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The City University of New York, Ph.D., 1975
Chemistry, biological

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HEXOSE TRANSPORT IN *ARTHROBACTER PYRIDINOLIS*

by

MARK E. SOBEL

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1975

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

May 12, 1975
Date

Chairman of Examining Committee

May 12, 1975
Date

Executive Officer

The City University of New York

ABSTRACT

HEXOSE TRANSPORT IN ARTHROBACTER PYRIDINOLIS

by

Mark E. Sobel

Advisor: Dr. Terry Ann Krulwich

The transport of glucose and fructose has been studied in *Arthrobacter pyridinolis*, an organism very limited in its ability to use hexoses and other carbohydrates as sole carbon source for growth. Fructose and rhamnose are the only known hexoses which this bacterium can utilize without special growth conditions. *A. pyridinolis* cannot grow on glucose as sole carbon source, although the cells possess catabolic enzymes of the Embden-Meyerhof and pentose phosphate pathways as well as a complete tricarboxylic acid cycle. Crypticity toward glucose is abolished by a period of growth in a medium containing malate, succinate, citrate, or fumarate in addition to glucose. Other carbon sources, which support as rapid growth as does malate (e.g. asparagine), do not enable the cells to use glucose. Malate, succinate, citrate, and fumarate abolish glucose crypticity only in the second phase of diauxic growth after the tricarboxylic acid cycle intermediate has been depleted. These organic acids also enhance the utilization of the α-glucosides sucrose and maltose. The mechanism whereby growth on certain tricarboxylic acid cycle

intermediates confers the subsequent ability to grow on glucose is related to an inducible transport system for glucose and α-glucosides which is repressed during growth on malate. Transport of 2-deoxyglucose, a nonmetabolizable analog of glucose, shows Michaelis-Menten kinetics with a K_m of 2.9×10^{-4} M and is competitively inhibited by glucose, α-methylglucopyranoside, and maltose. The 2-deoxyglucose transported into the cell is not phosphorylated, and no phosphoenolpyruvate (PEP): glucose phosphotransferase activity has been detected in cell extracts. The transport of 2-deoxyglucose is inhibited by respiratory chain inhibitors, uncouplers, and N-ethylmaleimide, indicating that glucose is transported by a respiration-coupled system.

When growth occurs on fructose as sole carbon source, *A. pyridinolis* utilizes an inducible four component PEP: fructose phosphotransferase which converts the sugar to fructose 1-phosphate. The PEP: fructose phosphotransferase was studied in membrane vesicles which are devoid of cytoplasmic components but retain the ability to carry out active transport in the presence of exogenously added energy sources. PEP-dependent uptake of fructose showed Michaelis-Menten kinetics with a K_m of 1.5×10^{-5} M.

Fructose uptake in membrane vesicles could be stimulated by malate almost as well as by PEP. It was also noted that phosphotransferase-negative mutants that could not grow on fructose as sole carbon source were able to utilize the hexose in the presence of malate. These observations led to

the conclusion that *A. pyridinolis* can transport fructose by either the phosphotransferase or by a malate-dependent system. Malate-dependent uptake of fructose exhibited Michaelis-Menten kinetics with a K_m of 5.6×10^{-7} M. A sugar-specific component of the malate-dependent system, which is distinct from components of the phosphotransferase, has been demonstrated. Inhibitors of electron transport, uncouplers, and N-ethylmaleimide inhibit malate-dependent fructose uptake but exert no effect on PEP-dependent transport of the sugar. Activity of a flavin adenine dinucleotide-linked malate dehydrogenase has been detected in membrane vesicles and whole cells and may be involved in the malate oxidation that is coupled to respiration-coupled transport.

Fructose transported by the malate-dependent respiration-coupled system is metabolized by adenosine 5'-triphosphate-dependent conversion to fructose 6-phosphate and subsequent isomerization to glucose 6-phosphate or phosphorylation by fructose 6-phosphate kinase to form fructose 1,6-diphosphate. On the other hand, fructose 1-phosphate formed by PEP: fructose phosphotransferase is converted by fructose 1-phosphate kinase to fructose 1,6-diphosphate for entry into the Embden-Meyerhof pathway.

In addition to repression of the glucose transport system during growth on malate, high concentrations of tricarboxylic acid cycle intermediates also appear to repress formation of the fructose-specific inducible components of the PEP: fructose phosphotransferase. This repression is

due to a primary effect of the organic acid rather than a secondary effect due to depression of inducer transport.

ACKNOWLEDGEMENTS

I am deeply grateful to Dr. Terry Ann Krulwich for introducing me to the world of science and for her guidance and encouragement throughout my graduate career. I also appreciate the constant support and understanding of my parents and friends during the preparation of this thesis. In particular, I would like to thank Ellen B. Wolfson, whose ancillary work on fructose transport expedited the completion of this work, and Dr. Jerome Schwartz for his electronmicroscopic studies of isolated membrane vesicles.

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LITERATURE REVIEW:
THE REGULATION OF BACTERIAL TRANSPORT SYSTEMS

In the 1950's, Cohen and Monod proposed, on the basis of evidence from studies conducted in their own and other laboratories, a theory of selective permeation of molecules across bacterial membranes (1). Their formulation involved specific proteins, or "permeases" which were proposed to exist in the membrane, capable of forming stereospecific, reversible complexes with different substrates. The complex of permease and its specific substrate could dissociate and associate on either side of the membrane. The effect of the permease was therefore to activate catalytically the equilibration of the concentrations of the substrate on either side of the membrane. It is clear today that bacterial transport systems are more complex than originally proposed by early workers in the field. Transport systems may involve more than one protein, and different carriers may mediate entry and exit of substrates (2,3). The term permease has either been abandoned or is reserved for the entire transport process, which may involve more than one protein in or around the membrane (4). The permease concept proposed by Cohen and Monod and their co-workers, however, focused attention on the role of specific proteins in bacterial transport processes. The existence of selective permeation systems mediating the entry of organic substrates into bacterial cells explained the capacity of cells to

accumulate certain molecules internally. Similarly, the absence of specific permeation systems explained the state of "crypticity" of certain cells toward particular substrates, i.e., their incapacity to utilize a given substrate even though they possess the relevant enzymes for catabolism. The transport of a substrate into the cells, then, can be considered to be the first in a sequence of enzymatic reactions which result in the utilization of that molecule. Moreover, it is often the rate-limiting reaction in the sequence (4). As such, the regulation of transport systems assumes major importance for the survival of a bacterial cell in a competitive environment.

