Purification and Initial Rate Kinetics of Acyl-Phosphate–Hexose Phosphotransferase from *Aerobacter aerogenes*†

Joseph P. Casazza and Herbert J. Fromm*

ABSTRACT: The enzyme acyl-phosphate–hexose phosphotransferase from *Aerobacter aerogenes* was purified to electrophoretic homogeneity. The molecular weight of the enzyme as determined on Sephadex gels is 150 000. The enzyme possesses potent phosphotransferase and phosphohydrolase activities. Initial rate kinetics were used to investigate the mechanism of acyl-phosphate–hexose phosphotransferase. These studies, which involved a number of different phosphoryl donors and substrate analogues, suggest that the kinetic mechanism is of the rapid equilibrium random Bi Bi type. A number of other enzymes that exhibit both transferase and hydrolase activities involve obligatory covalent enzyme–substrate intermediates in their mechanisms of action.

In 1967 Kamel and Anderson (1967) described the partial purification and properties of the enzyme acyl-phosphate–hexose phosphotransferase (EC 2.7.1.61) from *Aerobacter aerogenes*. They demonstrated that the enzyme was relatively nonspecific for both phosphoryl group donor and acceptor, and that the enzyme exhibits potent phosphatase activity both in the presence and absence of sugar acceptors. Kamel and Anderson (1967) have shown that the phophatase and phosphotransferase activities are related and are roughly of comparable magnitude. An enzyme with similar properties has been described from chicken breast muscle (Bergmeyer and Moellering, 1965).

The properties of acyl-phosphate–hexose phosphotransferase, in terms of activity and specificity, are similar in many respects to those reported for mammalian liver glucose-6-phosphatase (Nordlie, 1971). One of the major differences between the two enzymes is the observation that the bacterial system will not use nucleotides or inorganic pyrophosphate as substrates (Kamel and Anderson, 1967). In addition, whereas the bacterial system is soluble, liver glucose-6-phosphatase is associated with the endoplasmic reticulum of the cell (Hers et al., 1951).

Arion and Nordlie (1964) have proposed a kinetic mechanism for glucose-6-phosphatase that involves the participation of an obligatory phosphoryl-enzyme intermediate. In order to account for the sequential[1] rather than ping-pong initial rate data observed for the phosphatase, Arion and Nordlie (1964) suggested a modified ping-pong mechanism in which the phosphoryl-enzyme intermediate undergoes either hydrolysis or reaction with an acceptor other than water. Feldman and Butler (1972) have presented chemical evidence for a phosphoryl-enzyme intermediate in the glucose-6-phosphatase reaction. One of the obvious limitations of their proposal is the fact that, because of glucose-6-phosphatase's association with the microsomal fraction of the liver cell, this evidence, although suggestive of a covalent intermediate, is not considered to be conclusive. It is interesting that other enzymes that exhibit hydrolase and transferase activities are also believed to display the modified ping-pong Bi Bi kinetic mechanism proposed for glucose-6-phosphatase (Gross and Folk, 1973; Karkowsky et al., 1976; Schuber et al., 1976).

Because of the similarity in both activity and specificity of glucose-6-phosphatase and acyl-phosphate–hexose phosphotransferase, we thought it would be useful to investigate the kinetics of the bacterial enzyme as a model for the particulate phosphotransferase. If the kinetics implicated a phosphoryl-enzyme intermediate in the mechanism of action of acyl-phosphate–hexose phosphotransferase, its properties could be studied both kinetically and chemically. Another interesting facet of the bacterial enzyme concerns the mode of regulation of the phosphotransferase and phosphohydrolase activities. It seems improbable that the hydrolase activity associated with the enzyme would be permitted to manifest itself without rigorous metabolic constraints.

The results of the present report indicate that the kinetic mechanism of acyl-phosphate–hexose phosphotransferase is not ping-pong Bi Bi. In addition, ATP and free sugars may play a role in modulating the enzyme's phosphohydrolase activity.

Experimental Procedure

Growth of Cells and Preparation of Cell-Free Extracts. A. aerogenes (ATCC 9621) from a lyophilized culture were grown on a sterile 500-mL inorganic salt medium supplemented with 0.5% ribitol. The starter culture was divided equally and used to inoculate 10, 2-L inorganic salt medium flasks supplemented with 0.5% glucose and 0.1% yeast extract in a volume of 400 mL. The cells were grown under aerobic conditions with vigorous shaking at 37 °C and harvested, washed, and extracts prepared (Rudolph et al., 1968) using a French pressure cell.

Materials. ATP, acetyl phosphate, NADP, NAD, NADH, mannose-6-P, ribose-5-P, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes),[2] phosphoglucoisomerase, and acetate kinase were obtained from Sigma. Glucose was obtained from Pfanstiehl and recrystallized from hot ethanol. Glycylglycine, *N*-acetylglucosamine, hexokinase, pyruvate kinase, and lactate

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[1] The nomenclature is that of Cleland (1963) and Dalziel (1957).

[2] Abbreviations used: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DEAE, diethylaminoethyl; P_i, inorganic phosphate.

dehydrogenase were purchased from Calbiochem. Glucose-6-P dehydrogenase and phosphomannoisomerase were purchased from Boehringer-Mannheim. All reagents for gel electrophoresis and Cellex-D were supplied by Bio-Rad Laboratories. Sephadex G-100 and G-200 gels and blue dextran were purchased from Pharmacia. Gold label *p*-nitrophenyl phosphate was from Aldrich. Low ion water was obtained by passing distilled water through a Barnstead high capacity ion-exchange column and a Continental water deionizer. All other reagents were of highest possible purity.

