Glucose was varied in the range 111 \(\mu\text{M}\) to 1 \(\text{mM}\). Velocity was determined with the glucose 6-phosphate dehydrogenase assay.

3B. Plot of reciprocal of velocity \((v)\) versus the reciprocal of molar concentration of mannose 6-phosphate at 111 \(\mu\text{M} (\Theta)\), 142 \(\mu\text{M} (\nabla)\), 200 \(\mu\text{M} (\Box)\), 333 \(\mu\text{M} (\bigcirc)\) and 1 \(\text{mM} (\triangle)\) glucose. Mannose 6-phosphate was varied in the range of 222 \(\mu\text{M}\) to 2 \(\text{mM}\). Velocity was determined by the glucose 6-phosphate dehydrogenase assay.
A.  

\[ \frac{1}{V} \times 10^{-5} \]

\[ \frac{1}{(\text{Glucose}) \times 10^{-3}} \]

B.  

\[ \frac{1}{V} \times 10^{-5} \]

\[ \frac{1}{(\text{Mannose 6-phosphate}) \times 10^{-3}} \]
Figure 4. Plot of the reciprocal of velocity (v) (P$_i$ release) versus the reciprocal of molar concentration of mannose 6-phosphate. Mannose 6-phosphate was varied in the range 222 µM to 2 mM.
Figure 5A. Plot of the reciprocal of initial velocity (v) versus the reciprocal of molar concentration of glucose at 1.1 mM (●), 1.42 mM (▼), 2.00 mM (□), 3.33 mM (○) and 10.0 mM (△) ribose 5-phosphate. Glucose was varied in the range 111 μM to 1 mM. Velocity was determined with the glucose 6-phosphate dehydrogenase assay.

5B. Plot of the reciprocal of initial velocity (v) versus the reciprocal of molar concentration of ribose 5-phosphate at 111 μM (●), 142 μM (▼), 200 μM (□), 333 μM (○) and 1 mM (△) glucose. Ribose 5-phosphate was varied in the range 1.1 mM to 10 mM glucose. Velocity was determined with the glucose 6-phosphate dehydrogenase assay.
Figure 6. Plot of the reciprocal of velocity (v) (P_i release) versus the reciprocal of molar concentration of ribose 5-phosphate. Ribose 5-phosphate was varied in the range 166 μM to 1.5 mM.
where \( \phi_0 = (1/k_3 + 1/k_9) \) and \( \phi_A = (k_2 + k_3)/k_1k_3 \).

If an enzyme is of the modified Ping Pong type, all of the rate constants before the addition of the phosphoryl acceptor should be independent of what acceptor is used (i.e. water or any B). Therefore, if APHP does have an obligatory covalent intermediate, rate constants determined using data from the phosphatase \( K_m \) determinations should be comparable to rate constants derived from the phosphotransferase \( K_m \) determinations. More specifically, the following relationships should hold.

\[
\phi_A(\text{transferase}) = \phi_A(\text{phosphatase}) \quad (4)
\]

\[
\phi_0(\text{phosphatase}) = \frac{\phi_A\phi_B}{\phi_{AB}(\text{transferase})} \quad (5)
\]

Kinetic data from \( K_m \) experiments for both the phosphatase and phosphotransferase activities, using several different phosphoryl donors, are shown in table 3. It can be seen that for the three phosphoryl donors used, the relationship expressed in equation 4 does seem to hold reasonably well, whereas the relationship expressed in equation 5 does not. The failure of the \( \phi_A \) identity to hold true is at variance with a modified Ping Pong mechanism.

It should be noted that the plots shown in figures 3 and 5 show no sign of substrate inhibition. Transferase experiments with glucose levels as high as 5 mM show no signs of substrate inhibition. Experiments following the phosphatase activity as a function of glucose also showed no signs of substrate inhibition at 5 mM glucose.
Table 3. Kinetic parameters for acyl-phosphate-hexose phosphotransferase$^a$