In general, three mechanisms by which substances cross membranes using specific proteins have been described. These are "facilitated diffusion," "active transport," and "group translocation." While both active transport and facilitated diffusion involve specific carriers, only the former results in the accumulation of the transported solute across an electrochemical or osmotic gradient. Thus, in active transport, metabolic energy is required. The study of transport in membrane vesicles has been instrumental in helping to understand the mechanism of how active transport is energy coupled. These vesicles, first prepared by Kaback and his co-workers (5,6), are devoid of cytoplasmic constituents of the intact cell, so their metabolic activities are restricted to those provided by the enzymes of the membrane itself. Significantly, these mem-

brane vesicles require exogenous sources of energy in order to carry out transport. In a recent review, Kaback (7) has described several systems in a variety of organisms in which the energy derived from the oxidation of specific compounds via membrane-bound dehydrogenases and respiratory chains is coupled to transport. Anaerobic transport systems have also been demonstrated to be coupled to the oxidation of particular substrates via sequences of electron-transfer reactions (8). The molecular mechanism by which the energy released by electron donors is coupled to active transport is not clear, but a chemiosmotic mechanism of some sort is thought to be involved in respiration-coupled transport. This subject has been recently reviewed by Harold (9) and Kaback (7,10). On the other hand, Berger and Heppel (11,12) have suggested that transport systems in which periplasmic binding proteins are involved have a different mechanism of energy coupling involving phosphate bond energy derived from either glycolysis or oxidative phosphorylation. Such binding proteins can be isolated from gram-negative bacteria by a procedure developed by Heppel and his co-workers (13), involving treatment of the cells with Tris-EDTA followed by cold osmotic shock. Although the role that binding proteins play in transport has not been fully elucidated, the activities of transport systems in which these proteins appear to be involved are sharply reduced by osmotic shock and are absent from membrane vesicles.

Like active transport, the third transport mechanism, group translocation, is metabolically dependent. However, while in active transport the substrates enter the cell without chemical alteration, in group translocation the transported molecule is covalently changed during its passage through the membrane.

There follows a review of selected mechanisms whereby bacterial transport systems, of various kinds, are regulated. Among the devices which microbial cells use to regulate their transport processes are controls exerted on enzyme formation and on enzyme activity. Included in the former category are the specific induction and repression of individual transport systems and the more general effects of catabolite repression. Although these controls resemble those which are used to regulate intracellular metabolism, several novel features exist. In many cases, transport systems appear to play a critical role in regulating the synthesis of catabolic enzymes. In addition, particular substrates may be transported by more than one permease, the different permeases functioning independently from each other.

I. Inducible transport systems.

Negative control of the lac permease and autocatalytic induction. Since Jacob and Monod (14) formulated their operon theory relating the function of structural

genes and regulatory genes, several transport systems have been described in which the structural gene(s) for the permease is part of an operon and production of a transport protein is coordinately controlled with the other genes comprising the operon. Probably the best studied operon is the group of genes coding for the formation of proteins involved in β-galactoside hydrolysis and transport in *Escherichia coli*, known as the *lac* operon. Indeed, the finding that the *lac* permease and β-galactosidase are coordinately regulated as a functional genetic unit was crucial in Jacob and Monod's development of the operon theory.

The *lac* operon is under "negative control"; i.e., a repressor substance coded for by the regulatory *i* gene prevents transcription of the operon's structural genes by binding to the operator of the operon. When present, an inducer can react with the repressor, thereby inactivating it and releasing the repression. The structural genes of the operon can then be expressed (14). The fact that some inducers of the *lac* operon are also substrates for the *lac* permease has been used to explain the kinetics of induction of enzymes of the operon. Novick and Weiner (15) exposed cultures of *E. coli* to saturating and suboptimal concentrations of the gratuitous (non-metabolized) inducer methyl-β-D-thiogalactoside (TMG) and followed the differential synthesis of enzymes of the *lac* operon. They observed

that whereas uninduced cells responded rapidly to a saturating concentration of inducer, the response to a lower concentration of inducer was slower. On the other hand, they noted that preinduced cells were able to maintain the induced state even at nonsaturating concentrations. These results were explained by the fact that in the presence of inducer, the chance entry of an inducer molecule into a cell begins an "autocatalytic" cycle resulting in rapid and full induction of that cell. That is, as inducer enters the cell (via a basal level of *lac* permease), a small number of repressor molecules are bound and inactivated, permitting the cell to synthesize molecules of permease. This in turn results in the accumulation of a greater amount of inducer, the binding of more repressor molecules and the synthesis of still more *lac* gene products. This cyclical process continues until the cell synthesizes *lac* gene products at a maximal rate. Thus, the response of an individual cell to inducer is "all-or-none." Once the induction process is initiated, transport units are formed that ensure rapid induction of that cell, and even low concentrations of inducer are then sufficient to maintain the induced state.

Early genetic studies of the *lac* operon revealed that cells of *E. coli* which contained a functioning *y* gene could transport lactose. Mutants deficient for lactose transport were found to have either a mutation in the *y*

gene or a regulatory or polar mutation which impaired expression of this structural gene (4,16,17). In 1965, Fox and Kennedy (18) isolated a membrane localized protein which was a component of the *lac* transport system. Fox and Kennedy made use of the fact that N-ethylmaleimide (NEM) irreversibly inhibited the transport system for lactose with high (but not complete) specificity without affecting other enzymes of lactose metabolism. The addition of β-D-galactosyl 1-thio-β-D-galactoside (TDG), another analog of lactose, prevented inactivation by NEM, indicating that the site of sensitivity to NEM had a high affinity for TDG. Fox and Kennedy then proceeded to label the *lac* permease specifically with radioactive NEM. They first incubated cells with unlabeld NEM in the presence of saturating amounts of TDG. After removal of the cold NEM and the TDG, the cells were treated with radioactively labeled NEM. In this manner, they found that an inducible, NEM-sensitive, TDG-binding protein essential for transport of lactose and its analog *o*-nitrophenyl-β-D-galactoside (ONPG) was in the particulate fraction of the cell. Kennedy and his co-workers designated this protein the M (for membrane) protein. They devised an assay for the presence of the M protein in cell-free fractions of *E. coli* based on its affinity for TDG and the protective effect of bound TDG in preventing its reaction with labeled NEM. Using this assay, Fox, Carter, and Kennedy (19) established

that the M protein was present only in y^+ strains, that it appeared in inducible cells only after induction, but was present in constitutive (i^-) strains even in the absence of inducer, and that none of a number of strains with amber or deletion mutations in the *y* gene contained the M protein. Most significantly, they isolated mutants with a temperature-sensitive M protein and correlated temperature-sensitivity of the lactose transport system with temperature-sensitivity for the binding of TDG. It was thus established that the M protein was the product of the *y* gene and that it was regulated coordinately with the other gene products of the *lac* operon.

Kennedy and his coworkers characterized the M protein as being highly hydrophobic and firmly bound to the membrane; it was extracted from the membrane using detergents such as sodium dodecyl sulfate (SDS) or Triton X-100. Indeed, the state of the membrane plays an important role in *lac* permease activity. Fox (20) has studied the effects of unsaturated fatty acids on *lac* permease activity in a mutant of *E. coli* which is defective in the synthesis of unsaturated fatty acids. He found that induction of *lac* permease activity, as measured by TMG accumulation and ONPG hydrolysis, was dependent upon the presence of a long chain unsaturated fatty acid. In contrast, the induced synthesis of the other enzymes coded by the *lac* operon does not show dependence on the fatty acid. In

addition, Fox showed that once induced, function of the *lac* permease is not as critically dependent upon the presence of the lipid. Apparently, when the auxotroph is deprived of unsaturated fatty acids, assembly of the M protein into the membrane is abortive. Temperature also influences assembly of the *lac* permease (21).

