TRANSPORT AND METABOLIC STUDIES IN TWO *ARTHROBACTER* SPECIES

by

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ABSTRACT

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Arthrobacter pyridinolis could use only two sugars, D-fructose and L-rhamnose, when present as sole carbon source in the growth medium. Previous studies determined that D-fructose, when present as sole carbon source, was transported by a phosphoenol(PEP):D-fructose phosphotransferase system. Alternatively, D-fructose could be transported by a respiration-coupled system with concomitant oxidation of L-malate. In order to elucidate the mechanism of transport of L-rhamnose in this bacterium, transport studies were conducted in whole cells and vesicles of the wild type and mutant strains. PEP:hexose phosphotransferase-negative mutants of A. pyridinolis failed to grow on L-rhamnose. Although PEP:Lrhamnose phosphotransferase activity could not be consistently demonstrated in extracts of rhamnose-grown cells, low levels of PEP-dependent uptake of L-rhamnose were found using isolated membrane vesicles from rhamnose-grown cells. This uptake was not inhibited by uncoupling agents or an inhibitor of the respiratory chain. Phosphotransferase-negative mutants could grow on L-rhamnose if L-malate was also present in the medium. L-Malate and succinate caused a five-fold stimulation of the rate of L-rhamnose uptake in membrane vesicles over that observed in the absence of additions. L-Malate-dependent L-rhamnose uptake had a K_m for L-rhamnose of 2.9×10^{-6} M. It was inhibited by uncoupling agents, inhibitors of the respiratory chain, and sulfhydryl reagents.

The finding that there existed in A. pyridinolis two systems for the transport of L-rhamnose raised a problem as to how L-rhamnose was metabolized in this organism. The respiration-coupled L-rhamnose transport system caused accumulation of free rhamnose within the cell. The PEP:L-rhamnose phosphotransferase system, on the other hand, caused uptake and accumulation of the compound as rhamnose 1-phosphate. It was found that A. pyridinolis contained an inducible L-rhamnose isomerase and L-rhamnulokinase, as well as a constitutive L-rhamnulose 1-phosphate aldolase for the metabolism of free L-rhamnose accumulated by the respiration-coupled transport system. In addition, it was found that A. pyridinolis contained a L-rhamnose 1-phosphate phosphatase, which cleaved the L-rhamnose 1-phosphate produced by the phosphotransferase system to free L-rhamnose. Mutants lacking this enzyme exhibited severe inhibition of growth in the presence of L-rhamnose plus any of a variety of carbon sources, presumably due to accumulation of toxic levels of L-rhamnose 1-phosphate within the cell. Thus, it was concluded that metabolism of L-rhamnose transported by the phosphotransferase system occurred by cleavage of L-rhamnose 1-phosphate to free L-rhamnose by a phosphatase. Metabolism of the free L-rhamnose accumulated by both systems then proceeded by the following sequence of reactions: L-rhamnose $\longrightarrow$ L-rhamnulose $\longrightarrow$ L-rhamnulose 1-phosphate $\longrightarrow$ dihydroxyacetone phosphate + L-lactaldehyde. The latter three steps resemble those which occur in E. coli.

A polyphosphate kinase was purified more than 700-fold from Arthrobacter atrocyaneus. Purification was accomplished by ammonium sulfate precipitation, DEAE-cellulose chromatography, and Sephadex G-200 gel filtration. This enzyme catalyzed the incorporation of the

γ-phosphate of ATP into long chain polymers of inorganic phosphate containing high energy anhydride bonds. The enzyme had a broad pH optimum at 6.0-7.0 and required the presence of histone and inorganic phosphate for maximal activity. Mn^{+2} or Mg^{+2} was necessary to form a metal ion-ATP complex, however, high levels of free metal ion or ATP caused inhibition of activity. At non-inhibitory concentrations of free Mn^{+2} and ATP, a K_m of 0.53 mM was calculated for the Mn-ATP^{-2} complex. ADP, PP_i, and NaF were effective inhibitors of the enzyme. The product of the reaction was characterized with respect to its stability towards acid, base, and proteolytic enzymes. Treated radioactive product was chromatographed in an Ebel's solvent and identified as polyphosphate by its behavior. This identification was necessary in order to distinguish the activity from that of a protein kinase, with which it might be confused. Physiological studies conducted in crude extracts of the cells indicated that levels of the polyphosphate kinase responded to variations in the levels of inorganic phosphate in the medium, suggesting that the role of this enzyme might be to store phosphate in the cell as polyphosphate when phosphate levels in the environment are low.

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LITERATURE REVIEW

Early studies of bacterial sugar transport were done in whole cells. A combination of genetic information and kinetic studies allowed early workers to establish that the transport of certain substrates into the cell was dependent on the presence in the cell of specific proteins, many of which were inducible. In 1955, Cohen and Rickenberg (1) first reported that Escherichia coli had a specific system for the accumulation of β-galactosides. Rickenberg et al. (2) more completely described the system, established its role in the metabolism of lactose in E. coli, and introduced the term "permease." The permease was defined as a protein, having the stereospecificity and kinetics of an enzyme, whose function was to catalyze the transfer of a substrate across the cell's osmotic barrier. Two properties of permease-mediated transport were proposed: that a specific complex of permease and substrate were formed in the transport process, and that the transport function was independent of intracellular metabolism of the substrate. It was further proposed that at equilibrium the accumulation of substrate could be described by the Michaelis-Menten equation. Initial rates of galactoside entry were proposed to be a function of external galactoside concentration by Kepes and Monod (3). By analogy with enzyme kinetics:

$$V_{in} = V_{in}^{max} \frac{G_{ex}}{G_{ex} + K_t}$$

G_{ex} is the external concentration of galactoside and K_t is the external concentration of galactoside which will result in ½ the

maximal rate of entrance (V_{in}^{max}).

A "transporter" model was proposed for lactose transport by Kepes (4) and also by Koch (5). Kepes proposed that the sugar must combine with a transporter in order to traverse the membrane barrier freely. He postulated that the permease protein catalyzed the reaction between the sugar and an activated transporter. This reaction was suggested to be irreversible and rate determining. The complex was thought to dissociate spontaneously inside the cell, requiring metabolic energy to reactivate the transporter. A revised model by Koch (6) postulated that entry of the sugar-carrier complex did not require energy, but that the substrate was actively extracted from the complex inside the cell, at the expense of metabolic energy. Kepes and Monod (3) found that exit of galactoside from the cell failed to exhibit saturation kinetics. This finding was consistent with the hypothesis of Rickenberg et al. (2) that exit of galactosides was not a carrier-mediated process. However, Kepes (4) later showed that PCMB, a sulfhydryl reagent which blocked uptake of galactosides also reduced exit of galactosides from pre-loaded cells. Koch (5) showed that the temperature coefficient of the exit process was high, like that of entry, indicating non-specific leakage was not involved. Winkler and Wilson (7) found that the K_t for exit of β-galactosides from the cell was dependent on the energy metabolism of the cell. When cells were poisoned with sodium azide and iodoacetate, exit of lactose and ONPG, a lactose analogue, from pre-loaded cells was a saturable process with a K_t similar to the K_t for entry. In unpoisoned cells, such as were used by Kepes and Monod (3), the K_t was considerably higher so that it resembled a non-saturable process with first order

kinetics dependent on the internal concentration of galactoside.

While the term permease implies a single protein involved in transport, subsequent studies of the transport of β-galactosides and other substrates have made it clear that transport is a far more complicated process than was initially proposed. In fact, transport systems have been described which are obligately linked to the intracellular metabolism of their substrate.

While looking for a bacterial kinase which would phosphorylate N-acyl D-mannosamine at the expense of ATP, Kundig et al. (8) discovered the PEP:hexose phosphotransferase system in E. coli. It was described as a three component system which used PEP and N-acyl D-mannosamine and formed pyruvate and N-acyl D-mannosamine 6-phosphate as products. No other phosphate donor was active in this system. The three protein components, Enzyme I, Enzyme II, and a phosphocarrier protein (HPr) catalyzed the following two reactions:

$$\text{HPr} + \text{PEP} \xrightarrow[\text{Enzyme I}]{Mg^{+2}} \text{HPr-phosphate} + \text{pyruvate}$$

$$\text{HPr-phosphate} + \text{N-acyl D-mannosamine} \xrightarrow[Mg^{+2}]{\text{Enzyme II}} \text{N-acyl D-mannosamine 6-phosphate} + \text{HPr}$$

Genetic evidence in many laboratories implicated the phosphotransferase system in the phosphorylation and transport of a variety of sugars in many organisms. Simoni et al. (9) reported that a mutant of Salmonella typhimurium which was pleiotropically defective in the ability to grow on nine sugars was lacking Enzyme I of the phosphotransferase system. Using mutants of the phosphotransferase system, the role of this system was also established in the transport of lactose in Staphylococcus aureus (10) and of mannitol and other sugars in Aerobacter aerogenes (11). Saier et al. (12) have also

reported a fructose PEP:phosphotransferase system in the photosynthetic bacteria, Rhodospirillum rubrum and Rhodopseudomonas spheroides. Romano et al. (13), having surveyed many organisms to detect the presence of a glucose PEP:phosphotransferase system, observed that, for glucose, this activity was found only in facultative anaerobes and not in strict aerobes.

Enzyme I was purified from E. coli. It was found to be a soluble protein, inactivated by sulfhydryl reagents, which catalyzed the transfer of phosphate from PEP to HPr. It was a constitutively produced protein and showed no sugar specificity (14).

Anderson et al. (15) characterized the phosphocarrier protein, HPr, from E. coli. It had a molecular weight of about 9500. It could accept one phosphate group on one of its two histidine residues, linked to N-1 of the imidazole ring. Kundig et al. (16) showed that HPr was released from E. coli W2244 by cold osmotic shock of the cells. Like Enzyme I, HPr in E. coli was both soluble and constitutive.

Enzyme II of the phosphotransferase system was sugar-specific and membrane-bound. Kundig and Roseman (17) purified the constitutive Enzymes II from E. coli which were specific for glucose, fructose, and mannose. Many other Enzymes II were inducible only in the presence of their specific substrate. E. coli membranes were treated with a mixture of urea and 1-butanol. The resultant soluble fraction, designated IIA, and the pellet were both required for phosphorylation of the three sugars. The pellet was further dissociated into a protein, IIB, and a lipid fraction. By electrofocusing, IIA was resolved into three proteins, each specific for one of the three

sugars. A lipid requirement was observed; this requirement could be satisfied by phosphatidyl-glycerol, which formed a complex with IIB. Components could be recombined to yield activity only when added in a particular order---IIB, cation, lipid, and IIA. A second constitutive system for glucose was found in E. coli. It used components analogous to those of the inducible phosphotransferase system for lactose transport in S. aureus, which is described below.

Simoni et al. (18, 19) and Hays et al. (20) characterized the phosphotransferase system induced by lactose or galactose in S. aureus. A cell extract was resolved into four fractions, including a membrane pellet containing Enzyme II and a soluble fraction containing HPr, Enzyme I and Factor III, which were purified by precipitation at pH 3.7 and DEAE-cellulose chromatography. This system was analogous, although not identical, to that of E. coli. The major difference was that instead of a membrane-bound IIA, the protein which donated the phosphate group to the hexose was a soluble protein, called Factor III. Factor III was a sugar-specific, inducible protein, which was stable at 100° C for 3 minutes. It did not influence the transfer of phosphate from PEP to HPr. Its molecular weight was 35,700 $\pm$ 900. It contained three identical subunits which formed three protein-phosphoryl linkages on the N-3 position of histidine imidazole rings. The lipid requirement for the system could not be studied because the Enzyme II was resistant to solubilization, and the total lipid could not be removed.

The reactions catalyzed by the lactose phosphotransferase system were as follows (21):

1) PEP + HPr $\underset{Mg^{+2}}{\overset{\text{Enzyme I}}{\rightleftharpoons}}$ phospho-HPr + pyruvate

2) phospho-HPr + 1/3 III^{lac} $\rightleftharpoons$ 1/3 (P_3-III^{lac}) + HPr

3) 1/3 (P_3-III^{lac}) + lactose $\xrightarrow{\text{Enzyme II}}$ lactose-P + 1/3 III^{lac}

Kinetic studies showed that III^{lac}, II^{lac} and the sugar (reaction 3) formed a ternary complex in which lactose was directly bound to II^{lac}.

Although the Enzyme I and HPr from E. coli and S. aureus differed, their reactions were analogous. E. coli HPr in reaction mixtures containing all other S. aureus components resulted in about 5% of the phosphorylation seen with S. aureus HPr. In the reciprocal experiment, no phosphorylation was demonstrated when S. aureus HPr was substituted for E. coli HPr in the E. coli system (19). Experiments indicated that Enzyme I of either system was able to transfer phosphate to the heterologous HPr, although at a lower rate, but reaction of heterologous HPr with the sugar-specific protein showed little or no activity. The HPr's from E. coli and S. typhimurium, which were more similar, were each active in the heterologous system (15).

As the phosphotransferase systems in various species are studied, it becomes increasingly apparent that there exist many variations on the same basic theme. For example, characterization of the fructose phosphotransferase system from Aerobacter aerogenes (22, 23) revealed that, rather than HPr, an inducible protein called "K_m factor" was required for activity with an induced Enzyme II for fructose. A constitutive Enzyme II for fructose was also found which had a lower affinity and did require HPr.

There were several problems inherent in studying transport in

whole cells; these problems hindered studies correlating the phosphotransferase system with transport, as well as studies of alternate transport mechanisms. The presence of intracellular pools of metabolites might interfere with measurements of uptake, and metabolism of substrates immediately following their transport could give misleading information about kinetics and accumulation. One way to avoid the latter problem was to work with a mutant that was blocked at the first step in the metabolism of the substrate after it entered the cell. In some cases, the metabolic pathway was not sufficiently understood to isolate such a mutant. Another approach to this problem was to use a non-metabolizable analogue of the substrate to study transport. This was effective where such a compound was known and available. A third and novel approach, which made possible a considerable amount of research on mechanisms of transport, was the use of membrane vesicle preparations. The use of such preparations was introduced by Kaback and Stadtman (24). The vesicles, made from osmotically lysed spheroplasts, were characterized and determined to be closed membranous sacs, which under certain conditions were able to catalyze active transport. The preparations were essentially devoid of whole cells, spheroplasts, ribosomes, nucleic acids, and cytoplasmic enzymes.

E. coli ML308-225 membrane vesicles prepared by freeze etching were observed in the electron microscope. The convex outer surface of the membrane had a fine granular surface. The inner surface had a much coarser granularity. All the vesicles observed in the preparations had the same appearance on freeze etching, indicating that none of the vesicles were inverted during their preparation and

isolation (25, 26). The D-lactic dehydrogenase was localized on the inside surface of E. coli membrane vesicles. When vesicles were prepared from a mutant lacking D-lactate dehydrogenase, the purified enzyme could restore activity on being added to the preparation. However, it bound to the outside of these vesicles as shown by the fact that vinylglycolate, a substrate for the D-lactic dehydrogenase (27), did not accumulate when added to reconstituted vesicles (43). When radioactive vinylglycolate was added to vesicles prepared from wild type E. coli, and autoradiographs were made from the vesicles, essentially all vesicles accumulated the vinylglycolate (S. Short, personal communication). This indicated that all the D-lactate dehydrogenase in all the vesicles was located on the inner side of the membrane, strongly suggesting that the vesicles were correctly oriented and not inverted.

Futai (28) studied the orientation of the membrane in E. coli vesicles prepared by varying procedures: EDTA-lysozyme, sonication, or French press. The marker enzymes used in this study, ATPase and ferricyanide reductase, were determined to be on the inner surface of spheroplasts and, therefore, the cytoplasmic membrane of the cell. Futai found that EDTA-lysozyme vesicles had 60% of their ATPase activity accessible to antiserum and 50% of the dehydrogenase activity accessible to ferricyanide. He concluded that only 50% of these vesicles were right-side out and that these account for all of the transport activity. Futai found that all of the sites for these markers were located on the outside surface of vesicles prepared with a French press or by sonication. They were judged to be less than 5% right-side out vesicles, and they failed to show D-lactate depen-

dent transport.

Vesicles prepared by passage through a French pressure cell by Hertzberg and Hinkle (29) have been shown to catalyze uncoupler sensitive phosphorylation of ADP with several substrates. Moreover, since they catalyzed uncoupler sensitive uptake of protons during respiration or ATP hydrolysis as opposed to proton extrusion observed in whole cells, they were determined to be inverted with respect to intact bacteria. Mycobacterium phlei membranes were also shown to be inverted by sonication (30). Tsukagoshi and Fox (31) found that mixing membranes of different origins during sonication resulted in vesicles with hybrid orientations. Kaback and Deuel (32) showed that vesicles sonicated or passed through a French pressure cell retained transport activity, suggesting that these vesicles retained their orientation. These conditions, however, were not identical to those employed by Futai (28) and other workers.

In a preparation of E. coli membrane vesicles, Kaback was able to show activity of the glucose phosphotransferase system (33). Transport of the glucose analog αMG into vesicles was dependent on the addition of PEP to the incubation mixtures. Membrane vesicles prepared from a mutant missing Enzyme I were unable to transport or phosphorylate αMG with or without PEP. In a double label experiment, intravesicular (pre-loaded) ^{14}C-glucose remained unphosphorylated while externally added ^{3}H-glucose was taken up and phosphorylated upon the addition of PEP. This indicated that phosphorylation was obligately linked to the transport process. Kaback noted that addition of exogenous HPr or Enzyme I was not necessary, although both proteins were soluble and HPr was easily released from whole cells by cold osmotic shock (16). The membrane preparations were

indeed found to have small but significant amounts of HPr and Enzyme I, which presumably could remain associated with the membrane because of the gentleness of the procedure used for preparing vesicles.

Although the phosphotransferase system provided a mechanism which accounted for the transport of certain sugars in certain bacterial species, there were known to be many sugars which were not phosphotransferase substrates, as well as amino acids and ions, whose transport must be catalyzed by a different mechanism. Moreover, the transport of at least some of these non-phosphotransferase substrates was known to be inhibited by inhibitors of respiration and/or uncouplers (1,2), which did not inhibit the phosphotransferase system.

Membrane vesicles were used to study proline transport in E. coli W6, a proline auxotroph (32). Kaback and Milner (34) reported that transport of proline was 20-30 fold stimulated by the presence of D-lactate. Succinate stimulated transport 8-10 fold and L-lactate, DL-αhydroxybutyrate, and NADH stimulated 3-4 fold. D-lactate or succinate was stoichiometrically converted to pyruvate or fumarate, respectively, by the vesicles. D-lactate was also found to stimulate the uptake of a variety of other amino acids. Most of the amino acids could be recovered unchanged. Proline transport was not stimulated by NAD^+. It was resistant to arsenate and unaffected by ATP or PEP.

E. coli ML308-225 membrane vesicles transported β-galactosides with initial rates which were stimulated 19-fold in the presence of D-lactate (35). No phosphorylated lactose was detected. Lactose

uptake was inducible. Membrane preparations from E. coli GN-2, an Enzyme I deficient mutant, were able to concentrate the non-metabolizable lactose analogue TMG in the presence of D-lactate. Initial rates of β-galactoside uptake increased with temperature up to 53° C, but the optimum temperature for steady state accumulation was at 18° C. The temperature dependence of D-lactate dehydrogenase activity corresponded almost identically to that of initial rates of uptake (36). Anaerobiosis, azide, amytal, cyanide, antimycin A, and HOQNO blocked transport, as did uncouplers and valinomycin. Addition of dinitrophenol caused loss of accumulated substrate. Uptake was sensitive to PCMB and NEM, but not to arsenate and oligomycin. The inhibition by PCMB was reversed by DTT, while that caused by NEM was unaffected. PCMB also inhibited efflux, exchange of external and intramembranal lactose, and the dinitrophenol-induced efflux. All these effects were reversed by DTT (36).

Determinations of the rates of oxygen uptake by membrane vesicles in the presence of substrates which stimulated transport revealed no correlation between the effectiveness of a compound as a respiratory substrate (succinate>D-lactate>NADH) and the ability of that compound to stimulate transport (D-lactate>succinate>NADH) (37). Absorption spectra indicated that D-lactate, NADH, and succinate donated electrons to the same cytochrome system in vesicles. This was interpreted by Barnes and Kaback (37) to mean that the coupling of transport to the dehydrogenase must be prior to the entry of electrons into the cytochrome system.

A model was suggested by Barnes and Kaback to account for the observed characteristics of lactose transport in E. coli. They

proposed that the membrane carrier proteins were electron transfer intermediates "localized" between the primary dehydrogenase and cytochrome b_1. A cycle of reduction and oxidation was postulated to account for reversible conformational changes in the carrier, leading to a decreased affinity of the carrier for the substrate when it was on the inside of the membrane and increased affinity when the carrier was on the outside (36, 37).

Lombardi and Kaback (38) further proposed that in the oxidation-reduction model for transport, the oxidized form of the carrier has a high affinity for the substrate, and the reduction of the carrier causes a conformational change resulting in translocation of the substrate to the inside of the membrane and a decrease in the affinity of the carrier for substrate. Reoxidation of the carrier by the cytochrome chain then completes the cycle.

HOQNO (inhibition site-cytochrome b_1), anaerobiosis, cyanide (inhibition site-cytochrome a_2), and, to a small extent, amytal caused efflux of accumulated substrate. PCMB and oxamate did not. Since only inhibitors which functioned after the proposed coupling site allowed efflux, Kaback and Barnes concluded that the same coupling site was responsible for efflux (36).