Acyl-Phosphate–Hexose Phosphotransferase Purification. Acyl-phosphate–hexose phosphotransferase was purified by the method of Kamel and Anderson (1967) with slight modification. Protein was concentrated using ultrafiltration techniques. In addition, the last two steps of Kamel and Anderson's purification procedure were reversed in order, and the diethylaminoethyl (DEAE)-cellulose chromatography procedure was altered. Protein from the second salt fractionation was dissolved and dialyzed against 20 mM Pipes (pH 6.7). Up to this point in the purification procedure, the results we obtained were essentially identical with those reported by Kamel and Anderson (1967). The protein was added to a DEAE column (2.5 × 40 cm equilibrated with 20 mM Pipes (pH 6.7)) and the acyl-phosphate–hexose phosphotransferase activity washed off. The wash volume was then concentrated by ultrafiltration and dialyzed against 20 mM Pipes (pH 6.7). The protein was then added to a second DEAE column (2.5 × 30 cm) equilibrated with 20 mM Pipes (pH 6.7). Acyl-phosphate–hexose phosphotransferase was eluted immediately with a linear 0 to 0.3 M NaCl gradient. Fractions were collected (4 mL) and those which contained the highest specific activity were pooled and concentrated to a 3.7-mL volume. The protein was added to a 2.5 × 26 cm Sephadex G-100 column. Four-milliliter fractions were collected. Homogeneous acyl-phosphate–hexose phosphotransferase with a specifc activity of 100.5 IU/mg appeared in the excluded volume. Purity of the enzyme during purification was followed with gel electrophoresis, using both a protein stain and an activity stain for P_i.

Gel Electrophoresis. Native gels were run using the procedure of Davis (1964). Protein was stained using Coomassie brilliant blue R-250. Activity staining was done in 2 mM acetyl phosphate, 8 mM glycylglycine pH 7.5, and 200 mM $CaCl_2$. After a 30-min incubation at 27 °C, a band of white calcium phosphate could be seen. Gels were then washed in distilled water and stored in 0.2 M $CaCl_2$. Sodium dodecyl sulfate electrophoresis was done using the system of Laemmli (1970). Sodium dodecyl sulfate gels were made 10% in acrylamide.

Molecular Weight Determinations. The molecular weight of acyl-phosphate–hexose phosphotransferase 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 × 55 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 h was maintained, and 3-mL fractions were collected. Blue dextran was read at 620 nm, egg albumin at 280 nm, and cytochrome *c* at 415 nm; acyl-phosphate–hexose phosphotransferase was assayed using the *standard assay*; alcohol dehydrogenase activity was measured by following the oxidation of NADH in the presence of pyruvate; pyruvate kinase was assayed by following pyruvate formation with a lactate dehydrogenase couple, in the presence of phosphoenolpyruvate, ADP, and NADH.

Definition of Enzyme Unit. A unit of acyl-phosphate–hexose phosphotransferase activity was defined as the amount of enzyme that catalyzed the phosphorylation of 1 μmol of glucose at 28 °C per min under *standard assay conditions.*

Enzyme Assays. The enzyme was routinely assayed by measuring NADP reduction using a glucose-6-P dehydrogenase couple in a Cary 15 or Cary 118 recording spectrophotometer thermostated at 28 °C. *Standard assay* conditions were 8 mM glycylglycine (pH 7.5), 4 mM acetyl phosphate, 2 mM glucose, 180 μM NADP, and 1.7 units of glucose-6-P dehydrogenase in a 3-mL volume.

Phosphatase activity was measured over a 10-min period according to the method of Berenblum and Chain (1938). Assays were carried out in a 1-mL reaction volume buffered at pH 7.5 with 8 mM glycylglycine. The assay mixtures were stopped by the addition of 0.2 mL of 21% $HClO_4$ and immediately placed on ice. Inorganic orthophosphate (P_i) determinations were made immediately after all reactions were terminated. Reaction blanks (in which enzyme was added after addition of $HClO_4$) were analyzed at each concentration of phosphoryl donor.

Michaelis constant determinations for phosphotransferase experiments were made under conditions identical with those used for the standard assay, except that the substrate concentration was varied. All other spectrophotometric assays were made in 20 mM glycylglycine (pH 7.5), with the exception of P_i inhibition studies. P_i inhibition studies were carried out in 40 mM glycylglycine with KCl added to maintain constant ionic strength.

p-Nitrophenol release was followed at 410 nm using an extinction coefficient of 18.1×10^3 M^{-1} cm^{-1}. Enzymatic transfer of P_i from *p*-nitrophenyl phosphate to glucose was demonstrated by saturating the enzyme with glucose and varying the *p*-nitrophenyl phosphate around Michaelis constant levels and following glucose-6-P formation. Glucose-6-P was determined by following NADP reduction from a glucose-6-P dehydrogenase couple at the *p*-nitrophenyl phosphate–*p*-nitrophenol isosbestic point. Data gathered in this manner gave standard Michaelis–Menten kinetics. A *p*-nitrophenyl phosphate–*p*-nitrophenol isosbestic point of 342.25 nm was determined by optically scanning a 50 μM *p*-nitrophenyl phosphate solution containing acyl-phosphate–hexose phosphotransferase in 20 mM glycylglycine (pH 7.5).