Initial studies on the binding site for NEM utilized NEM prepared from radioactively labeled maleic anhydride. Carter, Fox and Kennedy (22) isolated radioactive S-succinyl-cysteine from partial acid hydrolysates of M protein that had been incubated with the labeled NEM. This indicated that a reactive cysteine on the M protein is the site of reaction with NEM. Studies of the interaction of sugar substrates of the *lac* transport system with the M protein (22) revealed that only TDG and melibiose had a significant protective effect against NEM binding. Phenyl-β-D-galactoside, glycerol-β-D-galactoside, and allolactose were found to bind to the reactive cysteine with much lower affinity. Still another class of sugars, including ONPG, TMG, isopropyl-β-D-thiogalactoside (IPTG), and lactose itself, exhibited little or no protective effect. Significantly, these latter sugars did not compete with TDG in protecting against NEM binding. These results indicated that there are two binding sites on the M protein, only one of which binds NEM. This finding was confirmed by directly measuring the binding of

labeled sugars to the M protein (23). Kennedy concluded that Site I binds sugars of Class I such as lactose, whereas Site II, containing the reactive cysteine, binds NEM, TDG, melibiose, and several α-galactosides (23).

Kennedy has speculated that site II serves a regulatory function in the cell, and that if a metabolite accumulated in the cell during growth on lactose with affinity for this site, binding of lactose at site I would be reduced (4). Noting the affinity of α-galactosides for site II, he has suggested that the transport of lactose may be regulated by the internal level of some α-galactoside (23). UDP-galactoside and galactose 1-phosphate, however, were found to have little or no affinity for site II (23).

Boos (24) has noted that substrates of site II are not transported as well as sugars that bind to site I, and has speculated that the ability of substrates to protect the sensitive cysteine might be inversely related to their rate of transport. He further suggests that site II (sulfhydryl-sensitive) is involved in the mechanism by which energy is coupled to transport. In support of his suggestion, Boos (24) has cited the isolation by Wilson and his co-workers of two mutants of *E. coli* with a defect in the mechanism by which energy is coupled to the active transport of lactose and β-galactosides (25-27). The defect in one of these mutants, X71-54, was mapped in the *y* gene. These mutants exhibit an increased sensitivity

against sulfhydryl reagents in their transport activity, and a severely decreased ability to accumulate nonmetabolizable β-galactosides such as ONPG. The latter property is apparently due to a lower K_m for the exit of lactose and its analogs, leading to abnormally rapid exit of the transported substrate; Winkler and Wilson (28) had previously demonstrated that energy is coupled to β-galactoside transport by increasing the K_m of exit. It is interesting to note, the observation of Kaback that sulfhydryl-containing proteins appear to be involved in energy coupling of transport of several substrates, including lactose, to respiratory chain activity (29,30).

Induction of other transport systems for galactose and galactosides. The *lac* permease is only one of several systems which have been described in *E. coli* by which galactose can be transported. One of the more interesting of these systems was first described by Prestidge and Pardee (31) and was designated the TMG permease II. In addition to TMG, this system can transport galactose and the β-galactosides TDG and methyl-β-D-galactopyranoside, but not lactose or ONPG (4,32). The genetic regulation of the TMG permease II is coordinated with that of α-galactosidase (33). These enzymes can be induced by growth in the presence of galactinol, galactose, and the α-galactoside melibiose (4,32). The β-galactosides TMG and IPTG, which can induce the *lac* system, are not inducers of TMG

permease II (32). Buttin (33) has studied the conditions under which the TMG permease II is induced in several strains of *E. coli*. Induction in K-12 strains does not take place at 37°C or higher, although normal induction occurs at 30°C. Since the TMG permease II and α-galactosidase synthesized at low temperatures are not inactivated when the temperature is shifted to 40°C, Buttin concluded that induction is temperature-sensitive. In support of this conclusion, Buttin found that derivatives of a K-12 strain selected for their ability to grow on melibiose at 40°C were partially constitutive. In contrast to the temperature-sensitive induction in K-12 strains, induction of TMG permease II in *E. coli* B strains is normal at 37°C, and the ML strain lacks TMG permease II and α-galactosidase whether grown at 30°C or 37°C.

Probably of more physiological significance than the above systems for the effective concentration of galactose is the β-methylgalactoside permease, first described by Rotman (34). The β-methylgalactoside permease was initially characterized in *E. coli* strains defective in galactokinase (35). Early studies indicated that the β-methylgalactoside permease might be coordinately regulated with the *gal* operon. This operon codes for three enzymes involved in galactose metabolism: galactokinase (K), galactose 1-phosphate uridyltransferase (T), and UDP-galactose 4-epimerase (E) (36). Buttin (37) has found a specific regulator gene

Rgal for the *gal* operon. Unlike the *lac* operon, the *gal* operon is never completely repressed and hence relatively high levels of the enzymes it codes for appear in uninduced cells (36). Induction by galactose or fucose results in a ten-fold increase in enzyme levels (36). Rotman, Ganesan, and Guzman (32) and Wu, Boos, and Kalckar (38) found that the β-methylgalactoside permease was also induced by galactose or fucose. In addition, coordinate endogenous induction of the *gal* operon and the β-methylgalactoside permease was encountered in *E. coli* K-12 strains of the phenotype $K^- T^+ E^+$ (38). Such strains showed a derepression of the *gal* operon, i.e., the transferase (T) and epimerase (E) were synthesized at the fully induced rate even when galactose was absent from the medium (39,40). It was subsequently found that this apparent constitutivity was due to a metabolic "cul de sac" by which UDP-galactose (formed from endogenous UDP-glucose by the action of epimerase) was split to form free galactose in the cell and the galactose accumulated because of the galactokinase deficiency. Thus, sufficient intracellular galactose was produced to induce the *gal* operon (36). The β-methylgalactoside permease was also induced under these conditions (38). Wu and Kalckar (41) demonstrated that strains deficient in the epimerase did not endogenously induce either the β-methylgalactoside permease or the transferase because no UDP-galactose was formed from UDP-glucose. It was

further found that β-methylgalactoside permease-deficient strains did not endogenously induce the *gal* operon (38). Wu and Kalckar (38) demonstrated that whereas the cellular threshold concentration of galactose for induction of the *gal* operon was 2×10^{-4}M, galactose concentrations of only 0.8×10^{-4}M and 0.1×10^{-4}M were found inside β-methylgalactoside permease-defective and epimerase-defective cells, respectively. It was concluded that galactose can leak from cells and that the β-methylgalactoside permease was required for the "recapture" of the galactose. In an elegant study using mixed cultures, Wu and Kalckar (38) demonstrated that strains defective in the β-methylgalactoside permease with a phenotype K^- E^+ could "leak" sufficient galactose into the medium to induce the β-methylgalactoside permease of a test response strain with the phenotype K^- E^-.

In spite of all these early indications for coregulation of the *gal* operon and the β-methylgalactoside permease, it ultimately became clear that the two are not truly coordinately controlled. Rotman and Radojkovic (42) mapped the structural gene for the permease in the vicinity of the histidine region of the *E. coli* chromosome. Using mutants of the *Rgal* gene, Ganesan and Rotman (43) demonstrated that this regulator gene did not control the inducibility of the β-methylgalactoside permease.