Konings and Freese (39) reported that the addition of the artificial electron donor system, ascorbate-PMS, markedly stimulated NADH dehydrogenase-coupled L-serine transport in Bacillus subtilis vesicles. Konings et al. (40) found that the addition of ascorbate-PMS in an atmosphere of pure oxygen caused a stimulation of lactose transport in E. coli vesicles that was six-fold greater than the stimulation seen with D-lactate. PMS alone slightly inhibited

D-lactate or succinate dependent transport but stimulated NADH dependent transport to the extent that it equaled the stimulation observed in the presence of D-lactate. The stimulation by NADH-PMS was inhibited by amytal. Ascorbate-PMS stimulation was inhibited by all the same inhibitors that affected D-lactate stimulation, except oxamate.

Mutants of E. coli and S. typhimurium which lacked D-lactic dehydrogenase activity were isolated (41). These mutants, E. coli dld mutants and S. typhimurium LT-2, had wild type ability to transport glutamate, proline, and tyrosine in whole cells. Membrane vesicles of these cells were markedly defective in D-lactate dependent transport; however, succinate-dependent transport was enhanced to almost the level of D-lactate dependent transport in wild type vesicles. Succinate oxidation was not increased and ascorbate-PMS stimulation was unaffected in the mutant vesicles. Apparently, the absence of a defect in transport in whole cells was due to a compensatory increase in the coupling of succinate dehydrogenase to transport.

Another class of mutants was isolated which was unable to grow on D-lactate, succinate, fumarate, malate, or D-ribose, but had normal D-lactic dehydrogenase activity (41). These mutants, called electron transfer coupling mutants, etc, had normal levels of ATPase, although they resembled some ATPase defective mutants phenotypically. Whole cells of etc mutants were defective in respiration-coupled transport while retaining glucose PEP:phosphotransferase activity. All respiration-coupled transport was defective, although oxidation of D-lactate and other substrates supporting transport was unaffected.

In order to explain the defect observed in etc mutants, Hong and Kaback (41) proposed a modification of the earlier model by Barnes and Kaback. Rather than being integral parts of the electron transport chain, it was suggested that the carriers for transport were components of shunts off the main portion of the respiratory chain. Therefore, oxidation of D-lactate or succinate could occur in the absence of active transport, as in etc mutants or in the presence of uncouplers. The component which was defective in etc mutants might serve to shunt electrons to and from the main respiratory chain to the carriers. It was suggested that the etc component might function in oxidative phosphorylation as well as active transport, since the etc mutation resulted in very low aerobic growth yields on glucose and glycerol.

Kohn and Kaback purified the D-lactate dehydrogenase from E. coli by ammonium sulfate precipitation, sodium perchlorate extraction, and DEAE-cellulose chromatography (42). It was identified as a flavoprotein and was insensitive to sulfhydryl reagents. It was completely inhibited by oxamate, oxalate, and 2-hydroxy 3-butynoate. When the enzyme-bound FAD was removed by acid ammonium sulfate or trichloroacetic acid, activity could not be restored by added FAD, nor did added FAD enhance untreated enzyme activity.

Purified D-lactic dehydrogenase was able to reconstitute D-lactic dehydrogenase activity and D-lactate dependent transport in vesicles of dld mutants of E. coli (43). This confirmed the finding of Reeves et al. (44) that guanidine-HCl extracts from wild type membrane vesicles were able to reconstitute D-lactate dependent transport in membrane vesicles from mutants defective in D-lactic

dehydrogenase. Purified enzyme inactivated by the attachment of 2-hydroxy 3-butynoate to the flavin moiety did not bind to vesicles, implicating the flavin in binding. The enzyme apparently bound to the outside surface of vesicles, as vinylglycolate, a substrate for the D-lactic dehydrogenase (45), was not taken up by reconstituted vesicles. It was interesting that the dehydrogenase could restore transport activity to vesicles when bound to the outside while it is normally present on the inside.

The substrate-specific carrier proteins associated with the respiration-coupled system have also been investigated. A membrane-bound proline binding activity has been solubilized and purified from vesicles of E. coli extracted with the non-ionic detergent Brij 36-T (46). Proline binding was inhibited by PCMB and DTT, but not by uncouplers or electron transfer inhibitors. Binding activity was also seen for serine, glycine, lysine, and tyrosine, although these did not inhibit proline binding, which is apparently proline-specific.

Most attention, however, has centered on the β-galactoside carrier protein. A membrane-associated protein essential to the transport of lactose in E. coli was discovered by Fox and Kennedy in 1965 (47). They found that NEM could block accumulation of the lactose analogue ONPG, presumably by affecting the lactose carrier, without affecting other enzymes of lactose metabolism. Addition of the lactose analogue TDG prevented inactivation, indicating that the site of NEM sensitivity had a high affinity for TDG. Fox and Kennedy added unlabeled NEM to cells in the presence of TDG to react with proteins other than the lactose carrier. Then the cold NEM and TDG were removed and radioactive NEM was added. Separate preparations

of induced or uninduced cells were labeled with ^{3}H- or ^{14}C-NEM, respectively. It was found that an inducible, NEM-sensitive, TDG-binding protein essential for transport of lactose was in the particulate fraction of the cell. It was called the M (for membrane) protein. Studies of mutants by Fox et al. (48) led to the conclusion that the y gene of the lactose operon was the structural gene for the M protein.

The M protein could only be extracted from the membrane by detergents. Particulate protein labeled with radioactive NEM was extracted with SDS and fractionated on a Sephadex G150 column (49). The molecular weight of the M protein was determined to be 29,000.

When substrates of the lactose transport system were tested for ability to protect against inactivation by NEM, it was found that two classes of substrate existed, only one of which protected against inactiv ation by NEM (50). The explanation proposed for this observation was that the M protein had two binding sites (I & II) for sugars it transports, only one of which bound NEM (II). Direct tests of binding to the M protein showed that TDG and melibiose had a high affinity for site II, the NEM-binding site (51). It was also found that α-galactosides had a higher affinity for site II than β-galactosides.

Further confirmation of the role of the M protein in lactose transport in E. coli was provided by studies using the fluorescent β-galactoside analogue, 2-(N-dansyl)-aminoethylβ-D-thio-galactoside (dansyl-galactoside). This compound was not transported by vesicles, but it competitively inhibited lactose uptake. Reeves et al. (52) showed that dansyl-galactoside bound the β-galactoside carrier and

partially protected it against inactivation by NEM. These effects were not seen in vesicles lacking the β-galactoside transport system. Fluorescence changes induced by addition of D-lactate were reversed by substrates which bound the M protein. The number of binding sites determined for dansyl-galactoside agreed with the amount of M protein determined by Jones and Kennedy (49). These results indicated that the M protein described by Fox and Kennedy and the β-galactoside carrier protein for respiration-coupled transport in E. coli were the same protein.

Many other respiration-coupled systems have now been identified in a variety of organisms. Kerwar et al. (53) used membrane vesicles to study one of the systems for galactose transport in E. coli which was also stimulated by ascorbate-PMS or D-lactate. Amino acid transport in S. aureus vesicles was found to be coupled to respiration via an L-α-glycerol phosphate dehydrogenase. Uptake was stimulated by addition of L-α-glycerol phosphate or ascorbate-PMS (54,55). Konings and Freese (56) described a respiration-coupled L-amino acid uptake system in B. subtilis, stimulated optimally by L-α-glycerol phosphate, NADH, and reduced phenazine methosulfate. Lo, Rayman, and Sanwal showed catabolite repressible succinate transport in E. coli (57,58). A mutant lacking succinate dehydrogenase and fumarate reductase required the addition of D-lactate or ascorbate-PMS to membrane vesicles to show succinate transport. The respiration-coupled transport of D-lactate, L-lactate, and succinate was studied in E. coli, B. subtilis, and a Pseudomonas species by Matin and Konings (59). At the concentrations studied, no substrate was able to energize its own transport in membrane vesicles; the addition of ascorbate-PMS,

D-lactate, L-lactate, succinate, or NADH was necessary. Respiration-coupled transport of glucose in Azotobacter vinelandii (60) and of gluconate in Pseudomonas aeruginosa (61) were coupled to the oxidation of L-malate by an FAD-linked L-malate dehydrogenase.

While respiration-coupled transport of lactose in E. coli membrane vesicles was always observed to be inhibited by anoxia, anaerobic lactose transport did occur in whole cells. Vesicles prepared from cells induced for α-glycerol phosphate dehydrogenase and fumarate reductase were found to transport lactose under anaerobic conditions in the presence of DL-α-glycerol phosphate and fumarate. Anaerobic transport could also be coupled to formate dehydrogenase and nitrate reductase in the presence of formate and nitrate. Membrane vesicles for the studies of anaerobic transport were prepared by a revised, milder procedure. Loss of activity by excessive washing was attributed to the release of some loosely associated components (perhaps one or more of the required enzymes) and was thought to explain the failure to find anaerobic transport in vesicles prepared by the usual procedure (62).

There has been extensive controversy over the models proposed by Kaback and his colleagues to account for the data they have obtained from membrane vesicle studies. A rapidly expanding group in the field of bacterial transport invokes the chemiosmotic hypothesis of the type proposed by Mitchell (63) to account for the data known about energization of respiration-coupled (or ATP-driven anaerobic) active transport. Mitchell suggested that proton extrusion, caused by oxidative phosphorylation during aerobic growth or by a membrane-bound ATPase during anaerobic growth, results in a membrane potential

and a pH gradient across the bacterial membrane. The production of pH and/or charge gradients would require that the membrane be impermeable to protons and the relevant ions, that the membrane system be closed (as it is in vesicles or cells), and that various components of the electron transport chain be specifically localized within the membrane. The "proton motive force" was postulated to drive the uphill transport of a substrate by coupling the inward flow of protons with that of substrate, i.e., proton symport. The proton motive force (Δp) was defined as being the electrical potential across the membrane ($\Delta\psi$) plus the potential generated by the pH gradient (ΔpH), giving $\Delta p = \Delta\psi - 59\Delta pH$, at 25° C. Harold and his co-workers (64-67) have proposed that glucose fermentation in the anaerobe Streptococcus faecalis generates a membrane potential of 150-200 mV and a pH gradient, which was calculated to be sufficient to drive sugar transport. The loss of active transport of many substrates on addition of uncoupling agents could be due to the collapse of this gradient caused by the ability of uncouplers to render the membrane permeable to protons.

Studies have been conducted, in many laboratories on numerous strains of a variety of organisms, which lend support to the occurence of an electrical and pH gradient in aerobic and anaerobic cells as well as in membrane vesicles. The role of this gradient in active transport has been more difficult to establish. However, the available data strongly suggest that respiration-coupled transport and ATP-dependent transport in anaerobic cells depend on the formation of such a gradient.

E. coli, and other bacteria, can use oxidative processes to support transport without participation of ATP as has been shown in whole cells and membrane vesicles in many laboratories. Klein

and Boyer (68) showed that under anaerobic conditions whole cells were able to transport proline. When whole cells were incubated with high levels of arsenate and low levels of inorganic phosphate to reduce the ATP and PEP levels in the cells, aerobic transport proceeded normally, presumably supported by energy derived from oxidation, but anaerobic transport was eliminated. Both sources of membrane energization were sensitive to uncouplers as would be expected if they produced a common driving force for transport.

Prezioso *et al.* (69) studied a mutant of *E. coli* K12 deficient in Ca^{+2}, Mg^{+2}-ATPase activity, AN120 (*uncA*). Transport, in vesicles, of several respiration-coupled substrates in the presence and absence of D-lactate or ascorbate-PMS was comparable to that observed in vesicles of the parent strain. The same results were shown in whole cells. These results were consistent with the idea that transport was driven by an energized state whose energy could be derived from oxidation and would not involve ATP directly except when, during anaerobiosis, ATP was required to generate the energized state. Conditions which inhibited the ATPase, and an ATPase inhibitor, DCCD, also failed to inhibit aerobic transport in the parent strain.

Schairer and Haddock (70) isolated an ATPase deficient mutant of *E. coli* K12, A103c. Wild type and mutant whole cells showed comparable rates of TMG accumulation. However, when respiration was inhibited by KCN, wild type rates of uptake were reduced only 35% and uptake by the mutant strain was abolished. These results, and similar results of Yamamoto *et al.* (71), were consistent with the role of the ATPase in supporting transport when respiration is unable to do so.

Consistent with the results with ATPase negative mutants, were those of Singh and Bragg (72) who described a mutant of E. coli K12, SASX76, which required the addition of Δ-aminolevulinic acid (ALA) in order to form cytochromes. The uptake of phenylalanine in cytochrome-deficient cells was absent whether or not D-lactate was added. However, glucose was able to drive phenylalanine transport, indicating that ATP from glycolysis could support active transport in the absence of an oxidative source of energy.

Simoni and Shallenberger (73) had, at this time, isolated several mutants defective in aerobic metabolism. DL-54, which was deficient in aerobic transport in whole cells and vesicles, was found to be ATPase-negative. Simoni and Shallenberger suggested that the Ca^{+2}, Mg^{+2}-ATPase might somehow be involved in the coupling of energy derived from respiration to transport. However, Bragg and Hou (74) found that DL-54 had a phenotype that resembled that of membrane particles from which the ATPase had been removed. Such removal resulted in the loss of aerobic transhydrogenase activity and aerobic active transport of amino acids. Addition of a preparation of ATPase or DCCD restored these activities. Bragg and Hou proposed that the DL-54 modified ATPase might be loosely bound to the cell membrane and that the ATPase performs a structural role in stabilizing the high energy state (perhaps by preventing proton leakage) in the membrane. The etc mutants of Hong and Kaback (41), described earlier, may similarly be mutants whose ATPase, albeit active, is bound loosely or "incorrectly" to the membrane.

Another ATPase negative strain of E. coli K12, NR 70, was similar to DL-54 and had lost the ability to transport substrates

aerobically as well as anaerobically (75,76). The addition of the ATPase inhibitor, DCCD, allowed restoration of respiration-coupled uptake. It was found that NR 70 was defective, compared to wild type, in maintaining a functional proton gradient. It was further shown that DCCD increased the membrane resistance to the passive flow of protons. The fact that it simultaneously restored active transport, further implicated a proton gradient in membrane energization for transport. It was suggested, in line with the earlier suggestion of Bragg and Hou (74), that the ATPase was not obligatory for aerobic transport by virtue of its catalytic activity, but due to its structural role in the membrane.

West and Mitchell (77) measured the initial rate of lactose inflow into non-metabolizing cells of E. coli and the initial rate of effective inflow of protons. It was found that the rates of these two processes were the same, indicating a 1:1 coupling of their flow as expected by the chemiosmotic hypothesis. The presence of the uncoupler FCCP greatly increased the difference in translocation of protons and lactose, presumably by allowing protons to leak out through the cell membrane.

Two mutants of E. coli, ML and X71-54, were isolated and shown to have an increased ability to catalyze faciliated diffusion of β-galactosides accompanied by a decreased ability to accumulate β-galactosides against a concentration gradient. West and Wilson (78) proposed that the defect in these mutants lies in the mechanism of coupling of energy to active transport. Increased efflux of TMG was also demonstrated. The defect in X71-54 was mapped in the y gene of the lac operon, which codes for the lactose carrier, M protein. The

mutant carrier might be able to translocate lactose, but be unable to couple it to inflow of protons. In fact, the two mutants showed proton inflow which was considerably slower than β-galactoside inflow.

Streptococcus lactis has no oxidative metabolism and requires the addition of metabolic substrates to demonstrate active transport. Kashket and Wilson (79) found that addition of valinomycin to these cells caused them to accumulate TMG to 14 times the concentration in the medium and then slowly lose it over twenty minutes until the internal and external concentrations were equilibrated. When the external concentration of K^+ was increased, the extent of TMG uptake was decreased until no effect of valinomycin was seen at all. It was proposed that the slow loss of TMG after its accumulation must be due to the cessation of K^+ efflux. When cells were washed and resuspended in K^+-free medium, efflux of K^+ was reestablished and a second burst of TMG accumulation occurred. FCCP or tetrachlorosalycilanilide (TCS) did not cause accumulation and prevented valinomycin-induced accumulation. DCCD inhibited glucose supported TMG transport but not the valinomycin-induced accumulation. The authors concluded that the efflux of K^+ caused the entry of protons with TMG on the sugar transport carrier. Similar results for amino acid transport in S. faecalis were reported by Asghar et al. (80). Kashket and Wilson (81) were also able to induce TMG uptake in S. lactis by suddenly exposing the cells to a medium at pH 6. The pH gradient imposed was able to cause a TMG accumulation to a 20-fold higher concentration than was found in the medium. Adding TMG to a lightly buffered medium caused outward proton flow. Addition of valinomycin alone also caused alkalinization of the medium. Measurement of K^+ efflux and H^+ influx during valino-

mycin-induced TMG accumulation and accumulation of ^{14}C-methylamine to determine ΔpH were conducted in these cells. The proton motive force was calculated from these measurements, and it was found that there was a direct relationship between the proton motive force and TMG accumulation.

Thus far in this review, no mention has been made of the periplasmic binding proteins thought to be involved in the transport of some compounds. When certain Gram-negative cells were treated with EDTA and subjected to cold water shock (82) there was release of periplasmic proteins---proteins thought to be localized between the cell membrane and the cell wall. E. coli cells treated by this osmotic shock procedure were studied to determine its effect (83,84), and it was found that osmotically shocked cells were no longer able to actively transport galactose or leucine. It was also noted that specific binding proteins for galactose and leucine were released into the shock fluid during this procedure (85,86). The shock fluid was able to restore active transport to shocked cells.

The galactose binding protein was purified and characterized. It had a molecular weight of about 36,000. It bound one molecule of galactose per molecule of binding protein, and it was insensitive to sulfhydryl reagents (86-88). Boos and his co-workers presented evidence for the involvement of the galactose binding protein in active transport (89-92). Boos et al. (93) proposed that a substrate-induced conformational change of the binding protein was necessary for the in vivo functioning of the galactose transport system. Kinetic studies of the entry and exit of galactose by Parnes and Boos (94) led them to propose that the periplasmic galactose binding protein was involved

only in the entry process for galactose, not in exit, and that energy was coupled to the entry of this sugar.

An interesting study was done by Berger (95) in which the sources of energy for proline and glutamine uptake in starved cells were compared. While glutamine uptake was dependent on a periplasmic binding protein (96,97), proline transport was mediated by a tightly membrane-bound binding protein (46). Berger reported that proline transport could be driven by energy from either electron transport or glycolysis. The oxidative sources of energy were resistant to arsenate and did not require ATPase activity, but were sensitive to cyanide and uncouplers. Glycolytic energy was resistant to cyanide but was abolished by arsenate and uncouplers, and required an active ATPase. Glutamine transport, on the other hand, was proposed to be driven directly by phosphate-bond energy from oxidative or substrate-level phosphorylation. When energy was supplied by the oxidative pathway, it was sensitive to cyanide and uncouplers, and, unlike for proline transport, an ATPase was required. Glycolytically derived energy did not require an ATPase and was insensitive to cyanide and uncouplers. Both pathways were inhibited by the presence of arsenate. Berger also reported that preliminary evidence indicated that other shock-releasable systems gave results like those for glutamine transport. He proposed that the differences in the binding of these proteins to the membrane might also be reflected in fundamentally different mechanisms of energy-coupling. As yet, however, it is impossible to present a coherent model for the energization and mechanism of those transport systems involving the periplasmic binding protein.

Against the background of the above studies of transport in other

laboratories, Arthrobacter pyridinolis was chosen for study because various properties of the organism and related species suggested that novel regulatory phenomena involving both transport and metabolism might be found (98-100). A. pyridinolis was capable of growth on a wide variety of organic and amino acids, but it grew on only two sugars, D-fructose and L-rhamnose, as sole carbon source. It was unable to grow on glucose or α-glucosides as sole carbon source, although the necessary catabolic enzymes for growth on these substrates were present. This crypticity towards glucose was abolished by addition to the medium of malate or compounds which could be converted to malate. It was found that addition of malate was necessary for the operation of a transport system for glucose (101). The data indicated that glucose was transported by a respiration-coupled system that was coupled to malate oxidation, similar to the glucose transport system found in Azotobacter vinelandii (60). Owing to a metabolic peculiarity of the organism (99), A. pyridinolis was unable to accumulate significant levels of 4-carbon compounds, such as malate, during metabolism. This accounted for the requirement for added malate in the growth medium to allow transport, and hence growth on, glucose, maltose, or sucrose.

Since A. pyridinolis could grow on D-fructose as sole carbon source, it was suspected that fructose could be transported by a system which differed from that for glucose and was not dependent upon malate. Indeed, a phosphoenolpyruvate:D-fructose phosphotransferase system was found, and shown to be involved in transport and metabolism of this sugar in A. pyridinolis. Further description of this transport system was made possible by studies of mutants

deficient in fructose phosphotransferase activity. It was found that the system consisted of three soluble components, one of which was inducible, and one membrane-bound component, which was inducible (102,103). Membrane vesicles prepared from D-fructose-grown cells were able to take up fructose in the presence of PEP with a K_m for fructose of 1.5×10^{-5} M (103). The D-fructose phosphotransferase system of A. pyridinolis most resembled that of Staphylococcus aureus (18-20). By analogy, A. pyridinolis contained a soluble Enzyme I, a soluble phosphocarrier protein, an inducible, soluble Factor III, and an inducible, membrane-bound Enzyme II.