Enzymatic release of mannose from mannose-6-P was assayed by the method of Fromm et al. (1964). After a 10-min incubation period, 1 mL of a 1.1-mL reaction volume was stopped by addition to a 1 × 1.5 cm AG-1X-2 column. Columns were washed with 6 mL of water. The pass-through and wash volume were collected and a 3-mL aliquot was counted in 10 mL of Bray's solution (Bray, 1960).

The degree of *N*-acetylglucosamine phosphorylation by acyl-phosphate–hexose phosphotransferase was determined by incubating a reaction mixture containing 15 mM *N*-acetylglucosamine for 10 min in the presence of acetyl [^{32}P]phosphate and acyl-phosphate–hexose phosphotransferase. At the end of the 10-min incubation period, 0.2 mL of 21% $HClO_4$ was added to the reaction mixture and heated to boiling for 10 min to hydrolyze all the acetyl phosphate. P_i was quantitatively extracted into the upper phase of a water-saturated isobutyl alcohol–benzene mixture (Berenblum and Chain, 1938). After two additional back extractions with ammonium molybdate, the aqueous phase was counted in Bray's solution. The amount of [^{32}P]-*N*-acetylglucosamine-6-P was found to be less than 10% of the phosphate transferred to glucose under similar conditions.

Statistical Analysis. Statistical analysis was done according to the method of Siano et al. (1975). Weighting was assigned

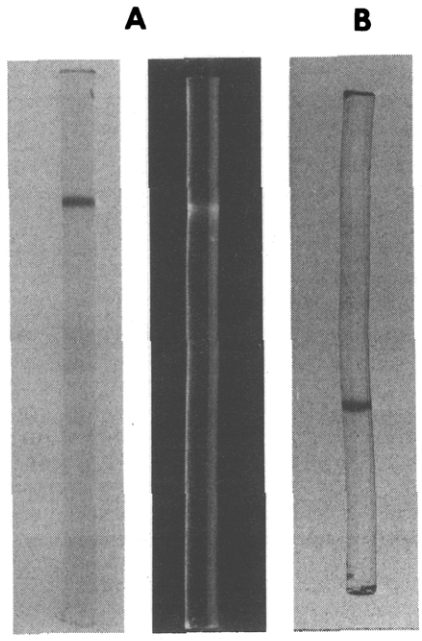


FIGURE 1: (A) Polyacrylamide gel electrophoretograms of purified acyl-phosphate–hexose phosphotransferase (specific activity, 100.5). The gel on the left was stained for protein, and the one on the right for phosphatase activity. Gel preparation, enzyme addition, and electrophoresis time were identical in the case of both gels. Other details may be found in Experimental Procedure. (B) Sodium dodecyl sulfate gel electrophoretogram of purified acyl-phosphate–hexose phosphotransferase (specific activity, 100.5). The enzyme was stained for protein. Other details are described in Experimental Procedure.

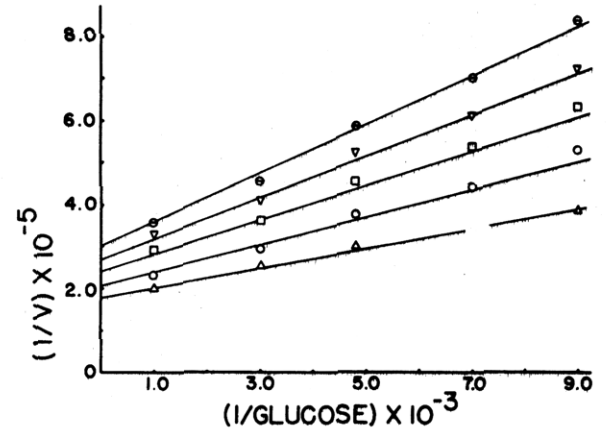


FIGURE 2: Plot of the reciprocal of initial velocity (v) vs. the reciprocal of molar concentration of glucose at 1.10 mM (⊖), 1.42 mM (▽), 2.00 mM (□), 3.33 mM (○), and 10.0 mM (△) ribose-5-P. Glucose was varied in the range 111 μM to 1.00 mM. Velocity was determined with the glucose-6-P dehydrogenase assay. Other experimental details are described under Experimental Procedure.

using the formula

$$W(1/V_i) = \frac{N V_i^{4-\alpha}}{\Sigma_i V_i^{4-\alpha}}$$

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

$$\sigma^2(V_i) = C V_i^{\alpha}$$

A log plot of σ^2, standard deviation, vs. log velocity allows evaluation of C and α. The α'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.

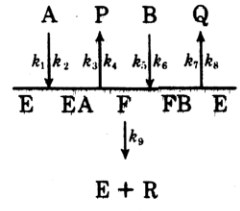
All velocity measurements represent initial reaction velocity. This portion of the product–time progress curve was determined either from continuous spectrophotometric tracings or by a series of stop-time determinations when the former procedure could not be used, i.e., for the isotope experiments. Velocity is reported in M min^{-1}.