Further characterization of the β-methylgalactoside

permease has involved the study of a galactose binding protein, first isolated by Anraku (44-47). Boos (48) demonstrated the close relationhsip (i.e., similar properties) of the galactose binding protein and the β-methylgalactosides permease. Lengeler *et al.* (49) confirmed the work of Ganesan and Rotman (43) in demonstrating that the regulatory gene of the *gal* operon, *Rgal*, does not function in the regulation of the β-methylgalactoside transport system. These workers also isolated a mutant strain exhibiting constitutivity for both binding protein synthesis and transport activity. They demonstrated the existence of a regulator gene, *mglR*, for the β-methylgalactoside permease which was distinct from the regulatory genes for the *lac*, *gal*, and other operons. The most convincing evidence that binding protein synthesis and transport activity are coregulated comes from the work of Boos (50), who isolated an altered galactose binding protein from a mutant of *E. coli* defective in β-methylgalactoside permease. The simultaneous alteration in the structure and activity of the galactose binding protein upon mutation of the wild type strain to a transport-negative strain and its reversion to a transport-positive strain demonstrated the essential role of the galactose binding protein in the function of the β-methylgalactoside transport system. Parnes and Boos (2) demonstrated that the binding protein mediated entry but not exit of galactose and suggested that other components of the β-methylgalactoside transport system

mediate exit.

In addition to the *lac* permease, TMG permease II, and β-methylgalactoside permease, *E. coli* cells contain a fourth permease for galactose, the "galactose permease" (43). Although this permease does not have as high an affinity for galactose as the β-methylgalactoside permease, it has been given its name for its ability to transport galactose and no galactosides (32). Arabinose is the only sugar other than galactose which the galactose permease can transport (32). The galactose permease can be induced by TMG, fucose, or galactose (32). Wilson (51) has recently studied the galactose permease in several *E. coli* K-12 strains. In strain D22, glucose was the only potent inhibitor of galactose uptake, although high concentrations of fucose also inhibited to some degree. This strain is defective in both the *lac* permease and β-methylgalactoside permease. Under the conditions of the study, the TMG permease II was not induced. The regulation of the galactose permease was studied in strain D22 and in two isogenic strains which had fully constitutive synthesis of the enzymes of the *gal* operon as a result of a mutation in either the *gal* regulatory gene, *Rgal*, or the *gal* operator locus. The results indicated that the galactose permease is regulated by the regulatory gene of the *gal* operon, *Rgal*, but is not part of the *gal* operon since it is not affected by a mutation in the *gal* operator locus.

In order to study the interaction of the β-methylgalac-

toside and galactose permeases, Wilson (51) introduced the β-methylgalactoside permease into strain D22 with the use of episomes. Strains containing such an episome were able to transport β-methylgalactoside and contained, in addition to the galactose permease, another system for the transport of galactose with a K_m of approximately 5×10^{-7} M, the value reported for the β-methylgalactoside permease. Such strains showed an increased rate of galactose transport over strain D22 at low but not high galactose concentrations. Wilson (51) distinguished between galactose transported by the β-methylgalactoside permease and that transported by the galactose permease by adding unlabeled β-glycerolgalactoside, which is a competitive inhibitor of galactose transport by the former system but has no effect on the galactose permease. He concluded that the β-methylgalactoside permease is inhibited about 33% at high galactose concentrations. Kinetic data of galactose transport in this strain could not be fitted by a model in which galactose transport was due to two independent systems. Instead, the data indicated that there was an interaction between the two permeases which affected the properties of both.

Several workers have reported that in addition to the above systems, galactose is a substrate of a transport system for arabinose in *E. coli*. Novotny and Englesberg (52), Schleif (53), and Brown and Hogg (54) have reported that galactose competitively inhibits arabinose uptake or the binding of arabinose to an arabinose binding protein. In addition, Messer (55) has recently reported that lactose

and TMG are low-affinity substrates for an arabinose permeation system. Despite some similarities between the galactose binding protein and the arabinose binding protein, and some cross-reactivity observed between antisera directed against them, Parsons and Hogg (56) have demonstrated that they are distinctly different proteins.

Positive control of the arabinose and maltose transport systems. The transport of arabinose in both *E. coli* B/r and K-12 strains is under the control of *araC*, the regulatory gene for the arabinose operon (53,57). However, as is the case for galactose transport, the gene(s) for arabinose transport are not part of the arabinose operon (53, 57,58). In contrast to the negative or repressor control of gene expression as seen in the *lac* and *gal* operons, Englesberg and his co-workers have demonstrated that the arabinose operon is under positive control; i.e., the *araC* gene product is required for expression of the arabinose operon and the arabinose permease(s) (57). In this system, arabinose (the inducer) interacts with the *araC* gene product and converts it to an activator of gene expression. Novotny and Englesberg (52) first described an uptake system for arabinose in *E. coli* B/r and documented an inducible, energy-dependent uptake system for arabinose with a K_m of 1.25×10^{-4} M. Uptake of arabinose by this system was abolished by mutations at a locus designated *araE*, which was located on the *E. coli* chromosome at a point other than the site of the arabinose operon. Hogg and Englesberg (58) isolated an

arabinose binding protein from *E. coli* B/r which bound arabinose with a K_m of 5×10^{-6} M. Studies of mutant strains defective in arabinose uptake indicated that the structural gene for the binding protein was not *araE*, although the binding protein was also under the control of the *araC* gene product. Brown and Hogg (54) conducted kinetic studies of initial rates of arabinose uptake in *E. coli* B/r and demonstrated two transport systems. The two systems could also be differentiated by substrate specificities. Mutants of each system were isolated and it was concluded that the higher affinity system involved the arabinose binding protein. Mutants lacking the low affinity system were *araE* mutants.

The structural gene(s) for the maltose permease in *E. coli* have also been demonstrated, by Schwartz and his co-workers, to be under positive control by the *malT* gene which is associated with the *malA* gene cluster (59). Three genes in the *malB* region of the *E. coli* chromosome, *malE, malF,* and *malK,* have been shown to be involved in the transport of maltose (59). Kellerman and Szmelcman (60) have recently purified a maltose binding protein from *E. coli* and demonstrated that its synthesis is under the same positive control as the *mal* operon. These workers also demonstrated that the *malE* gene is necessary for the biosynthesis of the binding protein. Mutational inactivation of the *malE* gene leads to a loss of maltose transport, however, some mutants have been isolated which are deficient in transport of mal-

tose but contain the maltose binding protein (81).