In the course of studying mutants which were deficient in phosphotransferase activity, it was observed that mutants that could not grow on fructose alone were able to grow on malate plus fructose to a much higher density than could be accounted for by growth on the malate alone. It was found that in the presence of malate, wild type cells could transport fructose by a respiration-coupled system, such as was previously found for glucose, as well as by the phosphotransferase system (104). Studies of the respiration-coupled system in membrane vesicles showed that malate stimulated fructose transport to 65% of the level of stimulation caused by PEP.

The respiration-coupled system for fructose transport exhibited a K_m for fructose of 5.6×10^{-7} M in membrane vesicles. The effects of inhibitors on both PEP- and malate-dependent transport were studied. Respiration-coupled transport was significantly inhibited by CCCP, DNP, HOQNO, KCN, and NEM. PEP-dependent uptake was only slightly affected, if at all, by any of these inhibitors or uncouplers (105).

Vesicles were found to have an FAD-linked L-malic dehydrogenase activity. This activity was also detected in sonicated whole cells and was found associated with the pellet fraction after centrifugation at 100,000 x g for one hour. No NAD-linked malic dehydrogenase was found (105).

Studies of wild type and mutant strains in vesicles and whole cells showed that the respiration-coupled system required the presence of a sugar-specific component (105). This component was inducible for glucose transport but constitutively produced for fructose transport. AP4374, a mutant lacking the fructose-specific component of the respiration-coupled system was unable to grow on fructose alone or in the presence of malate. It appeared that the presence of the respiration-coupled system for fructose was necessary for the induction of Factor III and Enzyme II of the fructose phosphotransferase system (106). These findings suggested that free fructose or fructose 6-phosphate must accumulate internally for induction of the fructose phosphotransferase system and that in AP4374 this accumulation was not possible, since respiration-coupled transport of fructose did not occur.

INTRODUCTION

A. pyridinolis can use D-fructose and L-rhamnose, but not a wide variety of other carbohydrates, when they are present as sole source of carbon in the growth medium. When present as sole carbon source, D-fructose is transported by a phosphoenolpyruvate:D-fructose phosphotransferase system (102,103). Alternatively, D-fructose can be transported by a respiration-coupled system with concomitant oxidation of L-malate (104,105). Activity of the respiration-coupled transport system in whole cells as well as in membrane vesicles depends upon the presence of exogenous L-malate. D-glucose, which is transported only by the respiration-coupled system, can be used only if L-malate or some precursor thereof is present in the medium (101). The ability of A. pyridinolis to use L-rhamnose as sole carbon source, without L-malate or a precursor of L-malate in the medium, suggested that this sugar might be transported either by the phosphotransferase system, or by some mechanism other than those used for fructose or glucose. The initial reactions of the known pathway for L-rhamnose metabolism in various species of bacteria are L-rhamnose ——> L-rhamnulose ——> L-rhamnulose 1-phosphate (112, 115-118, 121, 122). While the mechanism of L-rhamnose uptake in those species was not determined, the sugar was presumed to enter the cell as free L-rhamnose. Studies were conducted in whole cells of the wild type and mutants and in membrane vesicles in order to elucidate the mechanism of transport of L-rhamnose in A. pyridinolis.

MATERIALS AND METHODS

Bacteria and growth conditions. Arthrobacter pyridinolis and

mutant strains derived from it were used for all studies. The organism was routinely maintained in PYE medium (102) and experiments were performed after growth in a defined medium, MS (99). Carbon sources were added to 0.05 M unless otherwise specified, and cells were grown at 30° C on a shaker rotating at 200 rpm. Growth experiments were performed as described by Wolfson and Krulwich (99). Mutant strains AP243, AP253, and AP100 are deficient in phosphotransferase system activity. AP243 and AP253 are each deficient in one of the two constitutive components of the phosphotransferase system. AP100 is deficient in the inducible, fructose-specific Factor III of the system (103).

Enzyme assays and uptake experiments. Phosphoenol pyruvate: L-rhamnose phosphotransferase activity was assayed by the method of Tanaka, Lerner and Lin (107). Assays of sugar uptake in membrane vesicles were performed by filtration assay as described by Wolfson et al. (103), except that phosphoenol pyruvate-dependent uptake was measured at 30° C and uptake in the presence of all other compounds was measured at 23° C.

Preparation of membrane vesicles. Vesicles were prepared and isolated by the EDTA-lysozyme procedure of Kaback (25) with modifications described by Wolfson et al. (103). The vesicle preparations were free of whole cells and unlysed spheroplasts. No detectable levels of several soluble enzymes were found.

Chemicals. The L-isomers of malate and rhamnose and the D-isomer of fructose were used in all cases. [G-^{3}H]L-rhamnose was purchased from New England Nuclear Corp., and [U-^{14}C] D-fructose and [U-^{14}C]D-glucose were purchased from Biochemical and Nuclear Corp. and

Amersham-Searle, respectively. PCMB was purchased from Calbiochem. Rotenone was obtained from Dr. Arthur Cedarbaum. CCP, HOQNO, NEM and DMSO were obtained from Sigma Chemical Co. Ethyl methane sulfonate was purchased from Eastman Chemicals, and lysozyme was obtained from Boehringer-Mannheim. All other chemicals were obtained commercially at the highest purity available.

RESULTS

A PEP:rhamnose phosphotransferase system in A. pyridinolis. Wild type A. pyridinolis was able to grow on L-rhamnose as sole carbon source. AP100, a mutant which was deficient in the fructose-specific component of the phosphotransferase system, could use L-rhamnose as well as the wild type strain (Fig. 1). Mutants which were deficient in Enzyme I (AP243) or phosphocarrier protein (AP253), the non-specific, constitutive components of the phosphotransferase system, were unable to grow on L-rhamnose alone (Fig. 1). These results strongly suggested that L-rhamnose was transported by a PEP:phosphotransferase system similar to the one shown for D-fructose in A. pyridinolis (102, 103). However, direct measurement of L-rhamnose phosphotransferase activity was difficult to demonstrate. Extracts were prepared from cells at various points in their growth, and assays were conducted over a large range of protein concentration, rhamnose concentration, and PEP concentration, in the presence and absence of 20 mM sodium fluoride, and at several different temperatures. PEP:rhamnose phosphotransferase activity was only occasionally detected at significant levels, and no correlation with the conditions employed was apparent. Repeated efforts to establish optimal conditions for growth and preparation of extracts failed to increase the probability of seeing activity.

Membrane vesicles prepared from cells grown on L-rhamnose were able to catalyze PEP-dependent uptake of L-rhamnose (Fig. 2). In the presence of 100 mM PEP and 6 μM L-rhamnose, the activity reached 9.6 pmoles/min/mg of vesicle protein. While this was a low level of activity, it confirmed the presence of a PEP:phosphotransferase system for L-rhamnose in A. pyridinolis. PEP-dependent L-rhamnose uptake was

Figure 1. Growth of wild type and phosphotransferase-negative mutants of A. pyridinolis on L-rhamnose. Growth of the strains indicated on 0.05 M L-rhamnose was followed.

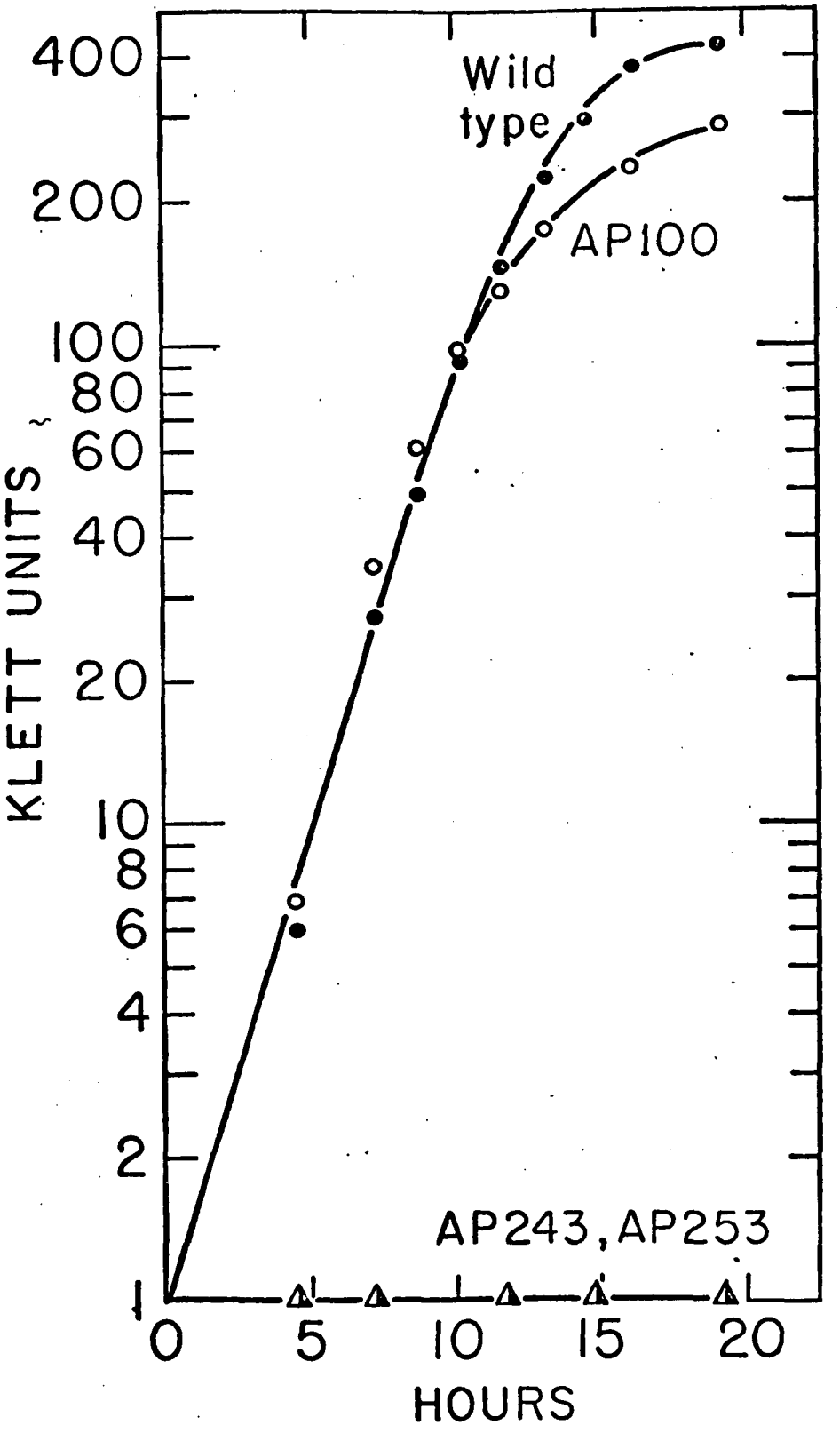
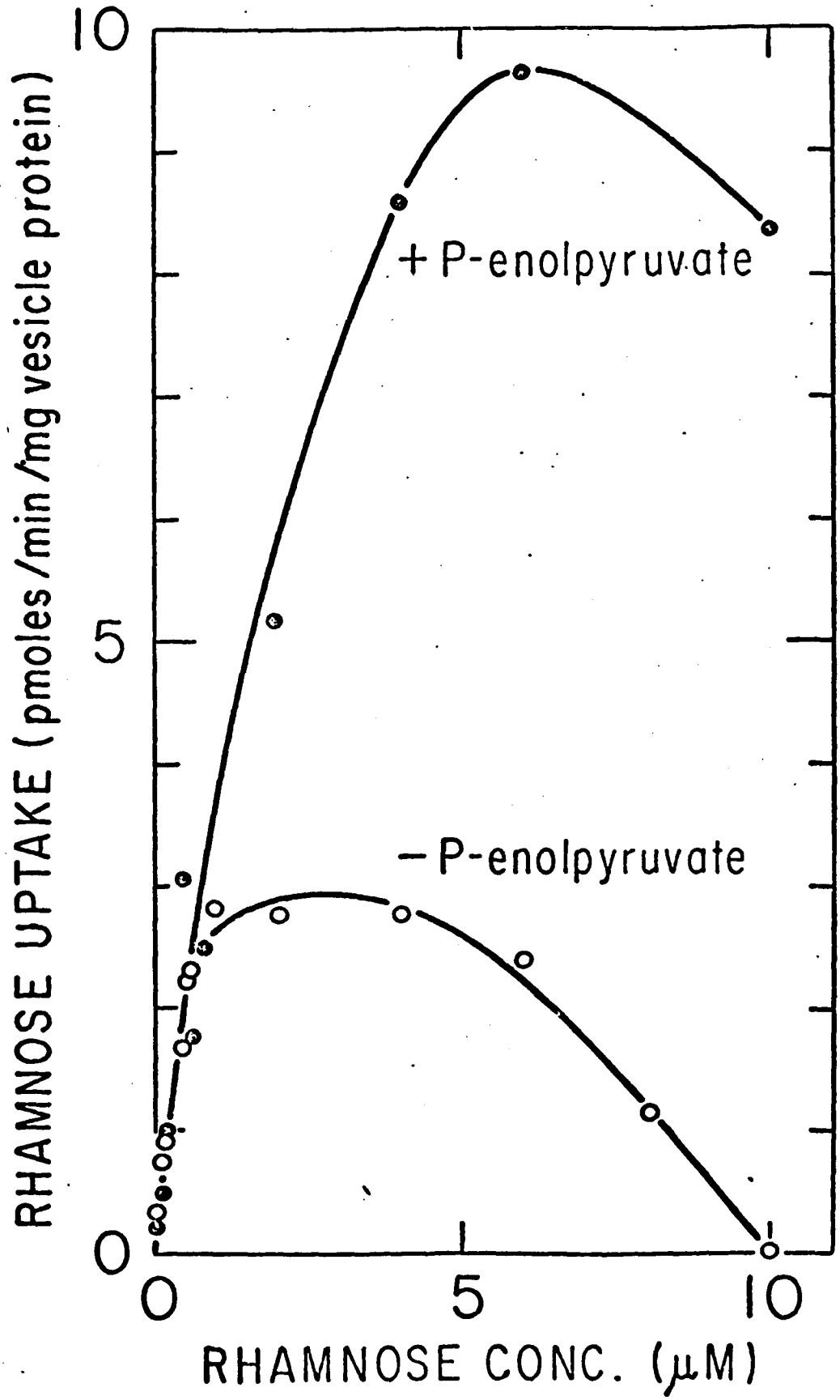

400
200
100
80
60
40
20
10
8
6
4
2
1
KLETT UNITS
Wild type
AP100
AP243, AP253
0
5
10
15
20
HOURS

Figure 2. Phospho-enol pyruvate-dependent uptake of L-rhamnose by membrane vesicles as a function of L-rhamnose concentration. Vesicles (0.03 mg) were incubated in a standard reaction mixture (103) either with or without 100 mM phosphoenol pyruvate. The indicated concentrations of radioactive rhamnose were added, and the incubations were terminated after 10 minutes.


10
5
0
0
5
10
RHAMNOSE UPTAKE (pmoles/min/mg vesicle protein)
RHAMNOSE CONC. (μM)
+ P-enolpyruvate
– P-enolpyruvate

negligible at 23° C; optimal activity was detected from 30° C to 40° C.

Respiration-coupled transport of L-rhamnose in A. pyridinolis. When the phosphotransferase-negative mutant AP243 was grown in medium containing 5 mM malate, as well as 50 mM L-rhamnose, it was able to grow to a much greater density than could be accounted for by its grown on 5 mM malate alone (Fig. 3). It seemed possible, therefore, that, like D-fructose, L-rhamnose could be transported by a respiration-coupled system, as well as a phosphotransferase system in A. pyridinolis. Indeed, malate-dependent uptake of L-rhamnose could be demonstrated in vesicles prepared from L-rhamnose-grown cells. The effect of various carbon sources on L-rhamnose uptake is shown in Table I. Optimal stimulation, about five-fold, was observed with either L-malate or succinate. Other carbon sources gave little or no stimulation of L-rhamnose uptake. Addition of FAD did not enhance the stimulation seen on addition of either malate or succinate.

The effect of temperature on rhamnose uptake by vesicles was determined in the presence of 4 mM malate. The optimal temperature for initial rates of uptake was approximately 23° C (Fig. 4). Subsequent experiments, therefore, were conducted at this temperature.

The effects of varying concentrations of malate and succinate were found to be almost identical. Saturation kinetics were observed with both, as illustrated in Fig. 5. The concentration of L-rhamnose was then varied in the presence of 4 mM malate. Michaelis-Menten kinetics were observed, and a K_m of 2.9×10^{-6} M was calculated for L-rhamnose (Fig. 6).

Inhibitor studies of both systems in vesicles revealed that the respiration-coupled system was inhibited by inhibitors of respiration

Figure 3. Growth of a phosphotransferase-negative strain of A. pyridinolis on rhamnose in the presence of malate. Cells of strain AP 243 were grown on 50 mM rhamnose (Δ——Δ), 5 mM malate (●——●), and 50 mM rhamnose plus 5 mM malate (○——○).

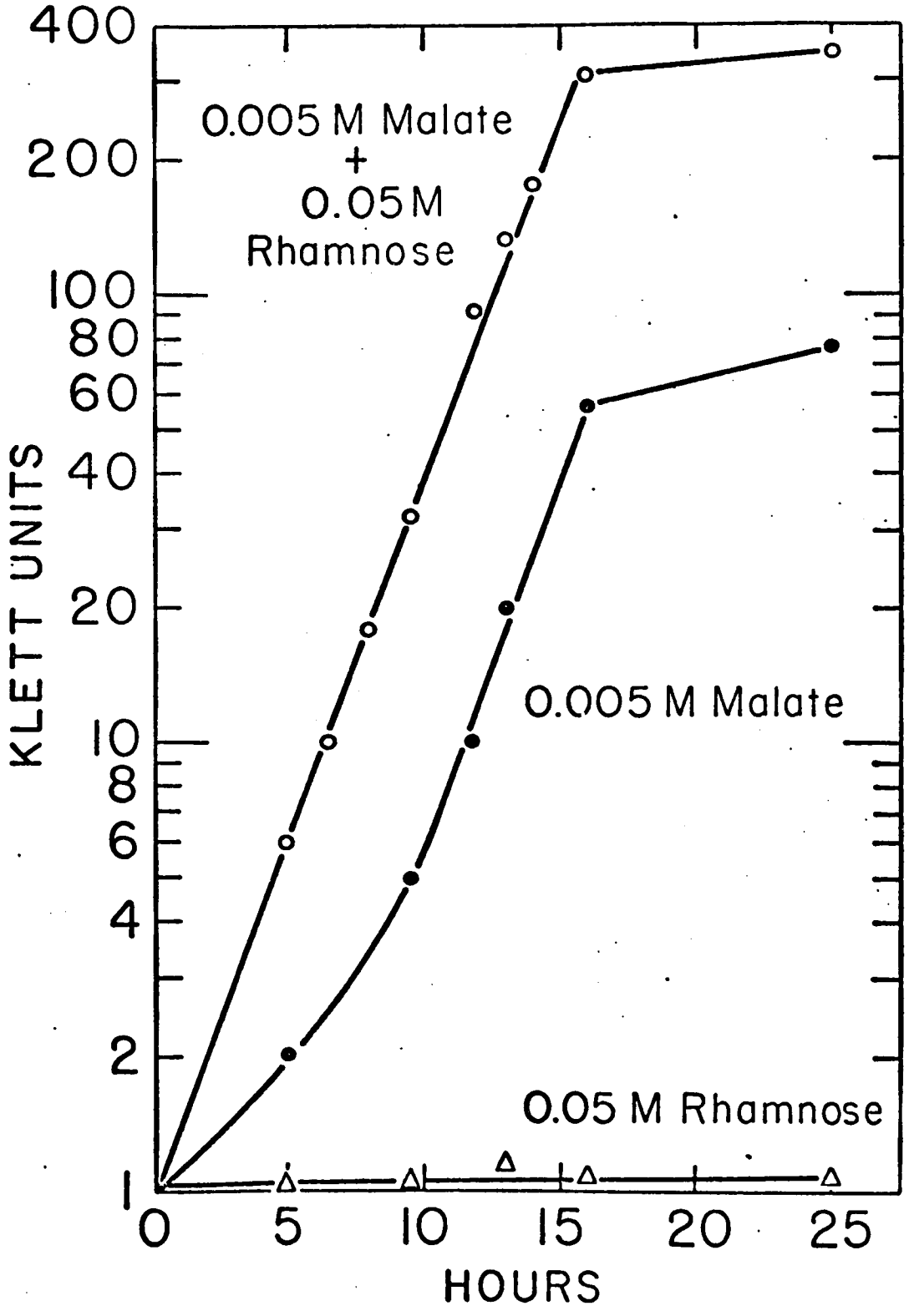

400
200
100
80
60
40
20
10
8
6
4
2
1
KLETT UNITS
0.005 M Malate
+
0.05 M
Rhamnose
0.005 M Malate
0.05 M Rhamnose
0
5
10
15
20
25
HOURS

TABLE I

UPTAKE OF RHAMNOSE BY VESICLES IN THE PRESENCE OF VARIOUS ADDITIONAL COMPOUNDS[a]

Addition	Rhamnose Uptake pmoles/ min/mg protein	Addition	Rhamnose Uptake pmoles/ min/mg protein
None	4.15	Malate	19.21
ADP	5.34	Malate + FAD	13.10
ATP	5.26	Succinate	21.94
FAD	6.62	Succinate + FAD	17.18
NADH	6.07	Fumarate	8.93
NADP	6.69	Cis-Aconitate	5.56
Citrate	7.37	Oxaloacetate	7.14
Isocitrate	5.98	3-Phosphoglycerate	6.07
D- actate	6.52	α-Glycerophosphate	4.87
Pyruvate	5.73	α-Hydroxybutyrate	3.68

[a]Vesicles (0.03 mg) were incubated at 26 C in a standard reaction mixture (103) with a final volume of 100 μl containing the additions indicated. FAD, when present, was at a final concentration of 50 μM; all other additions were at a final concentration of 5 mM. After 10 minutes of incubation, radioactive rhamnose was added to a final concentration of 2 M. The reactions were terminated 10 minutes after the addition of rhamnose.