Results

Purification of Acyl-Phosphate–Hexose Phosphotransferase. Kamel and Anderson (1967) reported a 770-fold enrichment of acyl-phosphate–hexose phosphotransferase over cell-free extracts in a six-step purification protocol. We found that, when the last two steps of the Kamel–Anderson purification procedure were modified somewhat and reversed in order, a pure preparation of the enzyme was obtained (for details see Experimental Procedure). Figure 1A illustrates disc gel electrophoretograms of the enzyme that were stained either for protein or phosphatase activity. The enzyme was also treated with sodium dodecyl sulfate, subjected to electrophoresis on sodium dodecyl sulfate gels, and stained for protein (Figure 1B). The enzyme appeared to migrate as a single protein band on sodium dodecyl sulfate gels. Acyl-phosphate–hexose phosphotransferase was judged to be homogeneous based upon the criteria described in Figures 1A and 1B. Chromatography on a Sephadex G-200 column was carried out in an attempt to determine the molecular weight of acyl-phosphate–hexose phosphotransferase. The value obtained for the molecular weight of the enzyme was 150 000.

Initial Rate Kinetics. Initial rate kinetics were undertaken to provide some insight into the kinetic mechanism of acyl-phosphate–hexose phosphotransferase. Figure 2 illustrates kinetic data in which acyl-phosphate–hexose phosphotransferase was studied with ribose-5-P and glucose as substrates for the enzyme's phosphotransferase activity. It was also observed that, when 1/velocity was plotted against 1/(ribose-5-P) at different fixed levels of glucose, the lines converged at a common point. In analogous experiments, mannose-6-P and acetyl phosphate were used as phosphoryl donors. The results obtained were found to be qualitatively identical with those depicted in Figure 2. It is clear from these findings that the double-reciprocal plots are convergent and that the kinetic mechanism is sequential. If we assume that the mechanism is of the modified ping-pong Bi Bi type (Arion and Nordlie, 1964) as shown in Scheme I, rate equations can be written for both

Scheme I



the phosphotransferase activity (eq 1) and the phosphohydrolase activity (eq 2), where A, B, P, Q, R, E, and F are phosphoryl donor, phosphoryl acceptor, dephosphorylated donor, phosphorylated acceptor, P_i, enzyme, and phosphoryl-enzyme, respectively.

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_A}{(A)} + \frac{\Phi_B}{(B)} + \frac{\Phi_{AB}}{(A)(B)} \qquad (1)$$

TABLE I: Kinetic Parameters for Acyl-Phosphate–Hexose Phosphotransferase.[a]

Substrate	Phosphotransferase					
	(Φ_A/E_0) (min)	(Φ_B/E_0) (min)	(Φ_{AB}/E_0) (M min)	K_a (mM)[b]	K_{iglu} (mM)[c]	K_{glu} (mM)[c]
Acetyl phosphate	1.74	1.61	3.09×10^{-4}	0.11	0.18	0.10
Mannose-6-P	14.7	35.5	4.82×10^{-3}	0.08	0.33	0.18
Ribose-5-P	157	20.4	4.13×10^{-2}	1.0	0.26	0.13

Substrate	Phosphatase		
	(Φ_0/E_0) (M^{-1} min)	(Φ_A/E_0) (min)	$\frac{\Phi_A\Phi_B\ (M^{-1}\ min)}{\Phi_{AB}E_0}$
Acetyl phosphate	3.57×10^4	9.38	9.07×10^3
Mannose-6-P	5.84×10^4	7.92	10.8×10^4
Ribose-5-P	2.60×10^4	19.6	7.76×10^4

[a] The phosphotransferase and phosphatase experiments for each donor were adjusted to a constant E_0. [b] The Φ values are related to the Michaelis constants as follows: $[\Phi_A/\Phi_0] = K_a$, $[\Phi_B/\Phi_0] = K_b$, $[1/\Phi_0] = V_{max}$. [c] K_{iglu} and K_{glu} are taken to represent the dissociation and Michaelis constants for glucose.

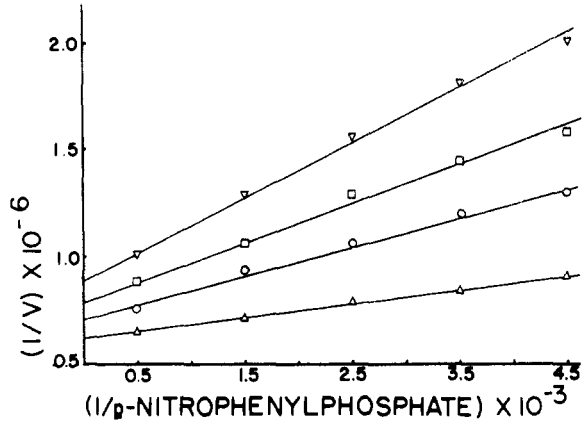


FIGURE 3: Plot of the reciprocal of initial velocity (v) (p-nitrophenol release) vs. the reciprocal of the molar concentration of p-nitrophenyl phosphate which was varied in the range 222 μM to 2.00 mM. Glucose was held constant at 0 μM (▲), 100 μM (○), 273 μM (□), and 900 μM (▽).

In eq 1 $\Phi_0 = (1/k_3 + 1/k_7)$, $\Phi_A = (k_2 + k_3)/k_1k_3$, $\Phi_B = (k_6 + k_7)(k_3 + k_9)/k_3k_5k_7$, and $\Phi_{AB} = k_9(k_2 + k_3)(k_6 + k_7)/k_1k_3k_5k_7$.

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_A}{(A)} \tag{2}$$

In eq 2, $\Phi_0 = (1/k_3 + 1/k_9)$ and $\Phi_A = (k_2 + k_3)/k_1k_3$.