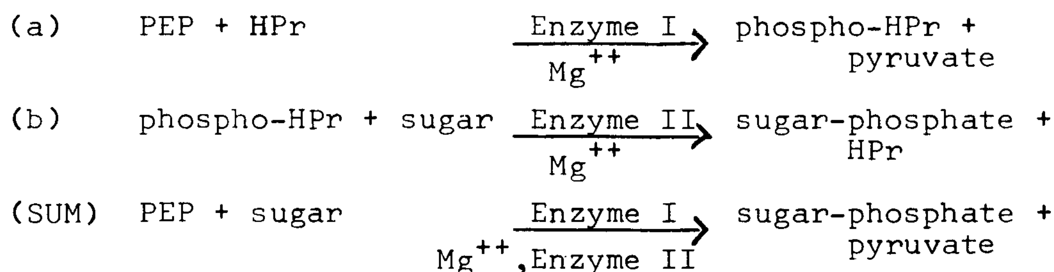
Exogenous induction of the hexose phosphate permease. An inducible transport system for hexose phosphates has been described in several species (61). Perhaps the most interesting feature of this transport system is its regulation, which has been termed "exogenous induction" (62). Although it had been known for some time that several bacterial species can utilize exogenous glucose 6-phosphate, it was generally assumed that the first step in the utilization of the phosphorylated sugar was dephosphorylation, probably via alkaline phosphatase localized at the periphery of the cell, followed by uptake of the free sugar. In 1951, however, Roberts and Wolffe (63) reported that when *E. coli* was grown on glucose 6-phosphate or fructose 6-phosphate as the sole carbon source, most of the organic phosphate of the cell came from the hexose phosphate substrate despite the presence of excess inorganic phosphate. It was thus suggested that utilization of hexose phosphates need not involve prior hydrolysis. This was indeed shown to be the case by the work of Fraenkel, Falcoz-Kelly and Horecker (64) who showed that a mutant unable to phosphorylate glucose could grow at a normal rate on glucose 6-phosphate. These workers also provided evidence that the transport system for glucose 6-phosphate is induced by that compound and not by glucose. Winkler (65) isolated hexose phosphate transport-negative mutants which could grow on hexoses but not on hexose 6-phosphates, thereby demonstrating that separate systems are

involved in the transport of the free sugars and the corresponding phosphates.

Winkler (62) used mutants blocked in both phosphoglucose isomerase and glucose 6-phosphate dehydrogenase to study the induction process. When grown in the presence of glucose these mutants accumulated large amounts of glucose 6-phosphate intracellularly but remained uninduced for hexose phosphate transport. When grown in the presence of glucose 6-phosphate, however, these mutants were induced for hexose phosphate transport. It was thus demonstrated that exogenous glucose 6-phosphate was necessary for induction of the hexose phosphate permease. It was also shown that the large amounts of glucose 6-phosphate accumulated intracellularly on growth of the mutant in the presence of glucose were not inhibitory to induction, since the hexose phosphate permease was induced upon addition of glucose 6-phosphate to glucose in the growth medium. Although Winkler (65) demonstrated that mannose 6-phosphate and fructose 6-phosphate could also induce the hexose phosphate permease, it was later found that fructose 6-phosphate was not an inducer in mutants of phosphoglucose isomerase, indicating that fructose 6-phosphate was not the direct inducer (62). Friedberg (66) demonstrated that part of the phosphoglucose isomerase is localized in the periplasmic space, making the fructose 6-phosphate in the medium susceptible to conversion to its glucose isomer.

Sugar transport via inducible phosphoenolpyruvate-

dependent phosphotransferase systems. In 1964, Kundig, Ghosh, and Roseman (67) reported the isolation of a multicomponent phosphotransferase system in *E. coli* which catalyzed the sequential transfer of phosphate from phosphoenolpyruvate (PEP) to a heat-stable, histidine-containing protein (HPr or phosphocarrier protein) to various carbohydrates. As initially described, the sequence of reactions was:



Although no difference in enzyme I or HPr was detected between glucose-grown and galactose-grown cells, more than one enzyme II was formed by the cells. It thus seemed that enzyme II was sugar specific. A close relationship between the PEP: hexose phosphotransferase (PTS) and carbohydrate permeases was first demonstrated by Kundig *et al.* (68). These workers osmotically shocked cells of *E. coli* and showed that they lost most of their HPr along with their ability to transport two glycosides. Genetic studies further clarified the role of the PTS in bacterial physiology. Tanaka, Fraenkel, and Lin (69) analyzed a pleiotropic carbohydrate mutant of *E. coli* and found it was lacking enzyme I of the PTS. Lin and his co-workers (70,71) also studied mutants of *Aero-*

bacter aerogenes unable to utilize mannitol and isolated mutants deficient in either enzyme I, HPr, or an inducible enzyme II specific for mannitol. Mutants deficient in either enzyme I or HPr showed a pleiotropic deficiency in utilizing not only mannitol but also sorbitol, glucose, fructose, and mannose. The mutant deficient in enzyme II was unable to utilize mannitol, but could grow normally on other carbohydrates. None of the mutants could accumulate radioactive mannitol. A spontaneous revertant selected on a single carbon source was isolated from enzyme I- and HPr-deficient mutants. All the other pleiotropic defects were corrected in the revertant.

Simoni *et al.* (72), studying the PTS in *Salmonella typhimurium*, isolated an enzyme I-deficient mutant which was unable to utilize nine carbohydrates readily utilized by the parent strain. The physiological defect in the mutant was shown to be an inability to take up sugars from the medium, thus linking the PTS to sugar transport. It was thus concluded that phosphorylation by the PTS is required for the transport of its substrates. In support of this conclusion, the work of Winkler (65) was cited, in which he showed that during the first minute of transport of α-methylglucoside by *E. coli*, only the substrate's phosphate ester was detected.

Morse and his co-workers (73-77) isolated and studied pleiotropic transport mutants of *Staphylococcus aureus*. It was subsequently demonstrated that such mutants were defec-

tive in either enzyme I or HPr (78,79).

The most direct proof that the PTS is responsible for entry of sugars by phosphorylation, and that the sugars are not transported and then phosphorylated by the PTS, came from the work of Kaback (80) who studied the PTS in membrane vesicles. Such membrane preparations specifically required PEP for the uptake of sugars, which accumulated as phosphorylated derivatives. On the other hand, membranes prepared from a mutant strain lacking enzyme I of the PTS were unable to take up significant quantities of α-methylglucoside. The loss of ^{32}P from PEP was stoichiometrically related to the appearance of ^{32}P in α-methylglucoside-phosphate. Significantly, Kaback showed that ^{3}H-glucose added to the incubation medium was phosphorylated more rapidly than free ^{14}C-glucose in the intramembranal pool, suggesting that the PTS is a mechanism by which sugars penetrate the membrane. Kelker and Anderson (81) subsequently demonstrated vectorial phosphorylation (i.e., phosphorylation of a compound concomitant with its translocation across the membrane) in intact cells of *A. aerogenes*.

Roseman and his co-workers have studied the PTS in detail in both *E. coli* and *S. aureus*. In both organisms, it was shown that the PTS is more complex than originally described, containing a phosphocarrier protein in addition to HPr. In *E. coli*, Kundig and Roseman (82) fractionated the enzyme system into two constitutively-synthesized soluble constituents, enzyme I and HPr, and a membrane fraction.

Kundig and Roseman (83) then studied several enzymes II of the *E. coli* PTS which were synthesized constitutively by that organism. These workers isolated the membrane fraction from glucose-grown cells. Extraction of the membrane fraction with urea and 1-butanol yielded a protein fraction (II-A) which catalyzed the transfer of phosphate from PEP to glucose, fructose, and mannose in the presence of enzyme I, HPr, and the residual pellet. Fraction II-A was purified and subfractionated by isoelectric focusing to three proteins. Each protein II-A catalyzed the phosphorylation reaction with a specific sugar in the presence of the other PTS components. The remaining membrane fraction was treated with deoxycholate and was fractionated, giving another protein (II-B) which was purified free of lipid. The combination of protein II-B and a specific protein II-A was inactive in the phosphorylation reaction unless supplemented with phosphatidylglycerol and either Ca^{++} or Mg^{++}. Activity was also dependent upon the order of mixing of the four components of the membrane fraction.