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\phi_A/E_0$ (min)</th>
<th>$\phi_B/E_0$ (min)</th>
<th>$\phi_{AB}/E_0$ (M min)</th>
<th>$K_a$ (mM)$^b$</th>
<th>$K_{iglu}$ (mM)$^c$</th>
<th>$K_{glu}$ (mM)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl phosphate</td>
<td>1.74</td>
<td>1.61</td>
<td>$3.09 \times 10^{-4}$</td>
<td>0.11</td>
<td>0.18</td>
<td>0.10</td>
</tr>
<tr>
<td>Mannose 6-P</td>
<td>14.7</td>
<td>35.5</td>
<td>$4.82 \times 10^{-3}$</td>
<td>0.08</td>
<td>0.33</td>
<td>0.18</td>
</tr>
<tr>
<td>Ribose 5-P</td>
<td>157</td>
<td>20.4</td>
<td>$4.13 \times 10^{-2}$</td>
<td>1.0</td>
<td>0.26</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Phosphatase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\phi_0/E_0$ (M$^{-1}$ min)</th>
<th>$\phi_A/E_0$ (min)</th>
<th>$\phi_{AB}(M^{-1}$ min) $\phi_{AB}E_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl phosphate</td>
<td>$3.57 \times 10^4$</td>
<td>9.38</td>
<td>$9.07 \times 10^3$</td>
</tr>
<tr>
<td>Mannose 6-P</td>
<td>$5.84 \times 10^4$</td>
<td>7.92</td>
<td>$10.8 \times 10^4$</td>
</tr>
<tr>
<td>Ribose 5-P</td>
<td>$2.60 \times 10^4$</td>
<td>19.6</td>
<td>$7.76 \times 10^4$</td>
</tr>
</tbody>
</table>

$^a$The phosphotransferase and phosphatase experiments for each donor were adjusted to a constant $E_0$.

$^b$The $\phi$ values are related to the Michaelis constants as follows: $[\phi_A/E_0]=K_a$, $[\phi_B/E_0]=K_b$, $[E_0/\phi_0]=V_{max}$.

$^c$K$_{iglu}$ and K$_{glu}$ are taken to represent the dissociation and Michaelis constants for glucose.
Effects of Sugar on the dephosphorylation of the phosphoryl donor

Gross and Folk (1973) have shown, that for an enzyme which exhibits a modified Ping Pong mechanism, plots of 1/velocity vs. 1/donor, at different fixed levels of acceptor, yield a family of parallel line when velocity is defined as donor dephosphorylation per unit time. The rate equation for the release of the dephosphorylated donor for a modified Ping Pong mechanism is shown below.

\[
\frac{E_0}{v} = \frac{1}{k_3} + \frac{(k_2 + k_3)}{k_1 k_3 (A)} + \frac{(k_6 + k_7 k_5 (B))}{(k_6 k_9 + k_7 k_5(k_7 (B))}
\]

Figure 7 illustrates an experiment in which p-nitrophenylphosphate hydrolysis was measured at different fixed concentrations of glucose. The data presented here is not consistent with the modified Ping Pong mechanism.

Bergmeyer and Moellering (1965) reported that p-nitrophenyl phosphate is a substrate for the phosphohydrolase reaction but not for the phosphotransferase reaction. If this were true, p-nitrophenyl phosphate would be the only substrate known which is a substrate for only one of the two reaction catalyzed by APHP. Our data suggests that p-nitrophenyl phosphate is a substrate for both of the reactions catalyzed by APHP. Glucose 6-P formation was followed using the coupling enzyme glucose 6-phosphate dehydrogenase. The reduction of NADP was followed at 342 nm, the isobestic point for the conversion of p-nitrophenyl phosphate to p-nitrophenol and \( P_i \) at pH 7.5. The enzyme exhibited
Figure 7. Plot of the reciprocal of initial velocity (v) (p-nitrophenol release) versus the reciprocal of the molar concentration of p-nitrophenylphosphate which was varied in the range 222 µM to 2 mM. Glucose was held constant at 0 µM (▲), 100 µM (■), 273 µM (○) and 900 µM (▼).
normal saturation kinetics with respect to p-nitrophenol phosphate in the presence of 2 mM glucose. An apparent $K_m$ for p-nitrophenyl phosphate of 300 μM was determined from these data. Reaction blanks identical to the reaction mixtures used for the apparent $K_m$ determination, except for the absence of glucose 6-phosphate dehydrogenase, were used to insure that formation of the p-nitrophenolate anion did not affect the absorbance of the assay at 342 nm. No change in absorbance could be observed in the absence of coupling enzyme over the time period used for the assays. These data supply strong support for p-nitrophenyl phosphate serving as a substrate for the phosphotransferase reaction.