It can readily be shown that the following relationships hold for the mechanism shown in Scheme I

$$\Phi_{A(transferase)} = \Phi_{A(phosphatase)} \tag{3}$$

$$\Phi_{0(phosphatase)} = \Phi_A\Phi_B/\Phi_{AB(transferase)} \tag{4}$$

Kinetic data from the initial rate experiments provided the Φ values shown in Table I. It can be seen that, for the three phosphoryl donors, the Φ relationship shown in eq 4 holds reasonably well, whereas that shown in eq 3 does not. This latter finding is clearly at variance with the mechanism of Scheme I.

Effect of Sugar Acceptors on the Dephosphorylation of the Phosphoryl Donor. Karkowsky et al. (1976) have shown that, for an enzyme that exhibits a kinetic mechanism of the type described in Scheme I, plots of 1/velocity vs. 1/(donor) at different fixed levels of acceptor yield a family of parallel lines when velocity is defined as donor dephosphorylation per unit time. Figure 3 illustrates an experiment in which p-nitrophenyl phosphate[3] hydrolysis was measured at different fixed concentrations of glucose. In data not shown, very similar results were obtained when mannose-6-P was used in place of p-nitrophenyl phosphate.

The rate expression for the mechanism shown in Scheme I under the limiting conditions outlined is described in eq 5, where A and B are taken to be p-nitrophenyl phosphate and glucose, respectively.

$$\frac{E_0}{v} = \frac{1}{k_3} + \frac{(k_2 + k_3)}{k_1k_3(A)} + \frac{(k_6 + k_7 + k_5(B))}{(k_6k_9 + k_7k_9 + k_5k_7(B))} \tag{5}$$

The results of Figure 3 serve to exclude the modified ping-pong Bi Bi mechanism proposed for glucose-6-phosphatase (Arion and Nordlie, 1964) and other enzymes that exhibit both hydrolase and transferase activities (Gross and Folk, 1973; Karkowsky et al., 1976; Schuber et al., 1976).

Kinetic Studies with Substrate Analogues. Because the initial rate experiments seemed to exclude the modified ping-pong Bi Bi mechanism illustrated in Scheme I, it was concluded that the kinetic mechanism for acyl-phosphate–hexose phosphotransferase is of the classical sequential type. It was of interest to determine the kinetic mechanism for the enzyme. The simplest and least ambiguous approach to this problem 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. It is possible, using this protocol, to differentiate between ordered and random mechanisms and, in the former case, to obtain the substrate binding order.

Figures 4A and 4B illustrate inhibition patterns with N-acetylglucosamine, a linear competitive inhibitor of glucose. The results of Figure 4B show that N-acetylglucosamine is a linear noncompetitive inhibitor of acetyl phosphate. 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

[3] Bergmeyer and Moellering (1965) reported that p-nitrophenyl phosphate will not transfer its phosphoryl group to glucose; however, we found this phosphate ester to be a donor in the transferase reaction.

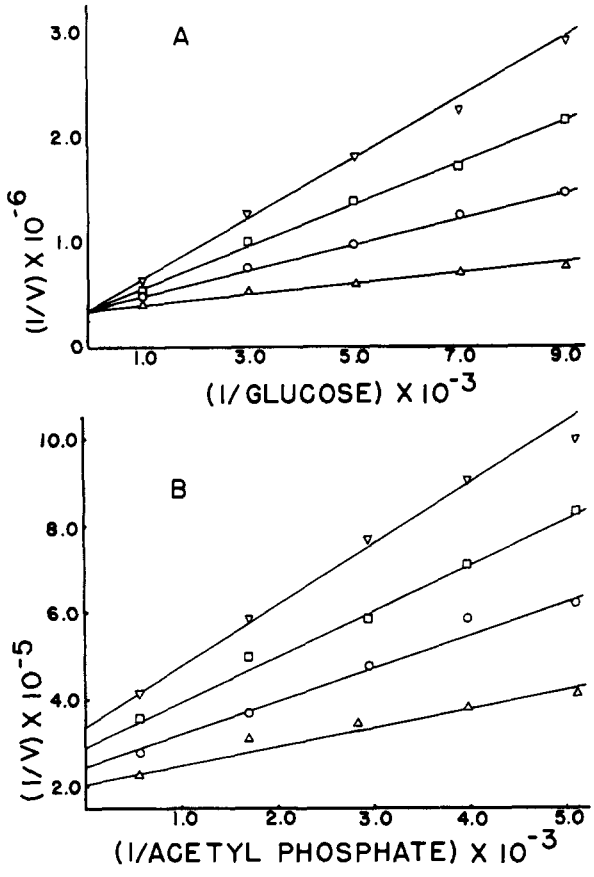


FIGURE 4: (A) Plot of the reciprocal of initial velocity (v) vs. the reciprocal of the molar concentration of glucose in the absence (△) and presence of 5 mM (○), 10 mM (□), and 15 mM (▽) *N*-acetylglucosamine. Glucose was varied in the range 111 μM to 1.00 mM and acetyl phosphate held constant at 44 μM. Velocity was determined with the glucose-6-P dehydrogenase assay. Other experimental details are described under Experimental Procedure. (B) Plot of reciprocal of initial velocity (v) vs. the reciprocal of the molar concentration of acetyl phosphate in the absence (△) 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-P dehydrogenase assay. Other experimental details are described under Experimental Procedure.