An analogous but not identical PTS was studied in *S. aureus*. Fractionation of the PTS in this organism showed that it contained four components. In addition to enzyme I and HPr, which were constitutively synthesized, a third soluble component was isolated (78,84). This component, which was sugar-specific and inducible, was designated factor III. The membrane fraction yielded an enzyme II which could not be further fractionated and appeared to be

analogous to protein II-B of the *E. coli* system. The factor III specific for lactose was purified and found to contain three identical subunits (85). Phosphorylation experiments indicated that up to three phosphoryl groups could be transferred to it. The reaction catalyzed by the PEP: β-galactoside PTS of *S. aureus* was studied by Roseman and his co-workers (86). The initial phosphoryl transfer from PEP to HPr was catalyzed by enzyme I. Only one phosphoryl group was transferred to each molecule of HPr. The phosphoryl group was next transferred to the sugar-specific factor III in a self-catalyzed reversible reaction which did not require any additional protein component. The phosphoryl groups in both phosphocarrier proteins are linked to the imidazole moieties of histidine residues, to N-1 in HPr and N-3 in factor III. The final step, formation of galactoside 6-phosphate, was catalyzed by the lactose-specific enzyme II. This enzyme was able to directly bind lactose in the absence of other components.

There appears to be considerable variety among the PEP-dependent phosphotransferases studied to date. Anderson and his co-workers (87,88), for example, in their characterization of the fructose PTS in *Aerobacter aerogenes*, found that an inducible protein called the "K_m factor" was required for activity with an induced enzyme II for fructose. HPr was not required for activity with this inducible enzyme II, although it was necessary for a second enzyme II with a low affinity for fructose which was constitutive. This consti-

tutive enzyme II was unaffected by K_m factor. The PEP: fructose PTS of the photosynthetic bacteria *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* is also unusual, containing only two components, one of which is tightly associated with the membrane (89).

In general, however, the net transfer of phosphate from PEP to a given sugar requires four proteins. Two PTS proteins, HPr and enzyme I, are required to phosphorylate all sugars transported by the PTS. In addition, phosphorylation of a particular sugar requires two sugar-specific proteins. One of these is the membrane bound II-B. The other is either factor III or enzyme II-A. Most of the sugar-specific protein pairs are inducible, while enzyme I and HPr are usually synthesized constitutively, or can be induced by a number of sugars (90). Roseman and his co-workers have mapped the genes specifying enzyme I and HPr in *S. typhimurium*, and have found that the two loci are linked (91). In addition, they have identified a promoter-like mutation, *ptsP*160, which results in a reduced expression of the genes specifying enzyme I and HPr in this organism (92). Pseudorevertants of strains carrying this mutation have additional lesions very closely linked to *ptsP*160 which are noninducible for HPr and enzyme I above a basal level. Presumably, strains carrying *ptsP*160 are defective in the normal induction mechanism for HPr and enzyme I. The existence of a promoter-like element governing the coordinate inducibility of both HPr and enzyme I suggests that the

genes coding for these two components constitute an operon. Synthesis of a sugar-specific factor III in this organism is not affected by the *ptsP*160 mutation, suggesting that the gene coding for this component has its own promoter. In *E. coli*, mutations affecting enzyme I and HPr have been found to lie close to each other by several investigators (93-95), and strict coordinate induction of enzyme I and HPr has been observed (94). It also appears, in at least two cases, that the genes coding for a sugar-specific enzyme II and the next enzyme in that sugar's metabolic pathway may constitute an operon. Induction by galactose 6-phosphate of the PEP: β-galactoside PTS enzyme II and β-galactosidase in *S. aureus* has been reported by Morse and his co-workers (96). The genes specifying these proteins are closly linked. In addition, Solomon and Lin (97) have isolated and mapped mutations of the genes specifying the mannitol-specific enzyme II and mannitol 1-phosphate dehydrogenase in *E. coli* and shown that these genes are closely linked. Furthermore, they have isolated a constitutive mutant which produces both of these enzymes in the absence of inducer. As will be shown below, *Arthrobacter pyridinolis* contains two transport systems for fructose. One is a PEP-dependent fructose PTS, while the other transport system is respiration-coupled. Wolfson and Krulwich (98) have demonstrated that a functional respiration-coupled fructose transport system is necessary for induction of factor III and enzyme II of the fructose PTS. They suggested that enzyme II and factor III may be coordin-

ately regulated. Their results also suggested that a sufficient concentration of either free fructose or fructose 6-phosphate must be accumulated intracellularly via active transport in order for induction of the PTS to occur.

There is little doubt that the PTS plays an important physiological role in the transport of carbohydrates in several organisms. In a survey of the distribution of the PEP: glucose PTS in bacteria, Romano and his co-workers (99) have concluded that the system is found in genera that are characteristically facultative anaerobes, but that it is absent in members of those genera that are strictly aerobic. As will be shown below, however, *Arthrobacter pyridinolis*, an obligate aerobe, utilizes the PTS for the transport of fructose. In addition, Levinson and Krulwich (100) have reported a PTS in *A. pyridinolis* for the transport of rhamnose. Cirillo and Razin (101) have reported that the PTS is present in several species of *Mycoplasma* and noted an apparent correlation between the presence of the PTS and a high affinity for sugar transport in such organisms. In addition to its role in transport, Adler and his co-workers have reported that the PTS plays a role in chemotaxis in *E. coli*, with specificity for the enzyme II correlating with the specificity for sugar taxis (102). The PTS has also been implicated as playing a role in catabolite repression, which will be discussed below.

II. Other controls on production of transport systems.

Catabolite repression and transient repression. The presence or absence of inducer is not the only factor which regulates the synthesis of inducible enzymes. Following the observation of several workers that the ability of bacterial cultures to utilize certain compounds was inhibited by the presence of glucose in the medium, Happold and Hoyle (103) studied the effect of glucose in tryptophan metabolism. By use of cell-free extracts of *E. coli*, these workers demonstrated that glucose does not inhibit already existing enzymes for tryptophan metabolism, but that such enzymes are not formed in the presence of the hexose. Epps and Gale (104) showed a similar effect of glucose on the formation of several amino acid deaminases in *E. coli*, and demonstrated that this "glucose effect" was not an incidental result of acid production during fermentation of the sugar. At about the same time, Monod (105) described the phenomenon of diauxie, i.e., bacteria in the presence of two carbon sources defer the formation of enzymes for using one until the other has been exhausted from the culture medium. Monod discovered, for example, that when cells of *E. coli* are supplied with a mixture of glucose and lactose, they utilize the glucose first and do not form the enzymes essential for the degradation of lactose (i.e., they do not induce the *lac* operon) until all the glucose is exhausted from the medium. The order of substrate utilization may in some cases be dicta-

ted by the affinity of a transport system for its substrates, or by the number of permeases available for entry of the substrates, as discussed by Lengeler and Lin (106).