Figure 4. The effect of temperature on malate-dependent rhamnose uptake by vesicles. Vesicles were incubated for 10 minutes at the temperature indicated in a standard reaction mixture (103) containing 4 mM malate. Radioactive rhamnose (2 μM) was added and incubation was continued at the same temperature for an additional 10 minutes before the reactions were terminated.

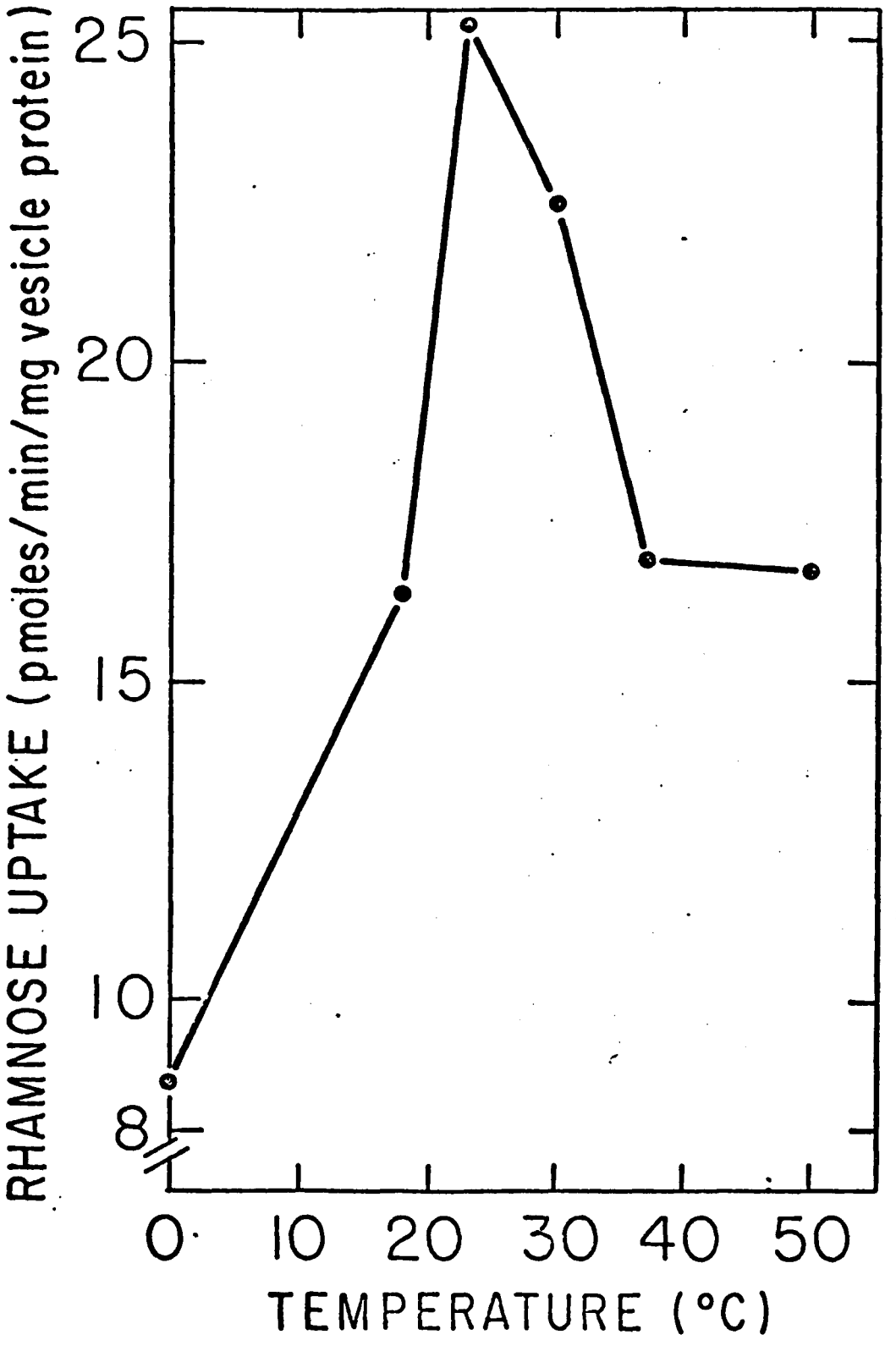

RHAMNOSE UPTAKE (pmoles/min/mg vesicle protein)
25
20
15
10
8
0
10
20
30
40
50
TEMPERATURE (°C)

Figure 5. The effect of malate or succinate concentration on rhamnose uptake by vesicles. Vesicles were incubated for 10 minutes at 23^0 C in the presence of the indicated concentrations of malate (o——o) or succinate (•——•). Radioactive rhamnose (2 μM) was added and the reactions were incubated for 10 minutes before being terminated.

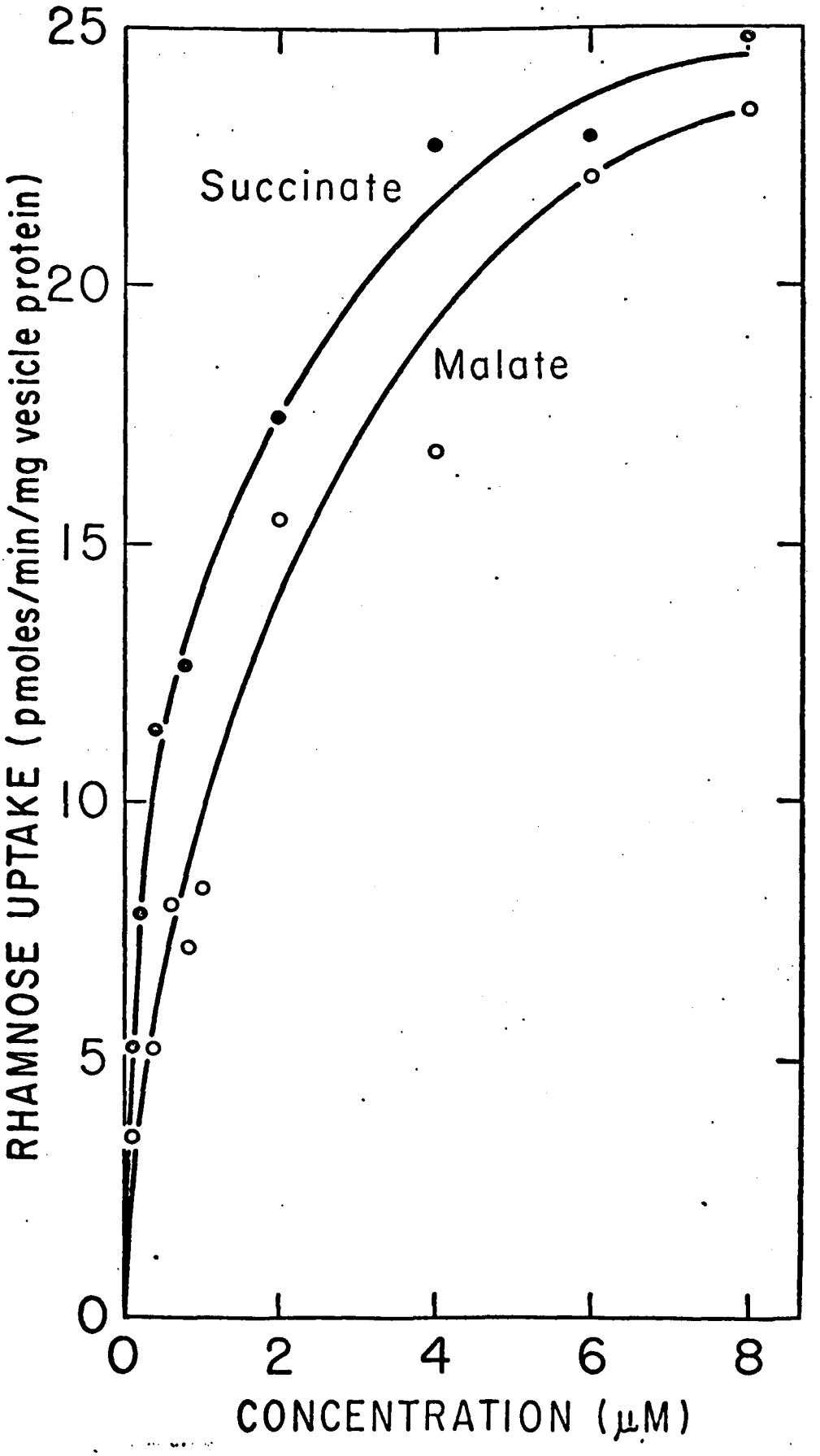
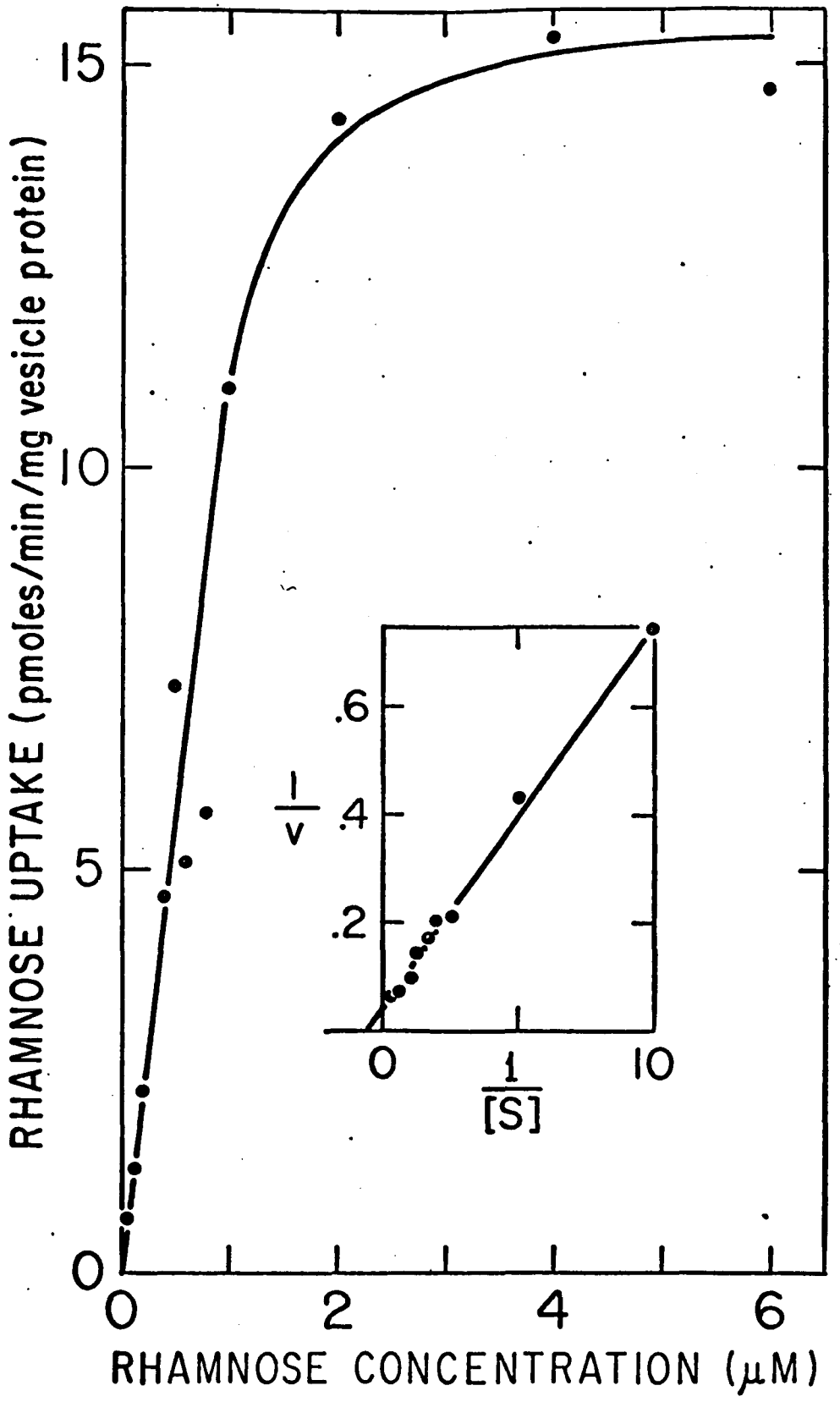

25
20
15
10
5
0
Succinate
Malate
RHAMNOSE UPTAKE (pmoles/min/mg vesicle protein)
0
2
4
6
8
CONCENTRATION (μM)

Figure 6. The effect of rhamnose concentration on the rate of malate-dependent rhamnose uptake by vesicles. Radioactive rhamnose, at the final concentrations indicated was added to vesicles that had been incubated for 10 minutes at 23^0 C in the presence of 4 mM malate. The reactions were terminated after an additional 10 minutes of incubation. Inset: a Lineweaver-Burk plot.


RHAMNOSE UPTAKE (pmoles/min/mg vesicle protein)
15
10
5
0
0
2
4
6
RHAMNOSE CONCENTRATION (μM)
1/v
.6
.4
.2
0
1/[S]
10

(rotenone, KCN, and HOQNO), uncoupling agents (DNP and CCP), and sulfhydryl reagents (NEM and PCMB). CCP, DNP, and HOQNO caused no inhibition of PEP-dependent uptake (TABLE II).

Previous studies of the respiration-coupled transport of D-fructose indicated that a sugar-specific component was involved in this transport system; the D-fructose specific component was thought to be constitutive, while those for rhamnose and glucose were inducible (105). This was confirmed in vesicles from rhamnose-grown cells by the finding that no malate-dependent glucose uptake was detectable, but malate-dependent fructose uptake was almost as great as rhamnose uptake.

DISCUSSION

The presence of a PEP:phosphotransferase system for L-rhamnose in A. pyridinolis was established by studies of mutants of the phosphotransferase system and PEP-dependent uptake in membrane vesicles from rhamnose-grown cells. These findings made it possible to explain the ability of A. pyridinolis to grow on L-rhamnose as sole carbon source: The only other sugar A. pyridinolis was able to utilize as sole carbon source, D-fructose, was also transported by the PEP: phosphotransferase system (102). Direct determination of PEP:rhamnose phosphotransferase activity by assaying cell extracts failed to produce consistent detectable levels of this activity. This could be due to the presence of an inhibitory protein in crude extracts which was lost during preparation of membrane vesicles, allowing detection of PEP-dependent uptake of rhamnose in vesicles. An alternate or additional explanation for which some evidence has been found (see section II), is that a phosphatase is active in crude extracts, cleaving the rhamnose

TABLE II

THE EFFECT OF INHIBITORS ON MALATE- AND P-ENOLPYRUVATE- DEPENDENT RHAMNOSE UPTAKE BY VESICLES[a]

Inhibitor	Concentration	%Malate- or P-enolpyruvate-Dependent Uptake	
		Malate	P-enolpyruvate
None	-	100	100
CCP[b]	1 μM	8.0	110
DNP	10 μM	22.3	165
HQNO[b]	10 μM	64.5	-
	100 μM	0	155
KCN	10 mM	11.1	-
NEM	1 mM	35.3	-
PCMB	10 μM	18.6	-
Rotenone	100 μM	5.0	-

[a]Incubations were carried out as described in the legend to Table I with the inhibitors, at the concentrations indicated, present during the 10 minutes prior to the addition of radioactive rhamnose. Malate was present at 4 mM, P-enolpyruvate at 100 mM, and rhamnose at 2 μM.

[b]CCP and HQNO were present as solutions in dimethylsulfoxide. Control incubations showed that this solvent caused no effect on rhamnose uptake at the level used (1% dimethylsulfoxide).

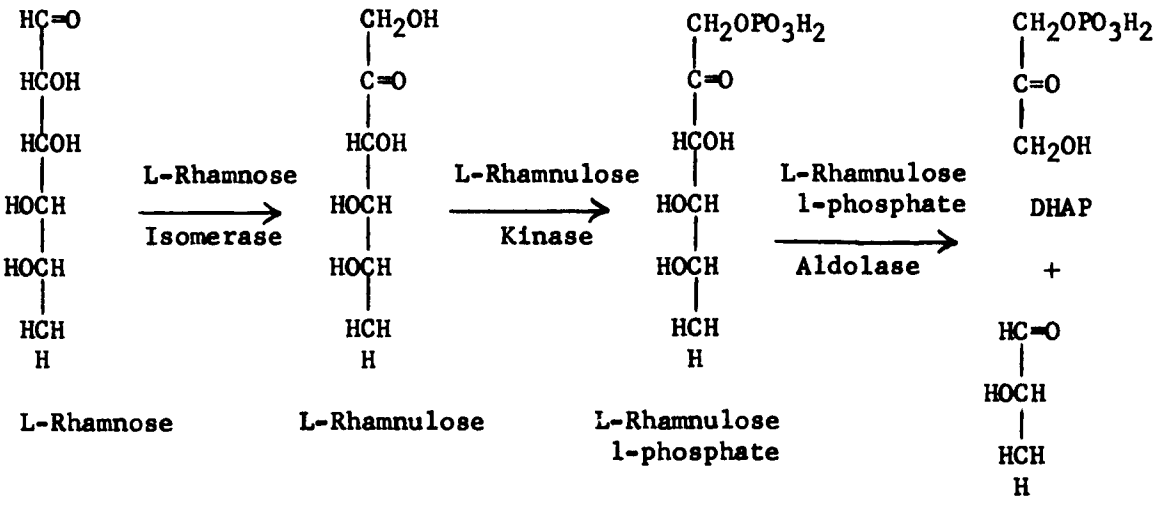
1-phosphate before it can be detected in our assay. This enzyme might not interfere with measurement of PEP-dependent uptake in vesicles. These findings suggested that more than one criteria should be used to determine whether or not a sugar is transported by the phosphotransferase system.

The mechanisms for rhamnose uptake in A. pyridinolis closely paralleled those for fructose uptake. A phosphotransferase system and a respiration-coupled system existed for both sugars. The activity for both systems for L-rhamnose uptake in vesicles was lower than that demonstrated for fructose in membrane vesicles from fructose-grown cells (105). While harvesting rhamnose-grown cells for vesicle preparations, it was observed that large amounts of slime material, possibly a carbohydrate polymer, were produced by the cells. This material was not found with fructose-grown cells, and it might decrease the amount of closed vesicles obtained from rhamnose-grown cells. Nevertheless, characterization of the two uptake systems in vesicles and correlation with the phenotype of phosphotransferase-negative mutants in whole cells supported the relevance of these activities to the functioning transport systems in the cell. It is interesting that the respiration-coupled system for fructose is constitutive, while that for rhamnose is inducible. This may reflect some difference in the regulation of transport of the two sugars.

II. METABOLISM OF L-RHAMNOSE IN *A. PYRIDINOLIS*

LITERATURE REVIEW

L-Rhamnose is a methyl pentose found in the cell wall of many bacteria (108), and in the glycosides of many plants (109). A survey by Eagon (110) of 33 species of bacteria, eighteen of which were able to utilize L-rhamnose or L-fucose demonstrated that the ability to use one methyl pentose did not confer ability to use the other, as only in nine cases were both sugars used. Neither sugar induced enzymes specific for the other. The pathway established in studies described below for L-rhamnose metabolism in E. coli was as follows:



Wilson and Ajl (111) identified the first step in the metabolism of L-rhamnose in E. coli B as the isomerization of this compound to L-rhamnulose. L-Rhamnulose was demonstrated in crude extracts of whole cells to which L-rhamnose was added, by chromatography in three solvent systems and by chemical comparison with synthetic rhamnulose in the cysteine-carbazole (112) reaction and in the methyl pentose test (113).

Further studies from the same laboratory showed that the L-rhamnulose

that was formed from L-rhamnose in crude extracts was then phosphorylated at the expense of ATP to form rhamnulose-1-phosphate (114). The phosphorylated sugar was identified by chromatography in two solvent systems. When isolated, the sugar phosphate could be hydrolyzed with acid phosphatase and the resultant free sugar was identified as free rhamnulose. The rhamnulose was thought to be phosphorylated in the one position becuase 80-90% of the phosphate was hydrolyzed in ten minutes in 1N HCl at 100° C. The fact that rhamnulose was phosphorylated six times faster than rhamnose suggested that rhamnose was not phosphorylated first and then converted to the ketose.

Takagi and Sawada partially purified several enzymes involved in the metabolism of L-rhamnose in E. coli B. A cell extract of E. coli B was subjected to $MnCl_2$ and ammonium sulfate precipitation, followed by heating to 50° C for 2 minutes. The unprecipitated protein was fractionated using ammonium sulfate, resulting in a 50-fold purification of the L-rhamnose isomerase activity (L-rhamnose ketol-isomerase, EC 5.3.1.14). The enzyme was specific for either L-rhamnose or L-rhamnulose. No other substrates were found. The presence of Mn^{+2} was essential for activity. Inhibition of activity was caused by PCMB and could be reversed by cysteine, suggesting the presence of critical sulfhydryl groups in the enzyme (115).