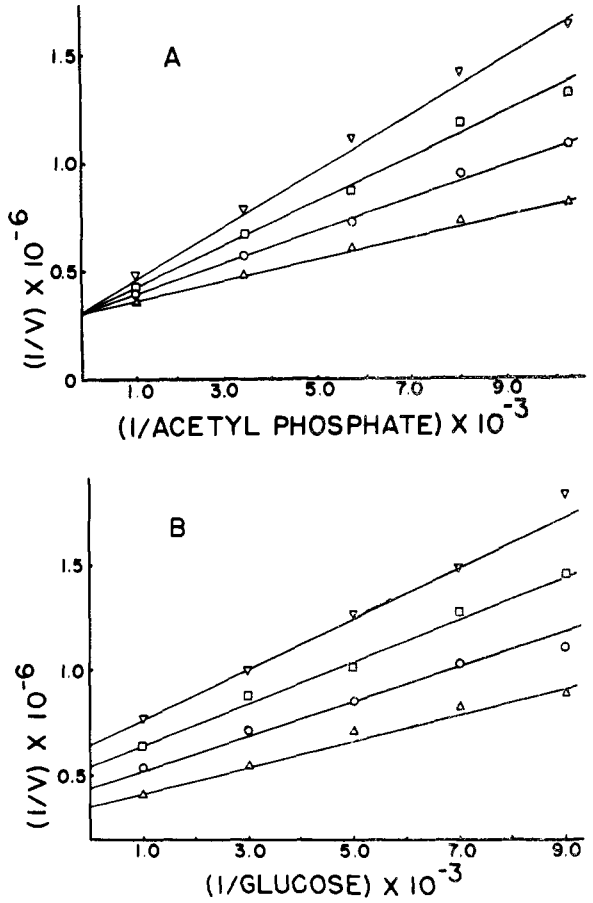


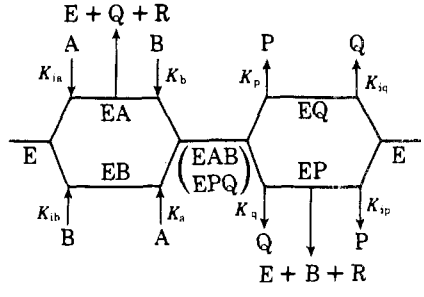
FIGURE 5: (A) Plot of reciprocal of initial velocity (v) vs. the reciprocal of the molar concentration of acetyl phosphate in the absence (△) and presence of 3 mM (○), 6 mM (□), and 9 mM (▽) P_i. Acetyl phosphate was varied in the range 97 μM to 880 μM, and glucose was held constant at 0.5 mM. Velocity was determined with the glucose-6-P dehydrogenase assay. Other experimental details are described under Experimental Procedure. (B) Plot of reciprocal of initial velocity (v) vs. the reciprocal of the molar concentration of glucose in the absence (△) and presence of 3 mM (○), 6 mM (□), and 9 mM (▽) P_i. 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-P dehydrogenase assay. Other experimental details are described under Experimental Procedure.

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 and exhibits less than 10% of the activity found with glucose as substrate. Because the assay system will not monitor *N*-acetylglucosamine-6-P formation, and because glucose effectively competes with the inhibitor, or 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 5A and 5B 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). In Scheme II, E, A, B, P, Q, and R are defined as enzyme, phosphoryl donor, phosphoryl acceptor, phosphorylated acceptor, dephosphorylated donor, and P_i, respectively. The *K*'s refer

Scheme II



to dissociation constants for the various steps illustrated in the mechanism.

The rate expression for dead-end inhibition by a substrate analogue (I) for substrate A is described by eq 6.

$$\frac{E_0}{v} = \Phi_0 + \frac{\Phi_A}{(A)}\left(1 + \frac{(I)}{K_{ii}}\right) + \frac{\Phi_B}{(B)} + \frac{\Phi_{AB}}{(A)(B)}\left(1 + \frac{(I)}{K_i}\right) \quad (6)$$

where Φ_0, Φ_A, Φ_B, and Φ_{AB} are $1/k_1$, K_a/k_1, K_b/k_1, and $K_{ia}K_b/k_1$, respectively. The rate constant k_1 is the forward direction unimolecular rate constant for the isomerization of

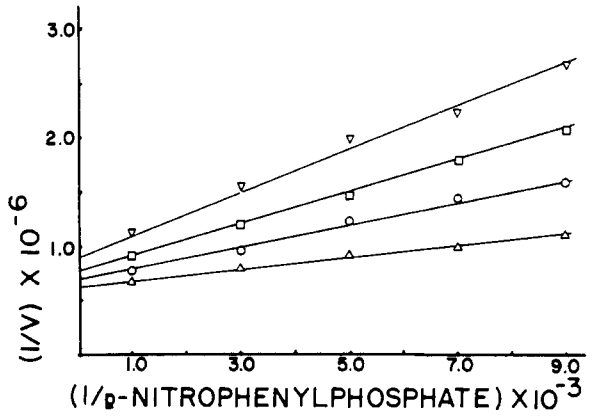


FIGURE 6: Plot of reciprocal of initial velocity (v) (p-nitrophenol release) vs. the reciprocal of the molar concentration of p-nitrophenyl phosphate in the absence (△) and presence of 0.3 mM (○), 0.6 mM (□), and 0.9 mM (▽) ATP. p-Nitrophenyl phosphate was varied in the range 111 μM to 1.00 mM. Other experimental details are described under Experimental Procedure.