Magasanik and his co-workers formulated the concept that catabolites which are formed rapidly from glucose accumulate in the cell and repress the formation of enzymes whose activity would augment the already large intracellular pools of these compounds. They suggested that "catabolite repression" is a more appropriate term for the "glucose effect" (107). Although growth rate alone is not an adequate predictor of a compound's ability to cause catabolite repression, the effect may be caused by compounds other than glucose which support a rapid rate of growth (108). Magasanik reasoned that the level of catabolites in the cell depends on the rate of their formation and on the rate of their utilization. Thus, any condition that leads to a decrease in the rate of biosynthesis of protein and nucleic acid without a comparable reduction in the rate of catabolism might lead to an intracellular accumulation of metabolites and to an increased repression of the catabolic enzymes. Under such conditions, compounds usually degraded too slowly to exert catabolite repression might become strong repressors (107).

The effect of catabolite repression is a permanent one. That is, repression persists until the preferred growth substrate is exhausted from the medium. In addition

to the catabolite repression observed during steady-state growth on glucose, a period of more intense repression has been observed to occur immediately after cells are exposed to glucose. This effect, which may last up to one generation, has been termed "transient repression" by Paigen (109). After the severe transient repression, induced enzyme synthesis starts at the reduced rate characteristic of glucose-adapted cells. In studies of transient repression of the *lac* operon Magasanik and his coworkers (110-112) have established that transient repression can be elicited by analogues of glucose which are not catabolized by the cell. In addition, mutants deficient in the ability to metabolize glucose and which are therefore resistant to catabolite repression by glucose are still susceptible to transient repression. They concluded that when transient repression is elicited by the addition of a new compound to the medium, the newly added compound need not be metabolized, and suggested that the actual passage of the compound through the cell membrane is responsible for transient repression.

Studies investigating the molecular basis of catabolite and transient repression have been reviewed by Magasanik (113), Paigen and Williams (114), and Perlman and Pastan (115,116). These studies indicated that neither catabolite nor transient repression involve the same repressor molecules involved in specific induction and re-

pression. Both catabolite and transient repression have been shown to be due, at least in part, to the lowering of the intracellular concentration of cyclic AMP. Cyclic AMP and a binding protein for the cyclic nucleotide have been shown to constitute a positive control system for the activation of catabolite-sensitive genes, stimulating mRNA synthesis by binding at the promoter region of an operon. Cyclic AMP may also play a role in stimulating enzyme synthesis at the translational level (117-121). Little is known about the factors that regulate cyclic AMP levels. Several workers have recently suggested that levels of cyclic AMP may not be a sufficient explanation for the effects of transient repression, and have suggested a role for cyclic GMP as the "effector" of catabolite repression (122,123). However, the physiological role that cyclic GMP plays in the bacterial cell is not known.

The PEP-dependent PTS apparently also plays a role in catabolite repression. Pastan and Perlman (124) reported that mutant strains of *E. coli* deficient in enzyme I or HPr of the PTS are more sensitive to repression of β-galactosidase by glucose or α-methylglucoside than are their parent wild type strains. The adddition of cyclic AMP prevented the repression by glucose, suggesting that the repression was due to the lowering of the intracellular concentration of cyclic AMP by glucose and α-methylglucoside. The studies of Pastan and Perlman (124) helped explain the

finding that several enzyme I and HPr mutants of *E. coli* were unable to utilize certain carbohydrates such as glycerol, lactose, maltose and melibiose (95,125-128) which are not substrates of the PTS in that organism (69). Morse and his co-workers characterized several pleiotropic mutants of *E. coli* (95,125-128) and found that they were indeed deficient either in enzyme I or HPr of the PTS. These mutants were also affected in the ability to synthesize tryptophanase (127). The reversion behavior of these pleiotropic mutants revealed two patterns. Some mutants reverted to complete restoration of the wild type phenotype and were shown to regain enzyme I activity (95,128), while other mutants showed a spectrum of partial wild type phenotypes and did not regain enzyme I activity (128). The reversion to partial wild type phenotype was apparently due to a second mutation at a distant site from the map location of the original mutation. These mutations were in several cases shown to map in the region of the operon coding for uptake of the affected carbohydrate, resulting in altered regulatory properties of the uptake system.

Roseman and his co-workers have extended the observation of hypersensitivity to catabolite repression in enzyme I and HPr mutants to *S. typhimurium* (94,129). They have isolated derivatives of such mutants resistant to repression (130) in *E. coli* and *S. typhimurium*. These mutants

are still deficient in enzyme I or HPr and cannot grow on PTS sugars, although they can utilize certain other carbohydrates which their parent strains cannot. The biochemical deficiency identified with the repression resistant mutation is the lack of the constitutive factor III specific for glucose (131).

Several permeases have been shown to be catabolite repressed by glucose. In *E. coli*, catabolite repression of the *lac* permease (113), L-α-glycerophosphate permease (132), citrate permease (133), arabinose permease (134) in addition to transport systems for fructose (134) and for lactate and succinate (135,136) has been reported. In addition, the citrate permease of *S. typhimurium* (137) as well as the transport systems for melibiose, maltose, and glycerol (131) are sensitive to catabolite repression. In *A. aerogenes*, glucose, succinate and mannitol repress induction of citrate transport (138), and in various *Pseudomonas* species, proline transport (139), lactate transport (135), and succinate transport (135,140) are repressed by glucose. Lactate and succinate transport in *Bacillus subtilis* is also catabolite repressed (135), as is the transport of citrulline in *Streptococcus faecalis* (141) and the transport of tartrate in *Penicillium charlessi* (142).

Regulation of glycerol utilization: "respiratory repression". Lin and his co-workers have described an active transport system in *E. coli* for L-α-glycerophosphate (G3P) which is induced by G3P (143-145). Depending

on the nature of the electron acceptor available, G3P can be converted to dihydroxyacetone phosphate by either of two dehydrogenases (146). Lin and his co-workers (132) have shown that the transport system for G3P, the aerobic and anaerobic G3P dehydrogenases, in addition to a transport system for the facilitated diffusion of glycerol and glycerol kinase, belong to a single regulon (*glp*). The *glp* regulon consists of several operons, all of which are negatively controlled by a repressor which is neutralized by G3P (147). The gene for glycerol kinase and the gene for the "glycerol facilitator" (responsible for the facilitated diffusion of glycerol) form one operon (148) while the gene for the G3P permease and the gene for the anaerobic G3P dehydrogenase appear to belong to another operon (146). The gene for aerobic G3P dehydrogenase is found in a third region of the chromosome, adjacent to the *glpR* locus that specifies the *glp* repressor (149). While synthesis of aerobic G3P dehydrogenase is more sensitive to specific repression, the other two operons are more sensitive to catabolite repression (132). In addition, a third kind of control, "respiratory repression," affects expression of the *glp* regulon (132). This latter effect is exerted by exogenous hydrogen acceptors. Thus, it was demonstrated by Freedberg and Lin (132) that the level of aerobic G3P dehydrogenase is maximal when the cells are grown with oxygen as the exogenous hydro-

gen acceptor, while the levels of anaerobic G3P dehydrogenase and the G3P permease are maximal when the cells are grown with fumarate as the exogenous hydrogen acceptor. Freedberg and Lin (132) have suggested a teleonomic explanation for this phenomenon, i.e., increased levels of G3P permease and anaerobic G3P dehydrogenase may be necessary to compensate for the lower efficiency of anaerobic energy generation. The differential sensitivity of the *glp* operons to specific repression, catabolite repression, and hydrogen acceptors suggests differences in the operator and promoter regions of the three gene clusters of the *glp* regulon.