A 13-fold purification of the L-rhamnulose kinase (ATP:L-rhamnulose 1-phosphotransferase, EC 2.7.1.5) was obtained by $MnCl_2$ and ammonium sulfate fractionation followed by mixing with calcium phosphate gel to which the activity was not adsorbed (116). A K_m of 4 mM for rhamnulose, 2.9×10^{-4} M for ATP, and 4.2×10^{-4} M for Mg^{+2} was

found. PCMB caused inhibition which could be reversed by addition of cysteine or glutathione. The reaction product was isolated and upon hydrolysis with acid phosphatase, was found to liberate rhamnulose and inorganic phosphate in a 1:1 ratio. The lability of the phosphate ester indicated it was located at the one position.

Chiu and Feingold partially purified L-rhamnulose kinase from E. coli K40 (117). The crude extract was subjected to $MnCl_2$ treatment and ammonium sulfate fractionation. After calcium phosphate gel treatment, the ammonium sulfate fractionation was repeated, and in a final step alumina gel was used to selectively adsorb the enzyme. A 34-fold purification was achieved to give a preparation free of ATPase, NADH oxidase, and L-rhamnose isomerase. The pH optimum was at pH 8.5. Michaelis-Menten constants of 8.2×10^{-5} M for rhamnulose and 1.1×10^{-4} M for ATP were found. A divalent cation, such as Mg^{+2}, was required, and other nucleotides could substitute for ATP. Other keto sugars were phosphorylated to a small extent. The phosphate ester product was purified and identified as rhamnulose-1-phosphate by periodate oxidation studies.

Englesberg studied L-rhamnose utilization in a mutant of Pasteurella pestis which had acquired the ability to use this sugar (118). The wild type strains were unable to do so. The results of these studies indicated that rhamnose was incompletely oxidized in this organism, and there was lactaldehyde in the cells which could account for at least part of the unoxidized rhamnose. In 1937, Kluyver and Schnellen (119) had proposed that Bacillus rhamnosifermentans split L-rhamnose during its metabolism to give glyceraldehyde and lactaldehyde in order to explain their finding of 1,2 propanediol as an end product of this

fermentation, because this compound could be formed by reduction of lactaldehyde.

In a paper by Sawada and Takagi (120), the 15-fold purification of L-rhamnulose-1-phosphate aldolase (L-rhamnulose 1-phosphate L-lactaldehye lyase, EC 4.1.2.b) from E. coli B was described,and the products of the reaction were identified as dihydroxyacetone phosphate and lactaldehyde. Crude extract was treated with 1% protamine sulfate. The supernatant was treated with alumina gel Cγ, and this supernatant was subjected to ammonium sulfate fractionation. The resultant preparation was slightly contaminated with L-rhamnose isomerase, L-rhamnulose kinase, and hexose diphosphate aldolase. The optimum pH for activity was 9.3. The K_m for rhamnulose-1-phosphate was 10 mM. Dihydroxyacetone phosphate was identified by reaction with α-glycerol phosphate dehydrogenase (L-glycerol 3-phosphate:NAD oxidoreductase, EC 1.1.1.8) and NADH, and by the presence of alkaline-labile phosphate. Formation of DHAP corresponded to loss of rhamnulose-1-phosphate. The reaction was reversible. When D-lactaldehyde was used instead of L-lactaldehyde in the reverse reaction, 6-deoxy D-sorbose was produced. Thus, the enzyme appeared to require or produce the trans configuration of hydroxyls at C3 and C4 of the ketose-1-phosphate.

Chiu and Feingold (121) also purified L-rhamnulose-1-phosphate aldolase from E. coli. After $MnCl_2$ precipitation and ammonium sulfate fractionation, the preparation was fractionated with acetone at -20° C and freed of acetone by passage through a Sephadex G100 column. After elution by increasing NaCl concentration from a DEAE-Sephadex A-50 column, the active fractions were precipitated with ammonium sulfate. This preparation yielded one band on acrylamide gel electro-

phoresis. The specificity of the enzyme required a ketose-1-phosphate with a D-configuration at C3 and the L- configuration at C-4. Aldehydes other than L-lactaldehyde, including D-glyceraldehyde, would react in the reverse reaction with DHAP. The specificity for DHAP was not studied, but was presumed to be analogous to that observed with hexose diphosphate aldolase and fuculose-1-phosphate aldolase.

The L-rhamnulose 1-phosphate aldolase was purified to homogeneity as determined by acrylamide gel disc electrophoresis, cellulose acetate electrophoresis, and agar gel immunodiffusion and immunoelectrophoresis (122). A molecular weight of $1.3\text{-}1.4 \times 10^5$ daltons was determined by density gradient centrifugation and Sephadex gel thin layer chromatography. A monovalent cation was required for activity, and the pH optimum was 7.5. A K_m of 0.3 mM was determined for L-rhamnulose-1-phosphate. Cleavage of fructose 1,6-diphosphate was only 1.8% of the cleavage seen with rhamnulose-1-phosphate as substrate. The enzyme was a class II aldolase, since it was activated by monovalent cations and was completely inhibited by the zinc chelator, 1,10-phenanthroline. Schwartz et al. (123) showed that the enzyme contained 2 g-atoms of zinc per mole of enzyme. Mercaptoethanol inhibited activity. Mercaptoethanol plus SDS converted the enzyme to inactive metal-free subunits of molecular weight 35,000. Electron micrographs of the crystalline aldolase showed the enzyme contained four subunits arranged in a square.

Power conducted studies on the genetic nature of the system for L-rhamnose metabolism in E. coli K12 (124). The genes for the enzymes were linked to the methionine-requiring, met B1 marker. Four classes of mutants were isolated which could not utilize L-rhamnose for growth.

The order of the genes for these four classes of mutation was established by reciprocal three factor crosses. In order of their map position were groups of mutants: lacking aldolase activity; lacking kinase activity; lacking isomerase activity; and having low or undetectable levels of all activities.

Studies of one bacterial species which had an incomplete pathway for L-rhamnose metabolism indicated that accumulation of an early product (s) of L-rhamnose metabolism was toxic for the cells. Salmonella typhosa was unable to use L-rhamnose as a carbon source. The presence of L-rhamnose caused inhibition of growth in this bacterium. Mutants resistant to this inhibition arose during growth in the presence of L-rhamnose but were still unable to use this sugar. The wild type strain contained L-rhamnose isomerase and L-rhamnulose kinase activity, but not L-rhamnulose-1-phosphate aldolase activity. A resistant strain, O-901, was missing both the isomerase and kinase activities (125). The loss of both enzymes was determined not to be due to the loss of ability to transport the sugar, to inhibition of activity, or to the necessity for production of rhamnulose to induce the kinase.

Inhibition of bacterial growth by bacteriostasis or bacteriolysis has been seen commonly in cells where a metabolite of some substrate is accumulated in the cell rather than being further metabolized. This was often found in mutants missing an enzyme of a metabolic pathway and was particularly noted when a sugar phosphate was the accumulated metabolite. An E. coli mutant lacking phosphoglucose isomerase and glucose 6-phosphate dehydrogenase accumulated high (50 mM) internal glucose 6-phosphate which caused inhibition of growth on those sub-

strates which required the use of fructose diphosphate aldolase. In vitro, FDP aldolase was inhibited by glucose 6-phosphate (126). E. coli ara-53 was deficient in L-ribulose 5-phosphate 4-epimerase. In the presence of L-arabinose, L-ribulose 5-phosphate accumulated and growth was inhibited. Inhibition could be overcome by addition of glucose. Resistant mutants lacked L-ribulokinase (127). Growth of a fructose 1-phosphate kinase deficient mutant of E. coli was inhibited by fructose or fructose 1-phosphate by an apparent pile up of the latter (128). The same effect was seen in a mutant of Aerobacter aerogenes (129). Mutants of mannitol 1-phosphate dehydrogenase in S. typhimurium (130) and in E. coli K12 (131) were sensitive to inhibition of growth by added mannitol. Nikaido found that mutants of Salmonella missing UDP-galactose 4-epimerase underwent severe bacteriolysis in the presence of galactose. A resistant mutant was negative for galactokinase activity (132). Accumulation of galactose 1-phosphate and UDP-galactose caused formation of spheroplasts in hypotonic medium. Only growing cells were lysed suggesting that a defect or deficiency in cell wall synthesis was caused. UDP transferase-negative mutants, which accumulated only galactose 1-phosphate, showed only bacteriostasis, not lysis (133). Lysis in a UDP-galactose 4-epimerase deficient mutant could be prevented by the presence of glucose. This protection was thought to be due to catabolite repression of the enzymes forming the toxic product by glucose (132, 134). An E. coli mutant which was deficient in galactose 1-phosphate uridyl transferase showed marked slowing of growth in the presence of galactose which could be overcome by addition of 0.5% yeast extract (135). Yarmolinsky et al. (136) found galactose sensitivity in E. coli mutants of galactose metabolism

that was analogous to the effects seen in Salmonella.

INTRODUCTION

Studies of L-rhamnose metabolism in E. coli (112, 115-118, 121, 122) revealed that L-rhamnose was metabolized via isomerization to L-rhamnulose, phosphorylation at the expense of ATP to form L-rhamnulose 1-phosphate, and cleavage by an aldolase to produce dihydroxyacetone phosphate and L-lactaldehyde. The finding that there existed in A. pyridinolis two systems for the transport of L-rhamnose raised a problem as to how L-rhamnose was metabolized inside the cells. The respiration-coupled L-rhamnose transport system caused accumulation of free rhamnose within the cell. Thus, rhamnose taken up by the respiration-coupled system in A. pyridinolis might be metabolized by the sequence of transformations known for E. coli. The PEP:rhamnose phosphotransferase system, on the other hand, caused uptake and accumulation of the compound as L-rhamnose 1-phosphate. Rhamnose 1-phosphate was not an intermediate in the known metabolic pathways for rhamnose metabolism, suggesting that an alternate pathway must exist for the metabolism of rhamnose transported by the phosphotransferase system of A. pyridinolis.

Four possible models for the metabolism of L-rhamnose in A. pyridinolis had to be considered in light of the two systems known for transport and the metabolic pathway known for E. coli. Three of these models are illustrated in Fig. 7. The fourth model was a variation of Model II in which I and I' were one enzyme with the ability to recognize either L-rhamnose or L-rhamnose 1-phosphate. Mutants were isolated which could not use L-rhamnose for growth, whether or not malate was also present. Identification of the lesions

involved and the resulting phenotypes made possible a description of the pathway for L-rhamnose metabolism in A. pyridinolis.

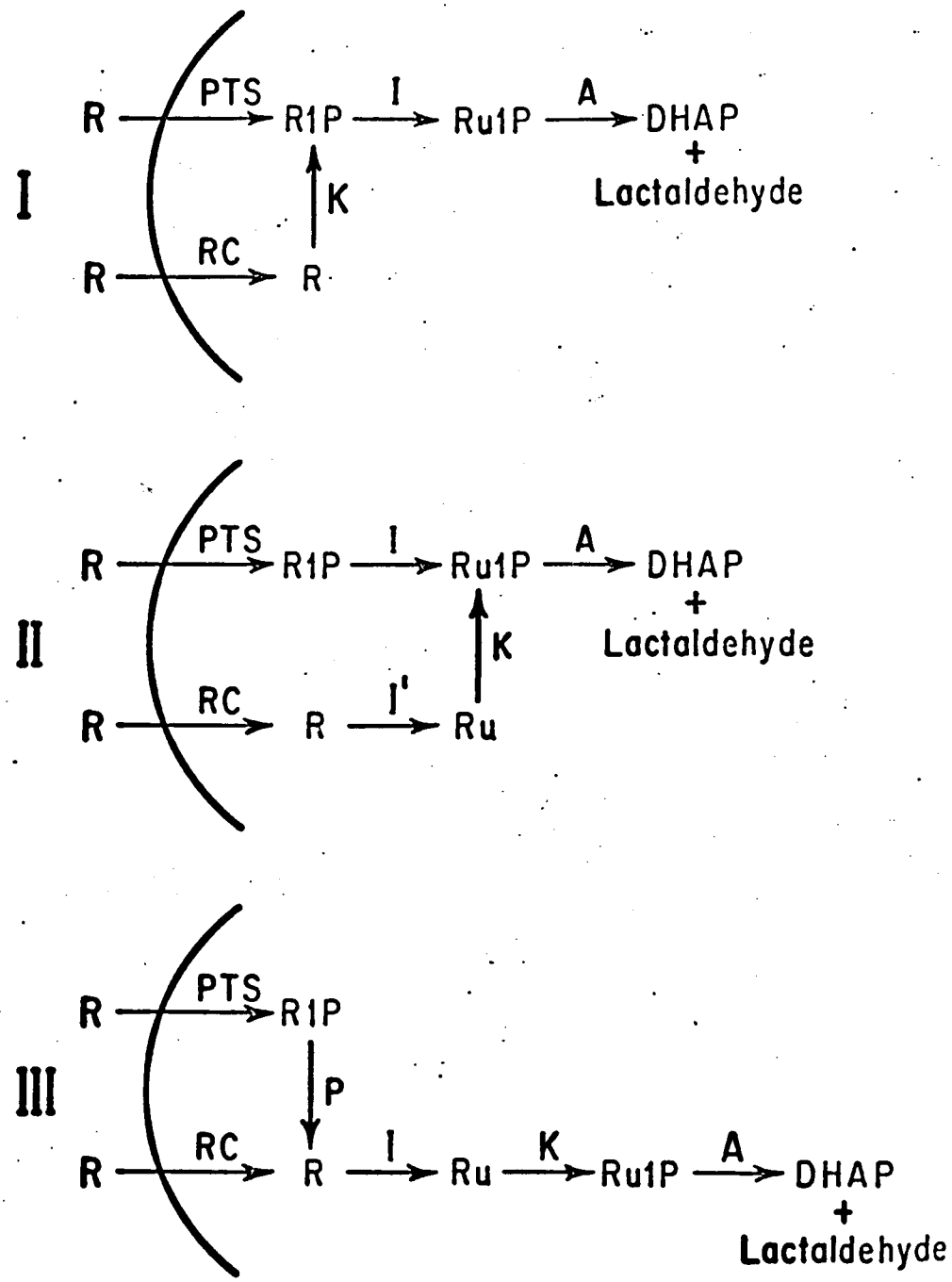
MATERIALS AND METHODS

Bacteria and growth conditions. Arthrobacter pyridinolis and mutants derived from it were used in all experiments. PYE medium was used to maintain cultures (102). MS was used as the defined medium (99), and carbon sources were added from separate sterile solutions to a final concentration of 0.05 M. Growth studies were conducted in 300 ml side-arm flasks as described by Wolfson and Krulwich (99).

Isolation of mutants. In order to isolate L-rhamnose negative mutants, cells of A. pyridinolis were treated with EMS as described by Wolfson and Krulwich (99). Cells were then washed and incubated overnight in MS plus L-rhamnose plus 40 μg per ml of pencillin G. Then the cells were washed and plated on PYE plates. The colonies formed were replica-plated onto L-rhamnose and D-fructose plates. Colonies which grew on D-fructose but failed to grow on L-rhamnose were identified and picked. These were further characterized by growth experiments done in the presence of 10 mM L-malate plus 50 mM L-rhamnose and 10 mM malate alone. Those that grew as well as the wild type on malate alone and showed no increment of growth in the presence of both carbon sources were further investigated to determine the lesions involved.

Enzyme assays. Crude extracts were prepared by sonic disruption as described by Wolfson and Krulwich (99). Before being assayed, extracts were dialyzed against a large volume of the buffer used in the assay. L-Rhamnose isomerase activity was determined by the assay

Figure 7. Schematic representation of possible models for the uptake and metabolism of L-rhamnose. Abbreviations used are: PTS-phosphotransferase system; RC-respiration-coupled transport; R-L-rhamnose, Ru-L-rhamnulose; RIP-L-rhamnose 1-phosphate; RulP-rhamnulose 1-phosphate; I - isomerase; K - kinase; A- aldolase.


I
R
PTS
R1P
I
Ru1P
A
DHAP
+
Lactaldehyde
K
RC
R
II
R
PTS
R1P
I
Ru1P
A
DHAP
+
Lactaldehyde
K
RC
R
I'
Ru
III
R
PTS
R1P
P
RC
R
I
Ru
K
Ru1P
A
DHAP
+
Lactaldehyde

procedure of Domagk and Zech (137), which was based on the appearance of L-rhamnulose, as measured by the cysteine-carbazole reaction of Dische and Borenfreund (111). L-Rhamnulokinase activity was determined in a mixture containing 25 mM Tris-HCl buffer, pH 8.5, 2.5 mM $MnCl_2$, 1.7 mM ATP, 1.7 mM reduced glutathione, 25 mM NaF and 50 µl of crude extract in a total of 0.18 ml. The reaction was initiated by the addition of 20 µl of 20 mM [G-^{3}H] L-rhamnose(10 µCi/ M). After incubation for ten minutes at room temperature, the reaction was stopped by addition of 0.3 ml of 1 M L-rhamnose. Samples of the reaction mixture were spotted on DEAE-cellulose discs (138). The discs were washed well in running water. After the discs were dried, the radioactivity was measured by liquid scintillation counting. A control sample was run in the absence of ATP. L-Rhamnulose 1-phosphate aldolase activity was determined by following the formation of DHAP in a reaction mixture (total, 0.5 ml) containing: 0.2 mM NADH; 10 mM NaF; 1 mM $MnCl_2$; 25 µM $MgCl_2$: 20 µg α-glycerol phosphate dehydrogenase; 12 mM L-rhamnulose 1-phosphate; and 25 mM Tris-HCl, pH 7.6. The reaction was started by the addition of 25-50 µl of crude extract which had been centrifuged at 150,000 x g for 60 minutes in a Beckmann L2-65B ultracentrifuge. This was done to remove most of the activity of membrane-bound NADH oxidase, which interfered with the assay. Control reactions from which L-rhamnulose 1-phosphate was omitted were conducted to correct for any residual NADH oxidase activity. The decrease in absorbance at 340 nm was followed in a Gilford Model 240 recording spectrophotometer at 37° C. The amount of substrate added was probably not saturating, as L-rhamnulose 1-phosphate was obtainable only in limiting quantities and the affinity of the enzyme for the

substrate was apparently very low. However, parallel assays of wild type and mutants were always performed and the results have been used for qualitative rather than quantitative interpretations. D-Fructose phosphotransferase activity was assayed as described by Sobel and Krulwich (102). Complementation assays were performed as described by Wolfson and Krulwich (106). Protein was determined by the method of Lowry et al.(139) using lysozyme as a standard. Specific activities are expressed as nanomoles or micromoles of product formed per minute per mg of protein.

Chemicals. In all cases, the L- isomers of malate and rhamnose and the D- isomer of fructose were used. [G-^{3}H]L-Rhamnose was purchased from New England Nuclear Corp. NADH, ATP, and α-glycerol phosphate dehydrogenase were purchased from Boehringer-Mannheim Corp. L-Rhamnose, pencillin G, reduced glutathione, and potassium thioglycollate were purchased from Sigma Chemical Co., and ethyl methane sulfonate (EMS) was obtained from Eastman Organic Chemicals. L-Rhamnulose 1-phosphate was generously donated by Dr. David Feingold. All other chemicals were obtained at the highest purity commercially available.

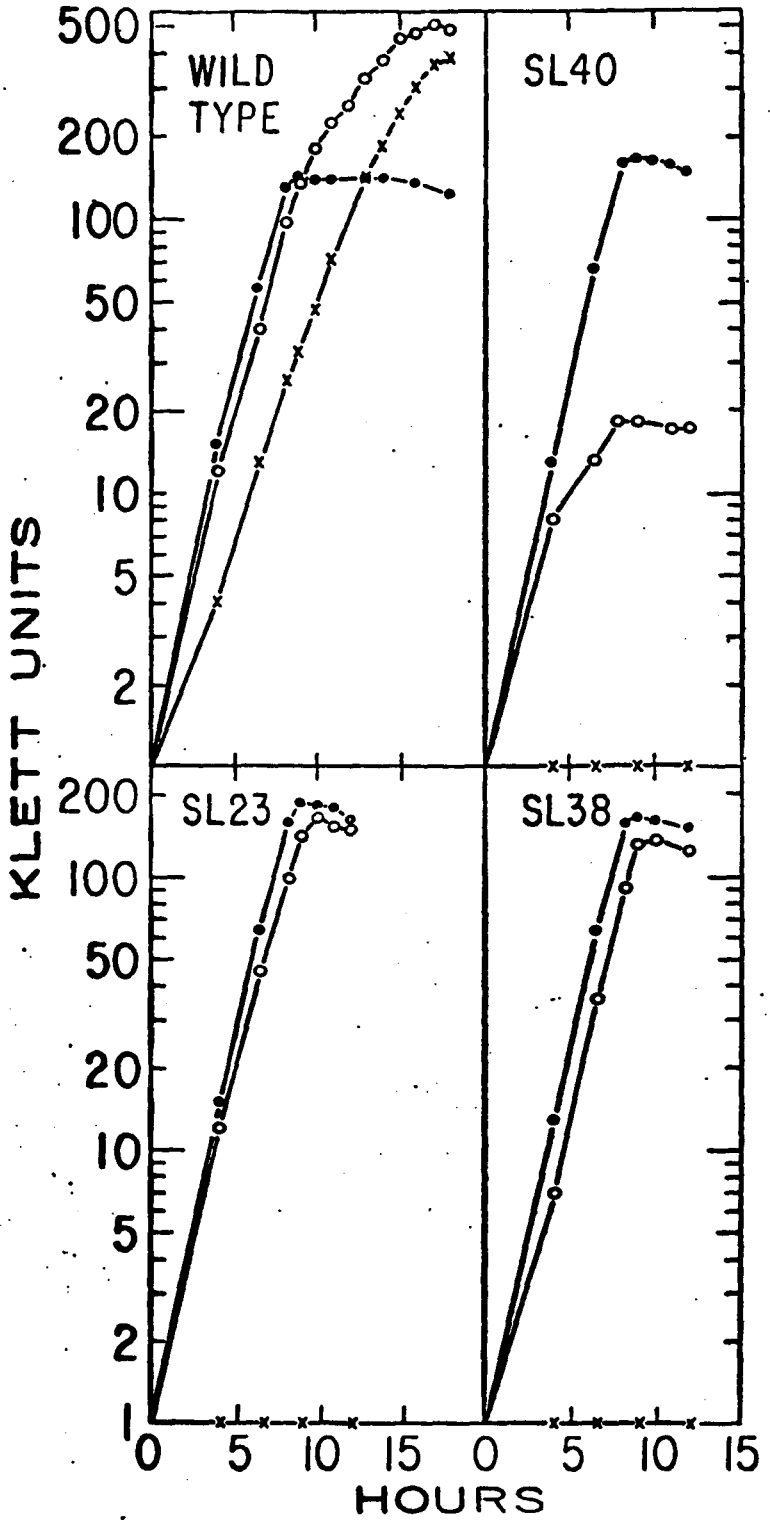
RESULTS

Crude extracts of wild type A. pyridinolis, prepared as described in MATERIALS AND METHODS, were found to contain L-rhamnose isomerase, L-rhamnulokinase, and L-rhamnulose 1-phosphate aldolase. The isomerase and kinase activities were induced only when the cells were incubated with L-rhamnose. The L-rhamnulose 1-phosphate aldolase, however, was present at comparable levels in cells grown on malate alone or in the presence of L-rhamnose.