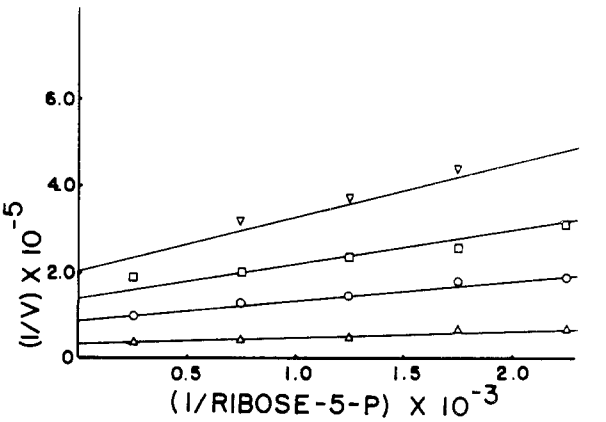


FIGURE 7: Plot of reciprocal of initial velocity (v) (P_i release) vs. the reciprocal of the molar concentration of ribose-5-P in the absence (△) and presence of 0.3 mM glucose (○), 0.6 mM glucose (□) and 1.0 mM glucose (▽). Ribose 5-phosphate was varied in the range of 444 μM to 4.00 mM. Other experimental details are described under Experimental Procedure.

the productive ternary complex. In eq 6, K_i and K_{ii} represent dissociation constants for the complexes EI and EIB (EB + I = EIB), respectively.

A very similar equation is obtained when a dead-end substrate analogue inhibitor for B is used. In this case, the $\Phi_B/(B)$ term, rather than the $\Phi_A/(A)$ term, is modified by the factor $(1 + (I)/K_{ii})$ shown in eq 6. The constant K_{ii} is now taken to represent the dissociation, EAI = EA + I.

The Effect of Glucose on the Dephosphorylation of p-Nitrophenyl Phosphate. The data of Figure 3 served to exclude the modified ping-pong Bi Bi mechanism shown in Scheme I as the mode of substrate interaction for acyl-phosphate–hexose phosphotransferase. It was of interest to determine whether these results are in harmony with the mechanism shown in Scheme II. If the rapid equilibrium random Bi Bi mechanism is used as the model for dephosphorylation of the phosphoryl donor in the presence of acceptor, the following rate equation is obtained

$$\frac{E_0}{v} = \frac{\Phi_B\Phi_{OH}}{[\Phi_B + \Phi_{OH}(B)]}\left\{1 + \frac{\Phi_0(B)}{\Phi_B}\right\} + \frac{\Phi_{OH}[\Phi_{AB} + \Phi_A(B)]}{[\Phi_B + \Phi_{OH}(B)](A)} \quad (7)$$

where Φ_{OH}, (A), and (B) represent the reciprocal of the unimolecular rate constant, k_{OH}, for the hydrolysis of EA, p-nitrophenyl phosphate, and glucose, respectively.

Data plotted to eq 7 may take many forms, but the question of interest is whether they are consistent with the results illustrated in Figure 3. If $\Phi_B > \Phi_{OH}(B)$ and $\Phi_{AB} < \Phi_A(B)$, the data shown in Figure 3 will be in accord with the mechanism of Scheme II.

Regulation of Acyl-Phosphate–Hexose Phosphotransferase. It is reasonable to assume that an enzyme such as acyl-phosphate–hexose phosphotransferase must be finely controlled, particularly with respect to its phosphatase activity, if *A. aerogenes* is to survive. We tested a number of compounds in order to appraise their ability to control the phosphatase activity of the enzyme. Figure 6 illustrates the effect of ATP on the hydrolase activity of the enzyme, and in Figure 7 is shown the effect of glucose on this same activity. The results indicate that ATP is a noncompetitive inhibitor of p-nitrophenyl phosphate, whereas glucose is a noncompetitive inhibitor of ribose-5-P. Although ATP does inhibit the transferase activity of the enzyme (data not shown), the effect is considerably less than that shown in Figure 7.

Equation 8 describes the rate equation to be expected for the phosphatase activity of acyl-phosphate–hexose phosphotransferase in the presence and absence of glucose using the model presented in Scheme II.

$$\frac{E_0}{v} = \Phi_{OH}\left(1 + \frac{(B)}{K_b}\right) + \frac{\Phi_A}{(A)}\left(1 + \frac{(B)}{K_{ib}}\right) \quad (8)$$

In eq 8, A, B, and Φ_A are taken to be ribose-5-P, glucose, and K_{ia}/k_{OH}, respectively.

According to eq 8, the linear family of lines in plots of $1/v$ vs. $1/(A)$ at different fixed levels of B should intersect on the *abscissa*, if $K_b = K_{ib}$. This identity is in reasonably good agreement with the data of Table I. If extrapolated, the lines in Figure 7 do in fact converge at a common point on the 1/(ribose-5-P) axis. The observation that the lines in Figure 6 do not converge at a common point on the abscissa indicates that ATP binding affects the binding of p-nitrophenyl phosphate to the enzyme and vice versa. Thus the data of Figures 6 and 7 suggest that the mechanism of inhibition of the hydrolase activity of acyl-phosphate–hexose phosphotransferase may not be the same with respect to ATP and glucose. This suggestion is consistent with the report (Kamel and Anderson, 1967) that adenine nucleotides are not substrates for the enzyme.

Discussion

The purpose of the present investigation was to study the kinetic mechanism of acyl-phosphate–hexose phosphotransferase as a model for enzymes that exhibit both hydrolase and transferase activities. We are aware that four enzymes that display these activities, glucose-6-phosphatase (Arion and Nordlie, 1964), transglutaminase (Gross and Folk, 1973), γ-glutamyltranspeptidase (Karkowsky et al., 1976), and nicotinamide adenine dinucleotide glycohydrolase (Schuber et al., 1976), exhibit sequential kinetics and are believed to have obligatory covalent enzyme intermediates in their kinetic mechanisms. Acyl-phosphate–hexose phosphotransferase seems to be an exception within this class of enzyme systems.