Because of Bergmeyer and Moellering's claim that p-nitrophenyl phosphate is not a substrate for the phosphotransferase reaction (Bergmeyer and Moellering, 1965), and because of the importance of this point, the same experiment was done with mannose 6-P as the phosphoryl donor. Figure 8 shows that mannose release exhibits the same pattern as p-nitrophenol release. In both experiments the lines are convergent. This is in contrast to the parallel lines suggested by equation 6. Changes in slope and intercept, in figure 8, are nonlinear with respect to glucose.

Neither the $\theta$ relationships discussed earlier, nor the above experiment give support for an obligatory covalent intermediate. The data in these experiments would seem to rule out the modified Ping Pong Bi Bi mechanism for APHP.
Figure 8. Plot of the reciprocal of initial velocity (v) (mannose release) versus the reciprocal of the molar concentration of mannose 6-phosphate which was varied in the range 212 μM to 1.9 mM. Glucose was held constant at 0 μM (□), 273 μM (△), 545 μM (○) and 909 μM (■).
Kinetic studies with substrate analogues

On the basis of the ϕ relationships and the phosphoryl donor product release experiments, it was concluded that the kinetic mechanism is of the classical sequential types. The simplest and least ambiguous method of determining the kinetic mechanism is to use dead-end competitive inhibitors for each substrate (Fromm and Zewe, 1962). In these studies, a competitive inhibitor for each substrate will give a particular inhibition pattern relative to the other substrate. Once the modified Ping Pong mechanism has been ruled out it is possible, using this protocol, to differentiate between ordered and random mechanisms, and in the former case obtain the substrate binding order.

Figures 9A and 9B illustrate inhibition patterns with N-acetylglucosamine, a linear competitive inhibitor of glucose. The results of figure 9B show that N-acetylglucosamine is a linear non-competitive inhibitor of glucose. If it is assumed that the phosphoryl donor can bind to the enzyme in the absence of glucose, which it must do to account for the phosphatase activity of the enzyme, the kinetic mechanism must be random. If the mechanism was ordered, the inhibition pattern relative to acetyl phosphate would be linear uncompetitive (Fromm, 1975).

It was observed that N-acetylglucosamine is a weak substrate for the phosphotransferase reaction. Phosphorylation of N-acetylglucosamine is less than 10% of the activity found with glucose as the acceptor. Because the assay system does not monitor N-acetylglucosamine 6-P formation and because glucose effectively competes with the inhibitor or
Figure 9A. Plot of the reciprocal of initial velocity (v) versus the reciprocal of the molar concentration of glucose in the absence (Δ) and presence of 5 mM (□), 10 mM (○), and 15 mM (△) N-acetylglucosamine. Glucose was varied in the range 111 μM to 1.00 mM and acetyl phosphate held constant at 444 μM. Velocity was determined with the glucose 6-phosphate dehydrogenase assay.

9B. Plot of reciprocal of initial velocity (v) versus the reciprocal of the molar concentration of acetyl phosphate in the absence (Δ) and presence of 5 mM (□), 10 mM (○), and 15 mM (△) N-acetylglucosamine. Acetyl phosphate was varied in the range 196 μM to 1.76 mM, and glucose was held constant at 1 mM. Velocity was determined with the glucose-6-phosphate dehydrogenase assay.
alternative substrate, it is not necessary to modify the theory of dead-end substrate analogue inhibition to account for the effects of N-acetylglucosamine.

Figures 10A and 10B describe the inhibitory effects of $P_i$ relative to the substrates acetyl phosphate and glucose. The data indicate that $P_i$ is a linear competitive inhibitor of acetyl phosphate and a linear noncompetitive inhibitor of glucose. These results are also consistent with a sequential mechanism of the rapid equilibrium random Bi Bi type, depicted in Scheme II, in which all steps equilibrate rapidly relative to the interconversion of the ternary complexes (Alberty, 1953). The $K$'s refer to dissociation constants for the various steps illustrated in the mechanism.

Scheme II

The rate expression for dead-end inhibition by a substrate analogue (I) for substrate A is described by equation 7

$$\frac{E_0}{v} = \phi_0 + \phi_a \left(1 + \frac{(I)}{K_{ii}}\right) + \frac{\phi_B}{(A)(B)} \left(1 + \frac{(I)}{k_i}\right)$$  (7)

where $\phi_0$, $\phi_a$, and $\phi_{AB}$ are $1/k_i$, $K_a/k_i$, $K_b/k_i$ and $K_{ia}K_b/k_i$, respectively.
Figure 10A. Plot of reciprocal of initial velocity (v) versus the reciprocal of the molar concentration of acetyl phosphate in the absence (○) and presence of 3 mM (△), 6 mM (□), and 9 mM (▽) P<sub>i</sub>. Acetyl phosphate was varied in the range 97 μM to 880 μM, and glucose was held at 0.5 mM. Velocity was determined with the glucose 6-phosphate dehydrogenase assay.