Several predictions could be made from the models proposed for L-rhamnose metabolism in A. pyridinolis (Fig. 7). According to models I, III, and IV, a mutant deficient in L-rhamnose isomerase activity would be unable to grow on rhamnose transported by either system. This would not be the case for Model II in which separate isomerases were proposed for L-rhamnose and L-rhamnose 1-phosphate. Using similar reasoning, the absence of an ATP-dependent kinase would not affect metabolism of L-rhamnose 1-phosphate, if Model I, II, or IV were correct. In all cases, the loss of the L-rhamnulose 1-phosphate aldolase would prevent growth on rhamnose via either pathway.

Among the mutants isolated which failed to grow on rhamnose whether or not malate was present, three classes were found, typified by SL23, SL38, and SL40. Growth curves of these mutants are shown in Fig. 8. As can be seen, SL23 and SL38 were unable to grow on L-rhamnose alone, and when 10 mM malate was present, growth proceeded only until the malate was consumed. Extracts of malate and rhamnose-grown cells of mutant strains were assayed for L-rhamnose isomerase, L-rhamnulokinase, and L-rhamnulose 1-phosphate aldolase. Specific

Figure 8. Growth of wild type and mutant strains of A. pyridinolis on L-rhamnose, L-malate, and L-malate plus L-rhamnose. Cells of the strain indicated were grown on 50 mM L-rhamnose (x——x), 10 mM L-malate (●——●), and 10 mM L-malate plus 50 mM L-rhamnose (o——o).


WILD TYPE
SL40
SL23
SL38
KLETT UNITS
500
200
100
50
20
10
5
2
1
0
5
10
15
HOURS

activities of each of the enzymes with respect to the wild type levels are shown in Table III. SL38 had no detectable L-rhamnulokinase activity. SL23 had no detectable L-rhamnose isomerase activity. L-Rhamnulokinase activity was not detectable by direct assay in SL23, since our assay used L-rhamnose as substrate and relied on its conversion to L-rhamnulose in the crude extract. In order to determine whether L-rhamnulokinase was present in the isomerase negative mutant, crude extract of SL23 was assayed in the presence of crude extract of SL38, which had no L-rhamnulokinase activity. The SL38 extract could convert the added rhamnose to the substrate for the kinase, thereby allowing the kinase activity of SL23 to be expressed. The results, as shown in Table IV, indicated that SL23 had wild type levels of L-rhamnulokinase.

The fact that an isomerase negative strain was unable to grow on L-rhamnose in the presence or absence of malate eliminated Model II, which involved two isomerases. More important, the isolation of a mutant which lacked only L-rhamnulokinase activity and was unable to grow on L-rhamnose either alone or in the presence of malate, eliminated Models I, II, and also the variation of Model II according to which one isomerase recognizes both substrates. This strongly suggested that, as shown in Model III (Fig. 7), L-rhamnose which was phosphorylated by the PEP:phosphotransferase system lost its phosphate once inside the cell and could then be metabolized by a sequence of steps resembling those which occur in E. coli.

Mutants of the class represented by SL40 showed an unusual pattern of growth. SL40 was able to grow on malate as well as the wild type strain did (Fig. 8). It was unable to grow on rhamnose

TABLE III

SPECIFIC ACTIVITY OF ENZYMES OF RHAMNOSE METABOLISM IN WILD TYPE AND MUTANT STRAINS OF A. PYRIDINOLIS[a]

Strain	Specific Activity/ Specific Activity of the Wild Type		
	Rhamnose Isomerase	Rhamnulokinase	Rhamnulose 1-phosphate Aldolase
Wild Type	1.00	1.00	1.00
SL23	<0.06	-	0.80
SL38	0.97	0.04	0.59
SL40	1.70	2.80	0.80

[a] Wild Type, SL23, and SL38 cells were grown in the presence of 50 mM malate plus 50 mM rhamnose. Cells of SL40 were grown on 50 mM malate and incubated with 50 mM rhamnose for three hours before harvesting. Assays were performed on crude extracts of the cells as described in MATERIALS AND METHODS.

TABLE IV

RHAMNULOKINASE ACTIVITY IN MUTANT STRAIN SL23 SHOWN BY COMPLEMENTATION[a]

Strain	Specific Activity (ηmoles Rul-P/mg protein/ min)
Wild Type	4.68
SL23	0.22
SL38	0.58
SL23 + SL38	4.78

[a]Cells were grown overnight on 50 mM malate and incubated in the presence of 2.5 mM rhamnose for three hours. Crude extracts were prepared, and assays were performed as described in MATERIALS AND METHODS.

alone. When both malate and rhamnose were present, SL40 was unable to use either compound for growth. It appeared that the addition of rhamnose to this mutant caused severe inhibition of growth. Similar results were seen when rhamnose was present in SL40 cultures growing on acetate, succinate, fructose, L-glutamate, D-gluconate or complex medium (PYE).

Crude extracts of SL40 contained L-rhamnose isomerase, L-rhamnulokinase, and L-rhamnulose 1-phosphate aldolase (Table III). It appeared that this mutant might contain a defective phosphatase, thereby allowing L-rhamnose 1-phosphate to accumulate in the cell. Sugar-phosphate accumulation was shown to cause severe inhibition of growth and, in some cases, lysis in other bacteria (118-136). The accumulation of L-rhamnose 1-phosphate in SL40 would account for the inhibition of growth on various substrates observed in the presence of rhamnose.

Since L-rhamnose 1-phosphate was not commercially available, and the L-rhamnose phosphotransferase system was not active enough to synthesize this compound in crude extracts, we were unable to assay eitherthe wild type or mutants for L-rhamnose 1-phosphate phosphatase activity. Therefore confirmation of the defect in SL40 was obtained by indirect means. Attempts were made to select a phosphotransferase-negative derivative of SL40 which, while still unable to use L-rhamnose alone because of the original lesion, would not accumulate rhamnose 1-phosphate in the presence of rhamnose. After mutagenesis of SL40 with EMS, a strain was isolated which was no longer able to grown on fructose alone, but was able to use fructose in the presence of malate, i.e., a phosphotransferase-negative strain (104). Such strains, e.g. SL4013, were still unable to use L-rhamnose alone, but had regained

the ability to use this sugar in the presence of malate and no longer exhibited the severe inhibition of growth seen in SL40 (Table V). A second group of strains was isolated after mutagenesis of SL40 by selecting for cells which were resistant to rhamnose inhibition. Strains of this type, typified by SL4002, were deficient in their growth on malate when compared to the wild type cells (Table V), and grew slightly more on rhamnose (or fructose) in the presence of malate than on malate alone.

DISCUSSION

The pathway of L-rhamnose metabolism in A. pyridinolis was determined by investigating mutants unable to use this sugar. One of four possible models for rhamnose metabolism (Model II) involved a separate isomerase for the rhamnose and rhamnose 1-phosphate which entered by the respiration-coupled and phosphotransferase systems, respectively. The finding that a mutant deficient in L-rhamnose isomerase activity was unable to grow on rhamnose via either transport system eliminated this model from consideration. According to two of the three remaining models (Model I and the variation of Model II), kinase activity would be required for only one of the two alternate pathways for metabolism. Mutant strain SL38, which lacked rhamnulokinase activity, was unable to use either pathway to metabolize rhamnose, eliminating these two models. In addition, the isomerase negative strain SL23 was unable to phosphorylate L-rhamnose using ATP, indicating the absence of an L-rhamnose kinase such as was proposed in Model I. It was therefore concluded that the two alternate pathways for rhamnose metabolism in A. pyridinolis occurred as described in Model III, by conversion of rhamnose 1-phosphate to free rhamnose by a phosphatase and further

TABLE V

GROWTH OF STRAINS OF A. PYRIDINOLIS ON RHAMNOSE IN THE PRESENCE AND ABSENCE OF MALATE[a]

Carbon Sources	Klett Units			
	Wild Type	SL40	SL4013	SL4002
1. 50 mM Rhamnose	385	0	0	0
2. 50 mM Fructose	277	269	0	0
3. 10 mM Malate	122	134	115	50
4. 50 mM Rhamnose				
+ 10 mM Malate	490	20	409	109
3-2[b]	368	-114	294	59
5. 50 mM Fructose				
+ 10 mM Malate	470	460	479	86
5-3[b]	348	326	364	36

[a]Cells were grown in 300 ml side-arm flasks as described in MATERIALS AND METHODS. Values represent growth after 18 hours at 30° C.

[b]Net growth on fructose or rhamnose after growth on malate.

metabolism by an isomerase, a kinase and an aldolase.

The requirement for a phosphatase to convert rhamnose 1-phosphate produced by the phosphotransferase system to free rhamnose was compatible with our consistent failure to show reproducible PEP:rhamnose phosphotransferase activity in L-rhamnose grown cells (140, see Section I). Also, cells deficient in L-rhamnose 1-phosphate phosphatase activity, as typified by SL40, showed severe inhibition of growth on a variety of substrates in the presence of rhamnose, presumably due to the accumulation of rhamnose 1-phosphate. Inhibition of growth and sometimes cell lysis due to the accumulation of sugar-phosphates has been shown to occur in many bacteria (118-136). Two types of mutant strains were isolated from SL40 which no longer showed inhibition by rhamnose. SL4013 apparently had lost its ability to phosphorylate rhamnose by the phosphotransferase system. It was unable to grow on rhamnose alone, but was no longer inhibited by rhamnose and was able to utilize this sugar in the presence of malate. The second strain, SL4002, might be deficient in the malate dehydrogenase necessary to couple the transport of rhamnose or fructose to respiration. This would account for the poor growth of the strain on malate. SL4002 also showed only a small, but reproducible, increment in growth when either rhamnose or fructose was present in the medium together with malate; presumably the respiration-coupled transport system was operative only to the extent that the requisite dehydrogenase was also active, i.e., to the extent to which the transport system was indeed energy-coupled. Similar, previously isolated mutants of wild type A. pyridinolis which grew poorly on malate, not only showed low activity of the respiration-coupled transport system, but also failed

to induce the fructose-specific components of the phosphotransferase system even in the presence of exogenous fructose. These and other data supported the conclusion that active transport of fructose into the cell by the respiration-coupled system was required for induction of the phosphotransferase system (106). SL4002, which might be deficient in the ability to couple transport to malate oxidation, no longer showed inhibition of growth in the presence of rhamnose and presumably did not accumulate rhamnose 1-phosphate. These observations suggested that this mutant could not induce the rhamnose-specific components of the phosphotransferase system, whose induction may require intracellular rhamnose, just as induction of the PEP:fructose phosphotransferase system required intracellular, actively transported fructose.

Both the rhamnose isomerase and the rhamnulokinase were inducible enzymes. A mutant lacking detectable rhamnose isomerase activity contained fully induced levels of rhamnulokinase activity. This indicated that L-rhamnose, rather than L-rhamnulose, was probably the inducer for the L-rhamnulokinase. Moreover, mutants deficient in phosphotransferase activity synthesized the enzymes necessary for growth on rhamnose in the presence of malate, eliminating rhamnose 1-phosphate as an inducer. It was not clear whether the isomerase and the kinase were coordinately induced with the rhamnose phosphotransferase system. It was interesting that the rhamnulose 1-phosphate aldolase was the only enzyme in the pathway that was not inducible and was also the only enzyme in which mutations were not found. Possibly, the aldolase employed was one that served some other purpose in the cell (e.g., fructose 1, 6-diphosphate aldolase) so that under the selection con-

ditions employed, a mutant lacking the enzyme would not be viable.

III. PURIFICATION AND CHARACTERIZATION OF A POLYPHOSPHATE KINASE FROM *A. ATROCYANEUS*

LITERATURE REVIEW

Polyphosphate is a polymer of inorganic phosphate with polyanhydride linkages, thermodynamically equivalent to the high energy bond of ATP. Metachromatic granules of polyphosphate were identified in corynebacteria by Ebel (141). In 1954 and 1955, reports of a reversible synthesis of polyphosphate from ATP were made by Hoffman-Ostenhoff et al. (142) and Yoshida and Yamataka (143). Muhammed purified a polyphosphate kinase (ATP:polyphosphate phosphotransferase, E. C. 2.7.4.1) from Corynebacterium xerosis (144). The enzyme was also identified in Aspergillus niger (145), Mycobacterium smegmatis (146), Chlorobium (147,148), and Clostridium (149).

In 1956, Kornberg et al. (150) reported the purification of an enzyme from E. coli which catalyzed the following reaction: $xATP + \alpha(PO_3^-)_n(primer) \longrightarrow xADP + \alpha(PO_3^-)_{n+x}$. The enzyme was purified using streptomycin sulfate and ammonium sulfate. The preparation was stable for weeks when frozen and could be thawed and refrozen without loss of activity. Heat treatment or incubation under acid conditions caused inactivation. Activity required Mg^{+2} and was stimulated by ammonium sulfate and other salts. The pH optimum was 7.2. Fluoride was an inhibitor. The product formed an acid-insoluble protein complex, so the reaction was stopped upon the addition of perchloric acid and bovine serum albumin. ADP inhibited activity completely at 8×10^{-5} M when the ATP concentration was 6-fold higher. Even when the ATP/ADP ratio was 24, 57% inhibition could be demonstrated. The K_m for ATP, using an ATP regenerating system, was 1.4 mM. PP_i caused 20%

inhibition at 1.6 mM. Radioactively labeled PP_i was incorporated into product, but P_i was not. The product of the reaction was characterized by the following properties: 1) it induced metachromasy; 2) it was non-dialyzable; 3) it formed an acid-insoluble complex with protein; 4) it was labile in acid or alkali; 5) it bound anion exchange resins; and 6) it was not degraded by nucleases.

Appreciable amounts of low molecular weight polyphosphate were not found. The fact that the product was precipitated by albumin at acid pH suggested that it was a polymer of very long chain length. Katchman and Van Wazer (151)observed that albumin precipitation was quantitative only when the average chain length of the molecules was 1600 phosphate residues.

The E. coli enzyme was purified more than 100-fold by Li and Brown (152) by ammonium sulfate precipitation, DEAE-cellulose chromatography, and Sephadex G-200 gel filtration. The catalytic properties of the enzyme were consistent with those found by Kornberg et al. (150). However, the presence of histone and inorganic phosphate in the reaction were necessary to see full activity. In the absence of histone, the reverse reaction could be demonstrated by adding enzyme, ADP, and radioactively labeled polyphosphate. Extensive characterization of the product was consistent with the findings of others that the product was a polyphosphate of very long chain length.

A polyphosphate kinase from Corynebacterium xerosis was purified by Muhammed (144). Activity of the enzyme was assayed by adding ADP and a ^{32}P-ATP generating system. It was found that 0.8 mM ADP was optimal for activity, but higher concentrations caused inhibition. The enzyme had a sharp pH optimum at 7.4. Mg^{+2} was essential for activity.

In contrast to the findings of Kornberg et al. (150), this enzyme was almost completely inhibited by the addition of ammonium sulfate to the assay. The product was isolated and characterized by criteria similar to those used for the E. coli enzyme (152, 153). No evidence was found for the role of a primer in the reaction, and no short chain intermediates were found. The reaction was measured in potassium phosphate buffer.

Accumulation of polyphosphate in microbial cells varies widely. The role of polyphosphate in the cell has not yet been determined, although several theories of its role have been proposed. Harold examined the possibility, in Neurospora crassa (153), that polyphosphate had a role analogous to the phosphagens found in animals and was closely linked to the levels of adenine nucleotides. However, his results and those of others indicated that polyphosphate was not a storage form of high energy phosphate for synthesis of ATP. Inhibition of oxidative and substrate level phosphorylation of ATP did not cause degradation of polyphosphate, although ATP pools were drastically lowered. This did not indicate a rapid equilibrium between polyphosphate and ATP, as was seen for creatine phosphate in mammalian muscle.

An alternative hypothesis for the role of polyphosphate was as a phosphorus reserve (154). It has been shown that polyphosphate can serve as a source of phosphorus for biosynthetic processes during phosphorus starvation (153, 155-160) and during spore germination (161). In this role, the energy content of polyphosphate would be irrelevant, but its structure would allow accumulation of phosphorus without affecting the levels of inorganic phosphate and adenine nucleotide pools.

Harold (156) studied accumulation of polyphosphate in Aerobacter

aerogenes, where it was found that large amounts of polyphosphate accumulated when nucleic acid synthesis was stopped by limiting a nutrient that was required for growth. Polyphosphate and nucleic acid synthesis apparently competed for intracellular phosphorus, since when nucleic acid synthesis resumed, polyphosphate was degraded and the inorganic phosphate appeared in RNA. In 1964, Harold (162) reported the occurrence in A. aerogenes of "polyphosphate overplus," a phenomenon described by Liss and Langen (163) in which polyphosphate was accumulated to very high levels upon the addition of inorganic phosphate to phosphate-starved cells. Harold presented evidence indicating that this phenomenon was due to derepression of the enzymes for polyphosphate metabolism during phosphate starvation. A ten-fold increase in polyphosphate kinase activity in phosphate-starved cells accounted for the accumulation observed. This rapid accumulation was independent of nucleic acid synthesis. Thus the evidence seemed to implicate polyphosphate as a phosphorus reserve in the cell, although the fact that the process has not been found to be indispensable in mutants (157, 162) has made it difficult to clearly establish whether this is, in fact, its role.

INTRODUCTION

During studies of enzyme induction in Arthrobacter atrocyaneus, the possibility was raised that this bacterium might produce a protein kinase which was involved in cellular regulation. Investigation of this possibility led to the discovery of an activity which incorporated the γ-phosphate of ^{32}P-ATP into a TCA-precipitable fraction, and which was dependent upon the presence of histone. It was tentatively proposed that this activity was a protein kinase (164), and a detailed investigation of this potentially interesting finding was begun. On further purification of the enzyme and in subsequent studies of the reaction product, it was shown that the enzyme was, in fact, a polyphosphate kinase, which donates the γ-phosphate of ^{32}P-ATP to form long chains of polyphosphate, a molecule containing phosphate joined by anhydride bonds and characterized by metachromasy when accumulated in cells. The enzyme has been purified 700-fold and characterized, and preliminary experiments have been conducted to determine its role in the cell.

MATERIALS AND METHODS

Organism and Growth Conditions. A. atrocyaneus (ATCC 13752) was maintained on PYE medium and was grown on minimal media MS (99) supplemented with 0.05 M sodium glutamate as carbon source for isolation of the polyphosphate kinase. Sixteen liters of cells were grown overnight in carboys, with vigorous aeration, at 30° C. Phosphate-free media for physiological studies contained 25 mM Tris, pH 7.2, 0.2% ammonium sulfate, 0.02% $MgSO_4$ plus trace salts (99).

Preparation of Crude Extracts. Cells were harvested by centrifuga-

tion, washed with 10 mM Tris, pH 7.2, and then resuspendend in this buffer. The cells were disrupted by sonication using a Heat Systems-Ultrasonics W185D Sonifier for a total of two minutes. The temperature was kept below 10° C. The extract this produced was then treated with DNase and RNase for one hour at 5° C. The cell debris was removed by centrifugation for ten minutes at 16,000 x g. When the extract was to be used for physiological studies, DNase and RNase treatment was omitted, and extracts were dialyzed against 10 mM Tris, pH 7.2 after centrifugation.