The weight of kinetic evidence in this report is clearly at variance with the modified ping-pong Bi Bi mechanism shown in Scheme I. On the other hand, the findings of this study are strongly suggestive of a rapid equilibrium random Bi Bi mechanism for acyl-phosphate–hexose phosphotransferase. Certainly, all the kinetic evidence now in hand is supportive of the kinetic mechanism outlined in Scheme II. Whether or not the kinetic mechanism is truly rapid equilibrium random remains to be ascertained. Rudolph and Fromm (1971) have shown, for example, from computer simulation studies, that kinetic data consistent with the rapid equilibrium assumption may be obtained even if the kinetic mechanism is steady-state random Bi Bi.

We have been able to purify acyl-phosphate–hexose phosphotransferase to homogeneity using electrophoretic criteria. The molecular weight of the enzyme as judged by gel filtration is 150 000. Preliminary studies with sodium dodecyl sulfate gel electrophoresis suggest the enzyme is a hexamer; however, this proposition will require additional investigation before this point can be made with certainty. It is of interest that Bergmeyer and Moellering (1965) in a footnote to their 1965 article reported crystallization of the phosphotransferase.

The subject of acyl-phosphate–hexose phosphotransferase regulation is of interest. It is obvious that the phosphatase activity of the enzyme must be regulated if the bacterium is to survive. We were encouraged to find that both ATP and glucose are inhibitors of the phosphohydrolase activity. Lowry et al. (1971) determined the intracellular concentration of ATP in *Escherichia coli* to be about 6 mM. If it is assumed that this figure is the approximate value for ATP in the closely related *A. aerogenes*, the hydrolase activity of acyl-phosphate–hexose phosphotransferase would appear to be markedly inhibited in vivo. In data not reported, it was observed that the transferase activity of the enzyme was also inhibited by ATP; however, transferase inhibition was approximately one-fifth of that recorded for the hydrolase activity.

Kamel and Anderson (1967) have suggested that acyl-phosphate–hexose phosphotransferase may play a role in the phosphorylation of sugars for which a kinase does not exist. Another possible role for the enzyme may be its potential ability to maintain the steady-state levels of phosphorylated metabolites by utilizing its phosphohydrolase and phosphotransferase activities coordinately, i.e., glucose-6-P could be utilized to phosphorylate glucose, while at the same time, glucose serves to inhibit the phosphohydrolase activity of the enzyme.

References

Alberty, R. A. (1953), *J. Am. Chem. Soc. 75,* 1928.

Andrews, P. (1964), *Biochem. J. 91,* 222.

Arion, W. J., and Nordlie, R. C. (1964), *J. Biol. Chem. 239,* 2752.

Askelöf, P., Korsfeldt, M., and Mannervik, B. (1976), *Eur. J. Biochem. 69,* 61.

Berenblum, M. L., and Chain, E. (1938), *Biochem. J. 32,* 295.

Bergmeyer, H. U., and Moellering, H. (1965), *Biochem. Z. 343,* 97.

Bray, G. A. (1960), *Anal. Biochem. 1,* 279.

Cleland, W. W. (1963), *Biochim. Biophys. Acta 67,* 104.

Dalziel, K. (1957), *Acta Chem. Scand. 11,* 1796.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Feldman, F., and Butler, L. G. (1972), *Biochim. Biophys. Acta 268,* 698.

Fromm, H. J. (1975), Initial Rate Enzyme Kinetics, Berlin, Springer-Verlag, p 97.

Fromm, H. J., Silverstein, M., and Boyer, P. D. (1964), *J. Biol. Chem. 239,* 3645.

Fromm, H. J., and Zewe, V. (1962), *J. Biol. Chem. 237,* 3027.

Gross, M., and Folk, J. E. (1973), *J. Biol. Chem. 248,* 1301.

Hers, H. G., Berthet, J., Berthet, L., and de Duvu, C. (1951), *Bull. Soc. Chim. Biol. 33,* 21.

Kamel, M. Y., and Anderson, R. L. (1967), *Arch. Biochem. Biophys. 120,* 322.

Karkowsky, A. M., Bergamini, M. V. W., and Orlowski, M. (1976), *J. Biol. Chem. 251,* 4736.

Laemmli, U. K. (1970), *Nature (London) 227,* 680.

Lowry, O. H., Carter, J., Ward, J. B., and Glaser, L. (1971), *J. Biol. Chem. 246,* 6511.

Nordlie, R. C. (1971), *Enzymes, 3rd Ed. 4,* 543–608.

Rudolph, F. B., and Fromm, H. J. (1971), *J. Biol. Chem. 246,* 6611.

Rudolph, F. B., Purich, D. L., and Fromm, H. J. (1968), *J. Biol. Chem. 243,* 5539.

Schuber, F., Travo, P., and Pascal, M. (1976), *Eur. J. Biochem. 69,* 593.

Siano, D. B., Zyskind, J. W., and Fromm, H. J. (1975), *Arch. Biochem. Biophys. 170,* 587.

Storer, A. C., Darlison, M. G., and Cornish-Bowden, A. (1975), *Biochem. J. 151,* 361.