10B. Plot of reciprocal of initial velocity (v) versus the reciprocal of the molar concentration of glucose in the absence (○) and presence of 3 mM (△), 6 mM (□), and 9 mM (▽) P<sub>i</sub>. Glucose was varied in the range 111 μM to 1.00 mM, and acetyl phosphate was held constant at 132 μM. Velocity was determined with the glucose 6-phosphate dehydrogenase assay.
The rate constant \( k_1 \) is the forward direction unimolecular rate constant for the isomerization of the productive ternary complex. In equation 7, \( K_1 \) and \( K_{11} \) represent dissociation constants for the complexes EI and EIB (EB + I = EIB), respectively.

**The effect of glucose on the dephosphorylation of p-nitrophenol phosphate**

The data from the dephosphorylated donor release experiments, figures 7 and 8, and the \( \phi \) relationships, serve to exclude the modified Ping Pong mechanism, shown in Scheme I, as the mode of substrate interaction for APHP. The data shown in figures 9 and 10 are in harmony with Scheme II, as suggested by the substrate analogue inhibition studies.

If the rapid equilibrium random Bi Bi mechanism is used as the model for the dephosphorylation of the phosphoryl donor in the presence of acceptor, the following rate equation is obtained

\[
\frac{E_0}{v} = \frac{\phi_B \phi_{OH}}{\frac{1}{\phi_B} + \frac{\phi_0(B)}{\phi_B}} + \frac{\phi_{OH}(\phi_{AB} + \phi_A(B))}{\frac{1}{\phi_B} + \frac{\phi_{OH}(B)(A)}}
\]

(8)

where \( \phi_{OH}, (A) \), and \( (B) \) represent the reciprocal of the unimolecular rate constant for the hydrolysis of EA, p-nitrophenol phosphate or mannose 6-P, and glucose respectively. Data plotted to equation 8 can take many forms depending upon the relationships of \( \phi_B, \phi_{OH}(B), \phi_A(B) \). The data shown in figure 8 are consistent with velocity plots generated from \( \phi \)'s for the transferase and phosphatase experiment. The plot generated is noncompetitive. Replots of the slopes and intercepts are nonlinear with respect to glucose.
Regulation of APHP

APHP has the novel ability to both phosphorylate a sugar, and then to convert this sugar phosphate to the free sugar and P\textsubscript{i}. The net reaction is the loss of one high energy phosphate. It is reasonable to assume that this futile cycle is not allowed to run uncontrolled, since this would quickly drain the bacterium's energy reserves. It seems, for \textit{A. aerogenes} to survive, the phosphatase activity would have to be under rigid control.

The fact that both glucose and water compete for the phosphoryl donor on the enzyme suggest one mode of control; as glucose concentrations rise, phosphatase activity should decrease. This is the case as shown in figure 11. Equation 9 describes the rate equation to be expected for the phosphatase activity of APHP in the presence and absence of glucose using the model presented in Scheme II, where \( A \) represents the phosphoryl donor and \( B \) represents glucose.

\[
\frac{E_0}{v} = \Phi_{OH} \left( 1 + \frac{(B)}{K_B} \right) + \frac{\Phi_A}{(A)} \left( 1 + \frac{(B)}{K_{ib}} \right)
\]

According to equation 9, inhibition of phosphatase activity, with respect to glucose, should be noncompetitive. Slope and intercept replots should be linear with respect to glucose. This is in agreement with the data shown. Extrapolation of the lines drawn in figure 11 shows that they intersect at a common point on the abscissa. In this case \( K_B \) should equal \( K_{ib} \). This is in reasonably good agreement with data presented in table 3.

It seems unlikely that glucose could be responsible for the com-
Figure 11. Plot of reciprocal of initial velocity (v) (P state release) versus the reciprocal of the molar concentration of ribose 5-phosphate in the absence (Δ) and presence of 0.3 mM glucose (○), 0.6 mM glucose (□) and 1.0 mM glucose (▽). Ribose 5-phosphate was varied in the range of 444 μM to 4.00 mM.
plete control of the phosphatase activity since the glucose levels in bacteria probably fluctuate greatly under different growth conditions. Screening experiments, with a wide variety of different metabolites, showed that both pyrimidine and purine nucleotides inhibit the phosphatase activity to some extent. ATP was by far the strongest inhibitor of phosphatase activity. Inhibition by ATP is noncompetitive as shown in figure 12.