Enzyme assays. The activity of the polyphosphate kinase was determined at 30° C in a mixture containing, except where noted, 6 mM potassium phosphate buffer, pH 7.0, 2 mM $MnCl_2$, 1 mg per ml Histone Type II from calf thymus, 0.4 mM (γ-^{32}P)ATP(7-28 dpm per pmole) and 8.8 µg per ml of enzyme in a total volume of 0.1 ml. ^{32}P-polyphosphate was determined by the paper chromatographic procedure of Li and Felmly (165) except that the paper strips were not dried before elution, and the solvent used was cold 10% trichloroacetic acid. One unit of activity is equivalent to that amount of enzyme which catalyzes the incorporation of one nanomole of (γ-^{32}P)ATP into product per minute. RNA polymerase activity was determined by the method of Bonner et al. (166). Protein was determined by the method of Lowry et al. (139).

Chemicals. (γ-^{32}P)ATP and $^{32}P_i$ were purchased from New England Nuclear Corporation. Histone Type II, protamine, casein, and bovine serum albumin were purchased from Sigma Chemical Co. Sephadex G-200 was purchased from Pharmacia, and DEAE-cellulose was purchased from Bio-Rad. All other chemicals were of the highest quality commerically available.

RESULTS

Purification of the Enzyme. The crude extract, prepared as described in MATERIALS AND METHODS, was fractionated using ammonium sulfate. Ammonium sulfate was added to 45% saturation with stirring at 0-5° C. The precipitated protein was harvested by centrifugation, was dissolved in 10 mM Tris buffer, pH 7.2, containing 1 mM EDTA, and was then dialyzed overnight against the same buffer. The ammonium sulfate fraction was then applied to a DEAE-cellulose column (1 x 24 cm) equilibrated with 5 mM potassium phosphate buffer, pH 7.0. The protein was eluted by 500 ml of a linear potassium phosphate gradient from 5 mM to 500 mM potassium phosphate, pH 7.0, containing 1 mM EDTA, 10 μM dithiothreitol, and 10% glycerol (Fig. 9). The active fractions, which were eluted at approximately 0.22 M potassium phosphate, were pooled and concentrated by addition of 45% ammonium sulfate. The precipitate collected was dissolved in 5 mM potassium phosphate containing 1 mM EDTA, 10 μM dithiothreitol, and 10% glycerol and dialyzed against this buffer overnight. The preparation was then applied to a Sephadex G-200 column (2.5 x 40 cm) equilibrated with the same buffer used for dialysis. A sharp peak of activity was eluted approximately at the external void volume (data not shown). The purification procedure is summarized in Table VI. The fractions eluted from the Sephadex G-200 column were used for all subsequent experiments. The enzyme preparation was found to be stable at 5° C for at least six months.

Characterization of the Enzyme. The time course of enzyme activity is shown in Fig. 10. The formation of product remained linear for 15 minutes. The behavior of the enzyme with varying pH is shown

Figure 9. Profile of DEAE-cellulose column. The enzyme fraction obtained after ammonium sulfate precipitation was applied to a DEAE-cellulose column (1 x 24 cm) equilibrated with 50 mM potassium phosphate, pH 7.0, and was eluted with a linear potassium phosphate gradient from 5 mM to 500 mM potassium phosphate, pH 7.0, containing 1 mM EDTA, 10 μM dithiothreitol, and 10% glycerol. Fractions were assayed as described in MATERIALS AND METHODS. o——o, specific activity; •——•, A_{280}; ——, potassium phosphate concentration.

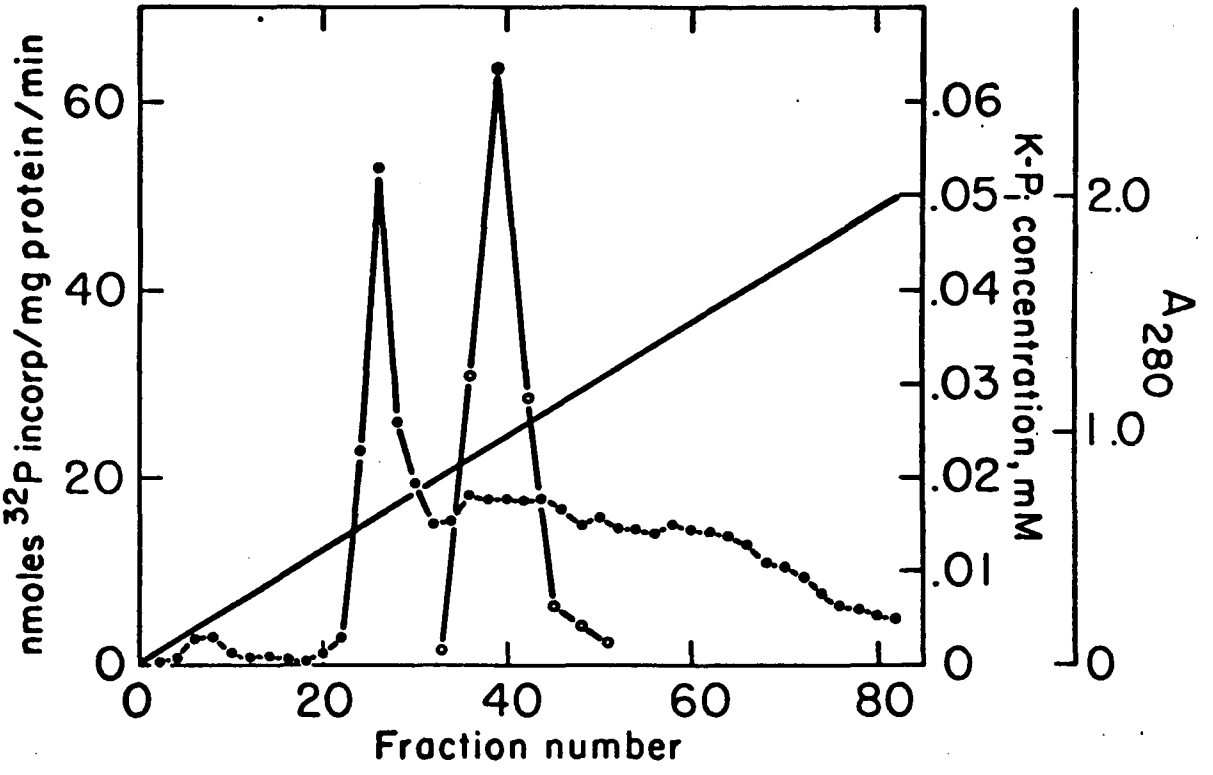

nmoles ^{32}P incorp/mg protein/min
60
40
20
0
0
20
40
60
80
Fraction number
.06
.05
.04
.03
.02
.01
0
K-P_i concentration, mM
2.0
1.0
0
A_{280}

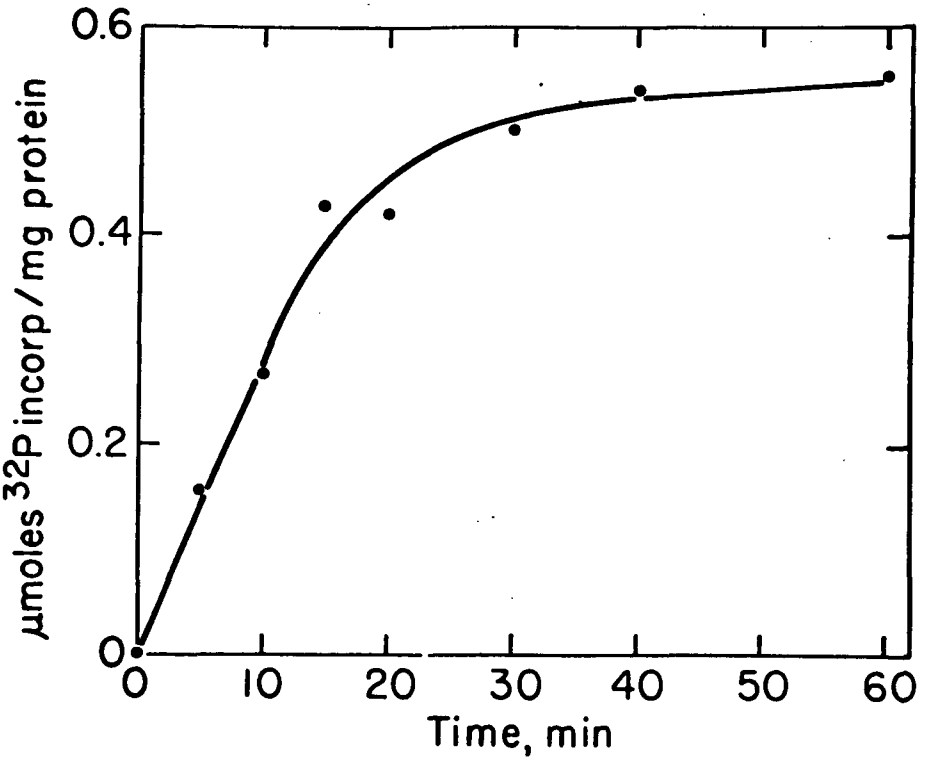
TABLE VI

PURIFICATION OF POLYPHOSPHATE KINASE[a]

Purification Step	Units/ml	mg/ml	Specific Activity (η moles/mg/min)	Purity
Sonicated Crude Extract	6.5	5.9	1.1	1
45% Ammonium Sulfate Precipitation	77.6	6.4	12.1	11
DEAE-cellulose Chromatography	33.2	0.3	99.3	89
Ammonium Sulfate Precipitation	790.6	1.7	472.0	425
Sephadex G-200 Chromatography	72.1	0.09	818.8	738

[a]The purification procedure is described in detail in RESULTS. The preparations were assayed as described in MATERIALS AND METHODS.

Figure 10. Time course of the polyphosphate kinase reaction. The enzyme was assayed as described in MATERIALS AND METHODS except that a final volume of 0.2 ml was employed. Aliquots of reaction mixture were removed at appropriate times and radioactively labeled product was determined as described.


0.6
0.4
0.2
0
μmoles 32P incorp / mg protein
0
10
20
30
40
50
60
Time, min

in Fig. 11. The enzyme exhibited a broad pH optimum at 6.0-7.0. The enzyme preparation was found to be free from RNA polymerase activity, although McConnell and Bonner (167) have reported that the two enzymes from E. coli may be purified together.

The response of polyphosphate kinase activity to varying concentrations of ATP, Mn^{+2}, and Mg^{+2} is shown in Figs. 12-14. Assays were performed as described in MATERIALS AND METHODS with the following modifications. In Fig. 12, the ATP concentration was varied while the $MnCl_2$ concentration and other variables were kept constant. In Figs. 13 and 14, respectively, the $MnCl_2$ and $MgCl_2$ concentrations were varied while the ATP concentration and other conditions were held constant. Values shown for concentrations of free Mg^{+2} or Mn^{+2}, free ATP, and Mn-ATP^{-2} or Mg-ATP^{-2} complexes were determined using stability constants of 10,960 M^{-1} for Mg-ATP^{-2} and 56,230 M^{-1} for Mn-ATP^{-2} as determined by Walaas (168). Free ATP concentrations greater than 8 μM appeared to inhibit the enzyme(Fig. 12). A K_m of 0.53 mM was calculated for Mn-ATP^{-2} from the rates of reaction at non-inhibitory levels of free ATP and Mn^{+2}. As shown in Fig. 13, 1.0-2.0 mM free Mn^{+2} was optimal for enzyme activity. At concentrations where both free ATP and Mn-ATP^{-2} were kept constant, an increase in the free Mn^{+2} concentration to greater than 2 mM caused inhibition of activity. Increasing free Mg^{+2} ion concentration caused a less sharp increase in activity than with Mn^{+2} (Fig. 14), and inhibition did not appear until approximately 6 mM free Mg^{+2} was present. This might be due either to a requirement for higher levels of free Mg^{+2} than Mn^{+2} for enzyme activity or to the five-fold lower stability constant for the Mg-ATP^{-2} complex relative to the Mn-ATP^{-2} complex.

The presence of phosphate ions was required for enzyme activity.

Figure 11. Dependence of the polyphosphate kinase reaction on pH. The assay was performed in a total volume of 0.1 ml, containing 1 mg per ml Histone Type II, 2 mM $MnCl_2$, 6 mM potassium phosphate, pH 7.0, 20 µl water, 0.4 mM ^{32}P-ATP, and 50 mM Tris-maleate-acetate buffer, pH 5-9.6. This mixture was incubated for 10 min at 30° C and radioactively labeled product was determined as described in MATERIALS AND METHODS.

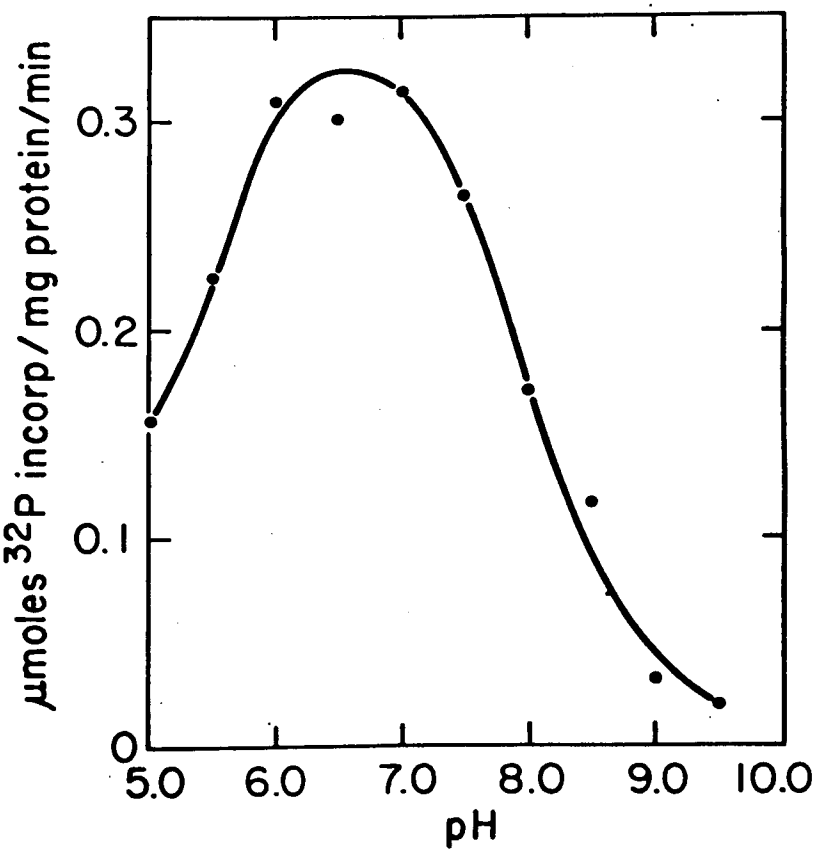

μmoles 32P incorp/mg protein/min
0.3
0.2
0.1
0
5.0
6.0
7.0
8.0
9.0
10.0
pH

Figure 12. Dependence of the polyphosphate kinase reaction on the ATP concentration. Incubations were performed as described in MATERIALS AND METHODS except that the concentration of ATP was varied. Values for Mn^{+2}, ATP, and $MnATP^{-2}$ were determined from the dissociation constant for the $MnATP^{-2}$ complex. ●——● specific activity; o——o, ATP concentration, Δ——Δ, Mn^{+2} concentration.

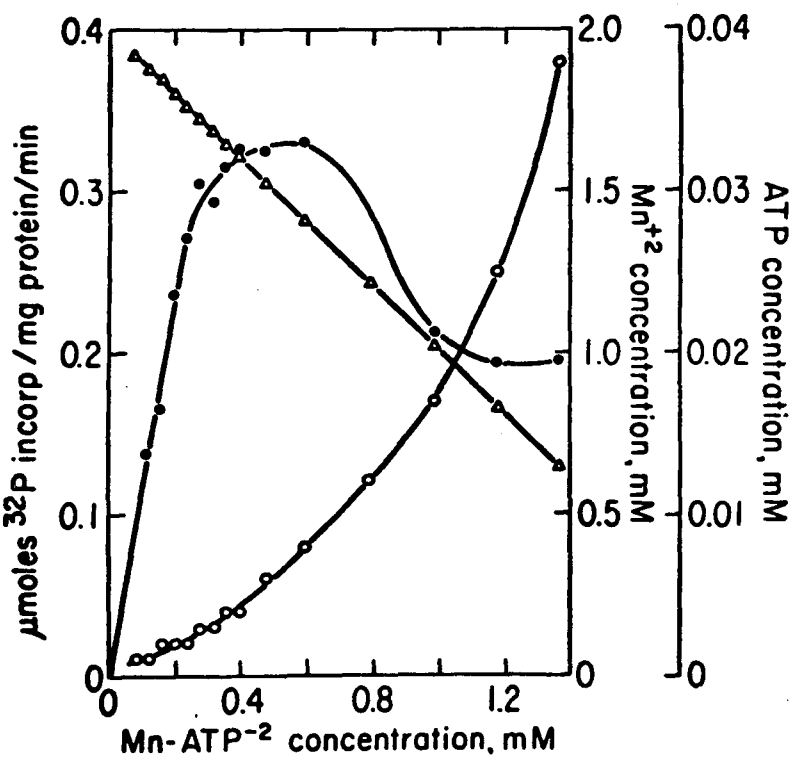

0.4
0.3
0.2
0.1
0
2.0
1.5
1.0
0.5
0
0.04
0.03
0.02
0.01
0
0
0.4
0.8
1.2
μmoles 32P incorp / mg protein/min
Mn+2 concentration, mM
ATP concentration, mM
Mn-ATP-2 concentration, mM

Figure 13. Dependence of the polyphosphate kinase reaction on the $MnCl_2$ concentration. Assays were performed as described in MATERIALS AND METHODS except that the concentration of $MnCl_2$ was varied. Values for Mn^{+2}, ATP, and $MnATP^{-2}$ were determined from the dissociation constant for the $MnATP^{-2}$ complex. ●·——● , specific activity; o——o , ATP concentration; Δ——Δ , $Mn\text{-}ATP^{-2}$ concentration.

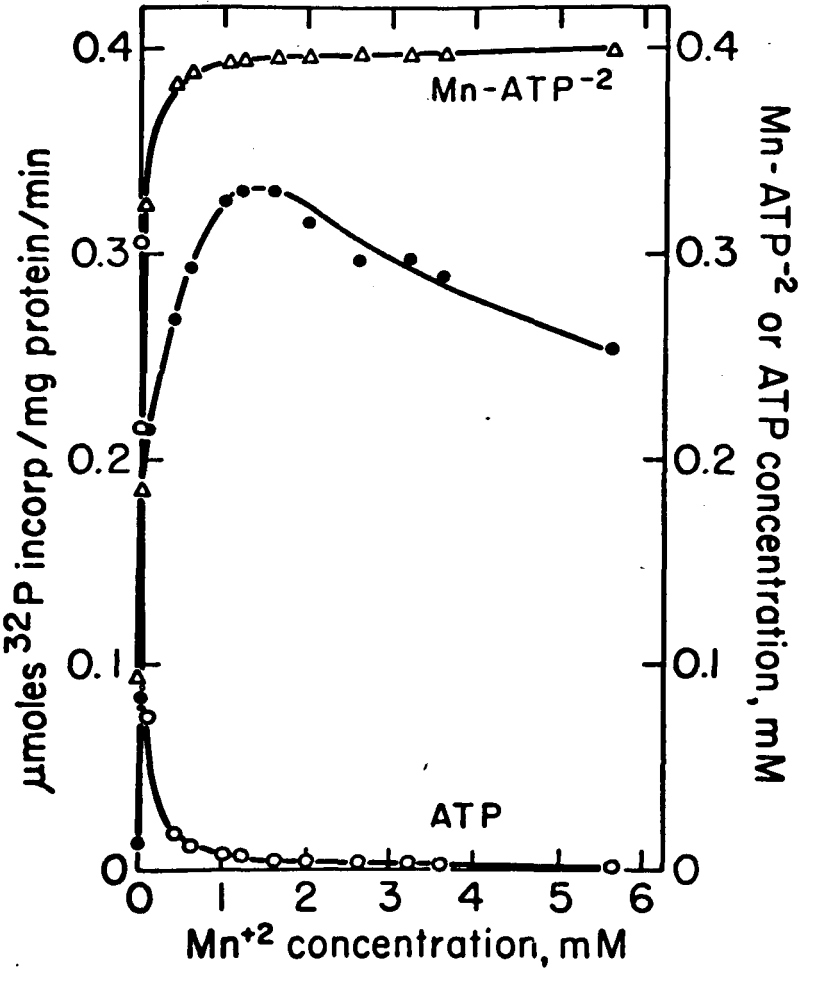
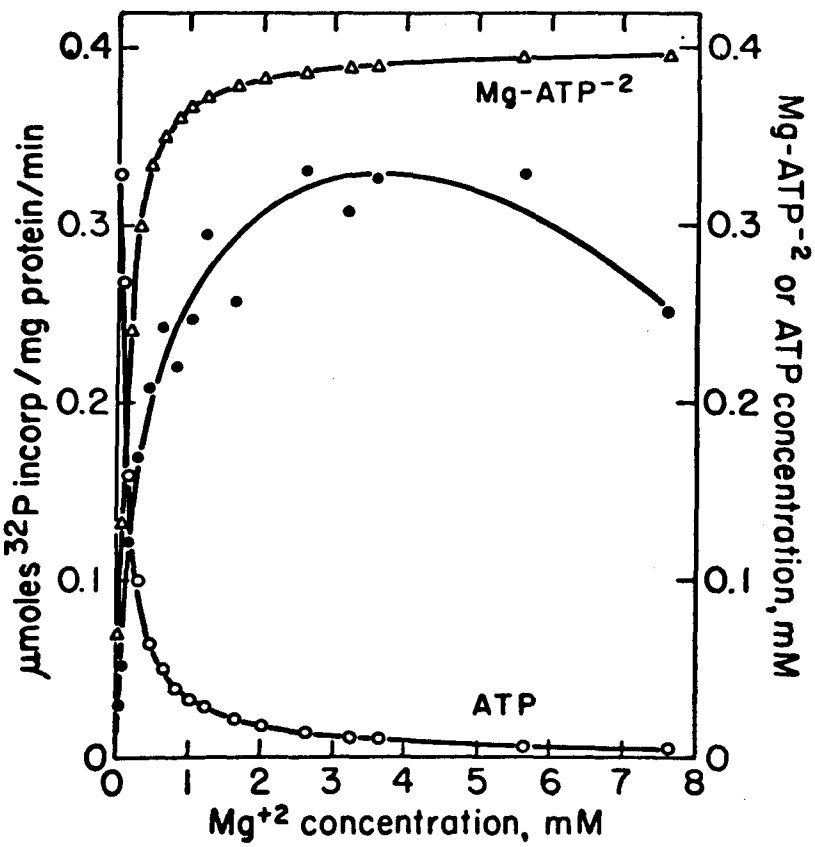

0.4
0.3
0.2
0.1
0
Mn-ATP^{-2}
ATP
µmoles ^{32}P incorp/mg protein/min
Mn-ATP^{-2} or ATP concentration, mM
0 1 2 3 4 5 6
Mn^{+2} concentration, mM

Figure 14. Dependence of the polyphosphate kinase reaction on the $MgCl_2$ concentration. Assays were performed as described in MATERIALS AND METHODS except that the $MgCl_2$ concentration was varied. Values for Mg^{+2}, ATP, and $MgATP^{-2}$ were determined using the dissociation constant for the $MgATP^{-2}$ complex. ●——●, specific activity; o——o, ATP concentration, Δ——Δ, $MgATP^{-2}$ concentration.


0.4
0.3
0.2
0.1
0
Mg-ATP⁻²
ATP
µmoles ³²P incorp / mg protein/min
Mg-ATP⁻² or ATP concentration, mM
0 1 2 3 4 5 6 7 8
Mg⁺² concentration, mM

The activity of the enzyme, as shown in Fig. 15, increased with increasing phosphate concentration up to approximately 5 mM. A K_m of 1.67 mM was calculated. Radioactively labeled phosphate was not incorporated into the product.

The enzyme was strongly inhibited in the presence of ADP, one of the reaction products. In Fig. 16 it may be seen that 4 mM ADP completely abolished activity or stopped the reaction when added after ten minutes. Also shown in Fig. 16 are the inhibitory effects of 1 mM PP_i or 20 mM NaF, similar to effects reported in E. coli (150, 152).

The dependence of the enzyme on the presence of histone is shown in Fig. 17. Other proteins, such as protamine, could substitute for histone to a lesser degree. Bovine serum albumin and casein could also substitute for histone. However, in contrast to the activity observed with basic proteins, enzyme activity in the presence of these two proteins could be detected only in the absence of inorganic phosphate (Table VII). The requirement for the presence of a protein, such as histone, might result in mistaken identification of the enzyme as a protein kinase. This possibility was eliminated for the enzyme from A. atrocyaneus by the characterization of the product.

Characterization of the Reaction Product. Determination of the chemical nature of the product was based on the known properties of the various phosphorylated protein residues and polyphosphate when treated by certain methods. It was initially thought that the reaction product might be either a phosphorylated protein, containing residues of acyl, serine, or imidazole phosphate, or a long chain polyphosphate. Serine phosphate ester bonds were stable to hydroxylamine treatment, but stable in acid at 100° C and in base (169, 170). Imidazole phosphate bonds

Figure 15. Dependence of the polyphosphate kinase reaction on the presence of inorganic phosphate. Assays were performed as described in MATERIALS AND METHODS, except that the inorganic phosphate concentration was varied from 0-10 mM. The reciprocal plot gave a K_m of 1.67 mM.

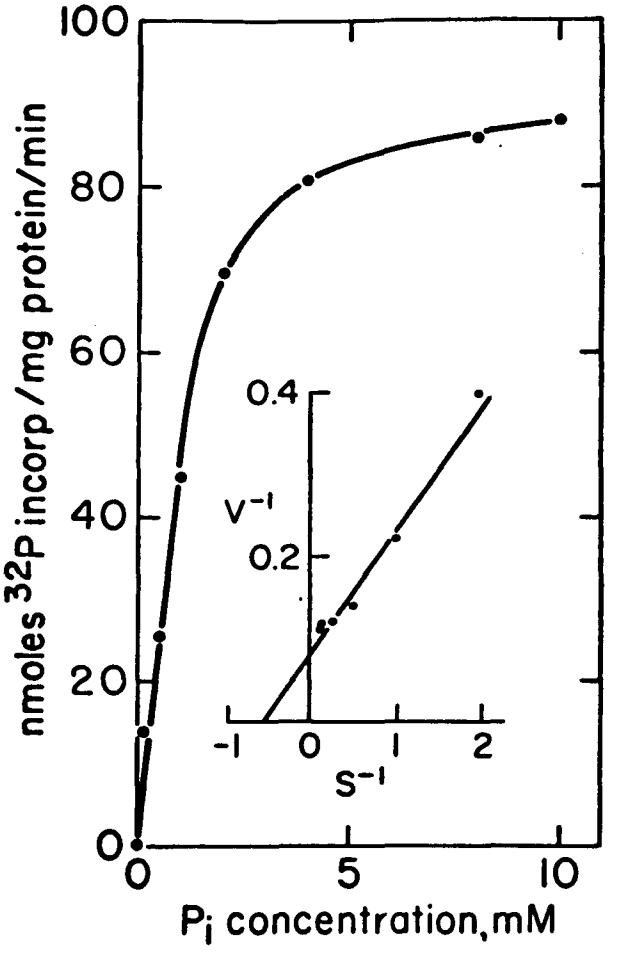

100
80
60
40
20
0
nmoles 32P incorp/mg protein/min
0
5
10
Pi concentration, mM
0.4
V-1
0.2
-1
0
1
2
S-1

Figure 16. Inhibition of the polyphosphate kinase reaction by ADP, NaF, and pyrophosphate. Assays were performed as described in MATERIALS AND METHODS in a volume of 0.2 mls with the following additions: 4 mM ADP (● ——— ●), 20 mM NaF (x——x), and 1 mM pyrophosphate (o ——— o) were added to each of three tubes at time zero. 4 mM ADP was added to one tube after 10 min (Δ ——— Δ). The control (▲ ——— ▲) contained no additions.

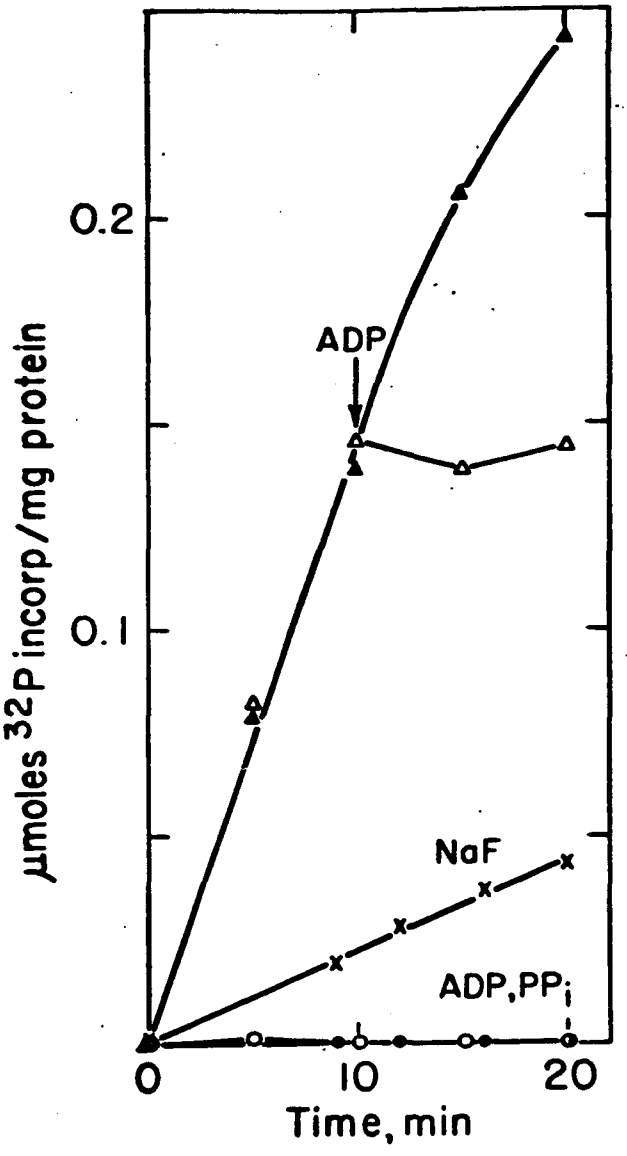

0.2
0.1
μmoles 32P incorp/mg protein
ADP
NaF
ADP,PPi
0
10
20
Time, min

Figure 17. Dependence of the polyphosphate kinase reaction on the histone concentration. Assays were performed as described in MATERIALS AND METHODS except that the histone concentration was varied from 0-2.0 mg per ml.

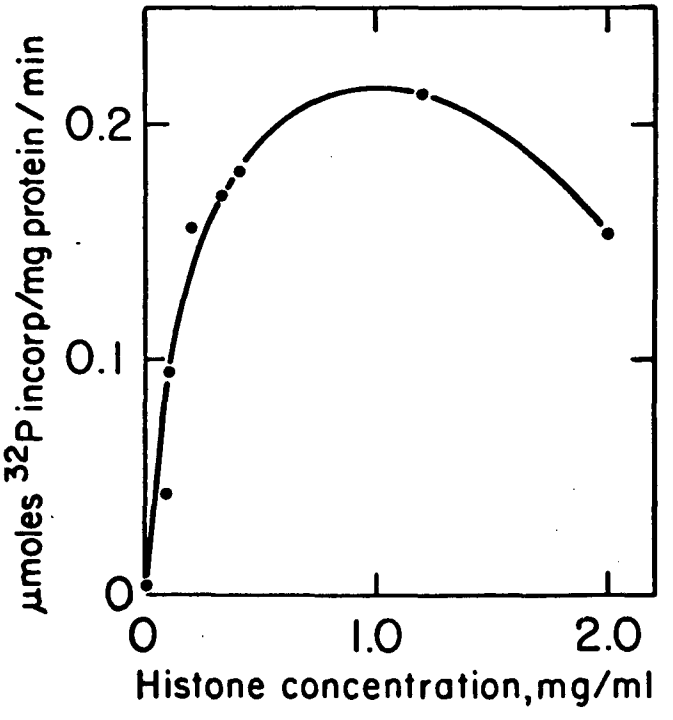

µmoles ^{32}P incorp/mg protein / min
0.2
0.1
0
0
1.0
2.0
Histone concentration, mg/ml

TABLE VII

EFFECT OF INORGANIC PHOSPHATE ON POLYPHOSPHATE KINASE REACTION WITH DIFFERENT PROTEINS[a]

Protein (1 mg/ml)	Inorganic Phosphate (6 mM) Added	Specific Activity (ηmoles/mg/min)
Histone	+	254.1
	-	19.9
Protamine	+	135.0
	-	0.6
Casein	+	0.5
	-	4.1
Bovine Serum Albumin	+	6.6
	-	59.0

[a]The 700-fold purified enzyme was assayed as described in MATERIALS AND METHODS except that different proteins were substituted for histone where indicated and assays were conducted with or without inorganic phosphate as shown.

were stable in base but labile in cold acid (14). Acyl phosphate bonds were labile to treatment with NH_2OH, base or acid at 100° C (171, 172). Long chain polyphosphate, having anhydride bonds like the acyl phosphate, was broken down to inorganic phosphate in acid at 100° C, in base at 40° C, and in hydroxylamine.

In order to obtain the reaction product for characterization, the assay was conducted for one hour and stopped by the addition of a solution of trichloroacetic acid to give a final concentration of 10%. The precipitate was harvested by centrifugation, resuspended in buffer, and washed twice with 10% trichloroacetic acid. It was then resuspended in buffer, neutralized, and dialzyed overnight at 5° C against a large volume of 5 mM potassium phosphate, pH 7.0. The product was then subjected to the following conditions: 1) 1.2 M HCl for 15 minutes at 100° C; 2) 0.5 M KOH for two hours at 40° C; 3) 1 M hydroxylamine for 30 minutes at room temperature; 4) 2.5 mg per ml papain for 30 minutes at 37° C. After treatment, each sample was spotted on strips of Whatman #1 filter paper. The strips were eluted for 16 hours with an Ebel's solvent containing 735 ml of isopropanol, 50 g trichloroacetic acid, 2.5 ml of 29% NH_4OH and 265 ml of water; 100 ml of the solvent was diluted with 25 ml of water before using (173). On elution with this solvent, long chain polyphosphate remained at the origin while inorganic phosphate migrated rapidly on the paper, separating it from product. Shorter polyphosphate molecules migrate from the origin with intermediate R_f values depending on chain length (173). After elution the strips were air dried and counted in a Baird Atomic strip counter. Radioactive ATP and inorganic phosphate standards migrated on the paper and separated well from each other (data not shown). Untreated radio-

active product remained at the origin after chromatography. It contained no residual radioactive ATP from the reaction, indicating that washing twice with trichloroacetic acid is sufficient to remove unreacted substrate. After chromatography of the sample treated with acid, no counts remained at the origin; the product was completely hydrolyzed to inorganic phosphate. After KOH treatment and elution with solvent, 19% of the counts remained at the origin. This suggested that the product was not protein containing imidazole phosphate bonds, which are stable to base under the conditions employed. Hydroxylamine caused the release of all but 8% of the counts from the origin. A serine phosphate ester product would be unaffected by this treatment. Seventy-two per cent of the product was unaffected by papain treatment. The loss of about 30% of the product after papain treatment might be due to some degradation of the histone needed to stabilize the product. However, a phosphorylated protein would show greater degradation after proteolytic treatment. This distinguishes the reaction product from an acyl phosphorylated protein which resembled polyphosphate on the basis of the other criteria tested. The labile nature of the product is in accord with results found by other investigators (150, 152, 174) and is compatible with the identification of the reaction product as polyphosphate rather than a phosphorylated protein.

Physiological studies. Experiments were performed which implicated phosphorus storage as the physiological role of the polyphosphate kinase. Activity of the enzyme was determined under different conditions in dialyzed crude extracts of the cells. An interesting observation was made about the stability of the enzyme. Extracts which were frozen and thawed once before assaying sometimes showed an increase of from three

to five fold over the activity of the same extract before freezing. One possible explanation of this observation was that the enzyme might be loosely associated with the cell membrane. Freezing and thawing may cause its release when sonication has not already done so.

To examine the effect of inorganic phosphate in the medium on polyphosphate kinase activity, A. atrocyaneus was grown on the phosphate-free medium described in MATERIALS AND METHODS with 50 mM malate as carbon source. Potassium phosphate was added to the three separate cultures at 5 mM, 25 mM, and 100 mM concentrations. Dialyzed crude extracts were frozen and thawed before assaying. The specific activities for cells grown in the presence of 5 mM, 25 mM, and 100 mM inorganic phosphate were 2.6, 1.4, and 0.3 ηmoles of ^{32}P incorporated into product per minute per mg protein, respectively. The data indicated a direct relationship between the availability of phosphate in the medium and the activity of the polyphosphate kinase.

To demonstrate induction of this enzyme, cells were grown on MS plus 50 mM malate overnight. These cells were harvested, washed, resuspended in phosphate-free medium and returned to the shaker. At 0, 10, 20, and 30 minutes, aliquots were removed and crude extracts were prepared, dialyzed, frozen and thawed for assay. It may be seen from Table VIII that an extremely rapid induction took place when cells were aerated in the absence of inorganic phosphate. In a similar experiment in which phosphate was always present, but the cells were starved for a nitrogen source, the enzyme was not induced, even after 60 minutes. This further implicated the enzyme in phosphorus metabolism.

TABLE VIII

INDUCTION OF POLYPHOSPHATE KINASE IN A. ATROCYANEUS[a]

Time of Induction (min)	Specific Activity (ηmoles/mg/min)
0	.08
10	3.52
20	3.88
30	4.78

[a]Cells were washed and resuspended in phosphate-free medium. After incubation with shaking for the indicated times, the enzyme was assayed in crude extracts of these cells as described in MATERIALS AND METHODS.

DISCUSSION

Studies of enzyme induction in A. atrocyaneus led to the purification and characterization of a histone-dependent polyphosphate kinase. The requirement for histone to detect enzyme activity might be misleading. Without purifying the reaction product, it might appear that the enzyme catalyzed the phosphorylation of histone. It was apparent that in investigations of protein kinase activity, one must first eliminate the possibility of the presence of a histone-dependent polyphosphate kinase. The additional finding that inorganic phosphate inhibited or activated the enzyme when different proteins were used might also be used as a criterion for polyphosphate kinase activity where it was suspected. This characteristic has also been found for the E. coli enzyme (Li and Brown, personal communication). A possible, but as yet unproved, role of histone orother proteins in the reaction might be to complex with the polyphosphate and prevent reversal of the reaction, thereby faciliating detection of the product. Depending on the protein, phosphate ions might interfere with this function or enhance it. Alternatively, the role of histone might involve some interaction with the enzyme itself such that the enzyme might be altered in the presence of inorganic phosphate.

The possibility that the inhibition by ADP occurred because ADP caused the equilibrium of the reaction to favor the reverse of polyphosphate synthesis must be considered. The data in Fig. 16 appeared to contradict this, since radioactivity was not lost from product on addition of ADP after ten minutes. However, if the role of histone was to complex with the product and prevent reversal of the reaction, it

might mask the effect of ADP inhibition on product which was already formed. The reversal of the polyphosphate kinase reaction by ADP has been shown in E. coli by Kornberg (175) and Li and Brown (152).

The activation by inorganic phosphate might be consistent with a role for the enzyme, which has been suggested by others, for the accumulation of a phosphorus reserve. It was shown in many organisms that polyphosphate could be utilized as a phosphorus source for nucleic acid and phospholipid biosynthesis during spore germination (161) or in cells starved for phosphorus (155, 158, 159, 176). Sufficient amounts of polyphosphate could accumulate to allow the cell mass to double several times under conditions of phosphate starvation. Studies of the pattern of polyphosphate synthesis were done in Aerobacter aerogenes by Harold (176) in wild type cells and in mutants of this bacterium deficient in polyphosphate metabolism. He found that under conditions of nucleic acid synthesis, polyphosphate synthesis was inhibited, possibly by competition for ATP, and degradation of polyphosphate was enhanced. When nutrient limitation caused a cessation of nucleic acid synthesis, inhibition of polyphosphate synthesis was relieved and polyphosphate degradation was inhibited. Kaltwasser (154) suggested that accumulation of polyphosphate regulated the intracellular levels of inorganic phosphate. Conditions under which polyphosphate was synthesized were those where macromolecular synthesis was inhibited by nutrient starvation or specific inhibitors while uptake of inorganic phosphate from the medium continued. If the physiological role of polyphosphate was to serve as a phosphorus reserve, then it would be advantageous to the cell to have a polyphosphate synthesizing enzyme which was sensitive to the levels of inorganic phosphate available to the cell.

Experiments designed to investigate the physiological role of the polyphosphate kinase strongly implicated involvement in the phosphate metabolism of the cell. The evidence indicated that the levels of inorganic phosphate in the medium clearly correlated with levels of polyphosphate kinase in the cell, and induction of the enzyme occurred when inorganic phosphate was removed from the medium, suggesting that the role of the enzyme was to store phosphate in the cell at the expense of ATP when phosphate was available only in limiting amounts in the environment.

ABBREVIATIONS

ADP-	adenosine 5' diphosphate
ATP-	adenosine 5' triphosphate
ATPase-	adenosine triphosphatase
CCCP-	carbonyl cyanide-m-chlorophenylhydrazone
DCCD-	N,N'-dicyclohexylcarbodiimide
DEAE-	diethyl aminoethyl
DHAP-	dihydroxyacetone phosphate
DMSO-	dimethyl sulfoxide
DNase-	deoxyribonuclease
DNP-	2,4-dinitrophenol
DTT-	dithiothreitol
EDTA-	ethylenediamine tetraacetic acid
EMS-	ethyl methane sulfonate
FAD-	flavin adenine dinucleotide
FCCP-	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FDP-	fructose 1,6-diphosphate
HOQNO-	2-heptyl-4-hydroxyquinoline-N-oxide
HPr-	histidine-containing phosphocarrier protein
α-MG-	methyl-α-D-glucopyranoside
NADH(NAD^+)-	nicotinamide adenine dinucleotide, reduced (oxidized)
NEM-	N-ethyl maleimide
ONPG-	o-nitrophenyl-β-D-galactoside
P_i-	inorganic phosphate
PCMB-	p-chloromercuribenzoate
PEP-	phosphoenol pyruvate

PMS- phenazine methosulfate

PP_i- inorganic pyrophosphate

RNA- ribonucleic acid

RNase- ribonuclease

SDS- sodium dodecyl sulfate

TCA- trichloroacetic acid

TDG- β-D-galactosyl-1-thio-β-D-galactopyranoside

TMG- methyl-1-thio-β-D-galactopyranoside

UDP- uridine 5' diphosphate

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