Preliminary studies have indicated that ATP may inhibit the phosphohydrolase activity more than the phosphotransferase activity. There is an interesting parallel here between reports by Nordlie that certain long chain fatty acids exert an activity discriminating effect on glucose 6-phosphatase activity. (Nordlie et al., 1967; Nordlie, 1968; 1969). Whether or not this discriminating effect of ATP is real or not, it does seem that ATP inhibition of the phosphatase activity has a physiological effect. ATP levels used in this experiment were well below those reported for E. coli (Lowry et al., 1971), a closely related bacteria.
Figure 12. Plot of the reciprocal of initial velocity (v) (p-nitrophenol) release versus the reciprocal of the molar concentration of p-nitrophenylphosphate in the absence (Δ) and presence of 0.3 mM (○), 0.6 mM (□), and 0.9 mM (▽) ATP. p-Nitrophenylphosphate was varied in the range 111 μM to 1.00 mM.
GENERAL DISCUSSION

The purification of APHP presented in this study represents a significant improvement over the method of Kamel and Anderson (1967). We have been able to purify APHP to homogeneity using electrophoretic criteria. The molecular weight of the enzyme was found to be approximately 150,000 using both gel filtration and short column equilibrium sedimentation. Preliminary studies with sodium dodecyl sulfate gel electrophoresis suggest the enzyme is a hexamer. This proposition will require additional investigation before the point can be made with certainty. Hopefully physical and chemical studies can be carried out on the protein to determine more of its properties.

The kinetic protocol described here could be of general use for determining the mechanism of two substrate systems exhibiting both a transferase and hydrolase activity. The kinetic evidence presented in this report strongly suggests that the kinetic mechanism presented is rapid equilibrium random Bi Bi as illustrated in Scheme II. Whether or not the kinetic mechanism of APHP is truly rapid equilibrium random Bi Bi remains to be ascertained. Rudolph and Fromm (1971) have shown, from computer studies, that kinetic data consistent with the rapid equilibrium assumption may be obtained even if the kinetic mechanism is steady-state random Bi Bi.

This enzyme is the first reported case of a modified rapid equilibrium random Bi Bi mechanism in the literature (Casazza and Fromm, 1977). This mechanism is clearly different from those reported for
other enzymes which catalyze both a transferase and a hydrolase activity. Glucose 6-phosphatase (Arion and Nordlie, 1964), transglutaminase (Gross and Folk, 1973), γ-glutamlytranspeptidase (Karkowsky et al., 1976) and nicotinamide adenine glycohydrolase (Schuber et al., 1976) are all believed to have obligatory covalent intermediates. The kinetic mechanism of APHP thus seems to be unique to this class of enzymes.

APHP regulation is of interest. It is obvious that the phosphatase activity of this enzyme must be regulated if the bacterium is to survive. Schemes for draining energy from both the ATP and acetyl phosphate pools are easily envisioned. It was encouraging to find that both ATP and glucose are inhibitors of the phosphatase activity. Lowry et al. (1971) determined the intracellular concentration of ATP in E. coli to be about 6 mM. If it is assumed that this figure is the approximate value for the concentration of ATP in the closely related A. aerogenes, the hydrolase activity of APHP would be severely inhibited. Of particular interest is the apparent discriminating inhibitory activity of ATP. Preliminary studies seem to indicate that ATP inhibits the phosphohydrolase activity to a greater degree than the phosphotransferase activity. This sort of inhibition is not without precedent. Nordlie has reported that certain fatty acids have the same effect on glucose 6-phosphatase (Nordlie et al., 1967; Nordlie, 1968; 1969). If this observation is supported by further work, it could raise some interesting questions. In particular, how ATP effects its discriminatory action.

The actual physiological role of APHP is still not known. Kamel
and Anderson suggested that the enzyme may be responsible for phosphorylating sugars for which a specific kinase may not exist (Kamel and Anderson, 1966a; 1966b). If indeed ATP does show a significant activity discrimination in its inhibition of APHP, this would be somewhat supportive of Kamel and Anderson's hypothesis. Otherwise these studies have shed no additional light on the true physiological role of APHP.
BIBLIOGRAPHY


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