III. Inducer exclusion.

As noted earlier, the extent of catabolite repression is dependent upon the particular carbon source and the physiological conditions of the culture (107,113, 114). Thus, in general, glucose causes more inhibition of inducible enzyme synthesis that does glycerol. Even with the same carbon source, however, catabolite repression may be more severe if uninduced cells are exposed to low rather than high concentrations of inducers. Work conducted in several laboratories has indicated that glucose can reduce the internal concentration of inducer, thus giving rise to incomplete induction. Cohn and Horibata (150-152) reported that glucose prevented the synthesis of both β-galactosidase and the *lac* permease when added together with a low concentration of TMG to uninduced

cells of *E. coli*. If, however, the glucose was added 15 minutes after addition of the TMG, the inhibition of enzyme synthesis was not as severe. Preinduction did not overcome the inhibitory effect of glucose in a mutant lacking the *lac* permease, indicating that the permease is essential in the induction process. It was also noted that β-galactosidase synthesis was less sensitive to glucose in a constitutive mutant than it was in the inducible parent strain. Increasing the concentration of inducer in the external medium resulted in a less severe inhibitory effect of glucose on β-galactosidase synthesis. This finding was also reported by Clark and Marr (153) and by Loomis and Magasanik (154) in their study of glucose-lactose diauxie. Drawing on their knowledge of the autocatalytic process of permease induction discussed earlier, Cohn and Horibata (152) suggested that glucose inhibits the entry of inducer (TMG), thus preventing induction of the *lac* operon.

Although it is impossible to completely overcome the repressive effect of glucose by raising the concentration of inducer or by preinducing cells (150-154), there is abundant evidence consistent with the hypothesis that one effect of glucose on inducible enzyme synthesis is exerted via inducer exclusion. Several laboratories have reported that glucose inhibits the transport of β-galactosides such as TMG (155-159). This finding was cited by Kepes (156)

and Koch (157) as evidence that a non-specific common carrier existed in the membrane in addition to specific permeases (156,157). However, the inhibition by glucose was observed only in cells grown on glucose. Winkler and Wilson (159) reported that the inhibition was noncompetitive in character. β-Galactosides did not inhibit the uptake of α-methylglucoside, indicating the lack of reciprocity between galactoside and glucose transport systems and making the "common carrier" hypothesis of Kepes and Koch unlikely. Boniface and Koch (158) studied the interaction between galactosides and glucose when the glucose permease and *lac* permease were introduced into the membrane together or separately, and found that glucose inhibition occurred only when the two permeases are synthesized together at sites permitting them to interact. Thus, *lac* permease units incorporated into the membrane in the absence of glucose remained resistant to inhibition by α-methylglucoside during subsequent growth on glucose, but permease units synthesized during growth on glucose were sensitive to inhibition.

Saier and Roseman (131) have reported that sugar substrates of the PTS in *S. typhimurium* catabolite repress the synthesis of enzyme systems required for the catabolism of melibiose, maltose, and glycerol. They suggest that a lowering of cyclic AMP caused by addition to the medium of these PTS substrates is not a sufficient explanation for

the severity of repression observed. They have invoked inducer exclusion as one of the factors involved in the repression of melibiose, maltose, and glycerol utilization, and have shown that sugar substrates of the PTS inhibit the permeases for melibiose, maltose and glycerol. The inhibition by PTS sugars resulted in a depression of the maximum velocity of TMG entry. Kinetic studies of the effect of PTS sugars on the melibiose permease showed that PTS sugars depressed the maximum velocity of TMG entry without affecting the K_m of TMG uptake. Inhibition by a particular PTS sugar required the enzyme II specific for that sugar. It thus appears that the inhibition does not result from competition between two sugars for a common transport carrier. As opposed to the studies of Winkler and Wilson (159) and Boniface and Koch (158), who studied the effect of glucose on TMG uptake via the *lac* permease in *E. coli*, Saier and Roseman (131) found that inhibition of TMG uptake via the melibiose permease in *S. typhimurium* did not require prior incubation of the cells with the PTS sugar. All PTS sugars were not equally active in inhibiting uptake, the most active being glucose. Interestingly, the presence of two PTS sugars in the medium did not inhibit TMG uptake to a greater extent than the more inhibitory of the two sugars alone.

Inducer exclusion has also been implicated in partially causing catabolite repression of serine deaminase in *E. coli*

(160). However, in studies of repression of tryptophanase, Freundlich and Lichstein (161) showed that inducer exclusion is not involved. Other studies have indicated that although exclusion of inducer entry may enhance the effect of catabolite repression in several systems, inducer exclusion plays no role in transient repression (113,114).

IV. Control of the activity of transport systems.

Catabolite inhibition and inhibition by sugar phosphates. Aside from its role in excluding entry of inducer, it is clear that the inhibition of transport by glucose or other sugars can play an important physiological role in the cell. Inhibition, rather than repression, offers the cell a mechanism for adapting rapidly to a changing environment. Paigen and his co-workers (162,163) have termed the inhibition of enzyme activity by glucose as "catabolite inhibition." In a study of catabolite inhibition of mannose utilization, McGinnis and Paigen (163) have noted that glucose generated inside the cell, e.g., by the splitting of lactose, can act to repress the further synthesis of the mannose-metabolizing enzymes but is incapable of inhibiting the utilization of mannose by the enzymes already present. These workers concluded that catabolite inhibition occurs when glucose is being actively transported across the cell membrane. They have also shown that the site of catabolite inhibtion of galactose and lactose utilization is either at the level of entry of substrate into the cell or at the level of the

first metabolic reaction, i.e., galactokinase or β-galactosidase, and suggest that the former alternative is more likely in light of the finding that catabolite inhibition requires the transport of glucose across the cell membrane.

Bag (164) has reported that in *Vibrio cholerae*, glucose inhibits the transport of galactose and fructose. All of these sugars are transported via a PEP dependent PTS in this organism. The studies of Saier and Roseman (131) on the inhibition by PTS sugars of the permeases for non-PTS sugars in *S. typhimurium*, and the findings of McGinnis and Paigen (163) and Bag (164) can be put into perspective by the studies of Kaback (165,166) on the PTS in membrane vesicles of *E. coli*. Kaback demonstrated that glucose 1-phosphate and glucose 6-phosphate noncompetitively inhibit the transport of glucose in such vesicles. Also inhibitory, but not as effective as glucose 1-phosphate, were mannose-1-phosphate, fructose 6-phosphate, fructose 1-phosphate, acetyl-phosphate, and xylose 1-phosphate. Evidence was provided for the existence of two inhibitory sites, each accessible from both sides of the membrane, one specific for glucose 1-phosphate and related sugar 1-phosphates, and the other specific for glucose 6-phosphate and related sugar 6-phosphates. The inhibition exerted by glucose 6-phosphate and either glucose 1-phosphate or mannose 1-phosphate was always less than would

have been expected from the additive effects of the inhibitors when used independently. Fructose uptake was inhibited by glucose 1-phosphate, fructose 1-phosphate, and fructose 6-phosphate. Glucose 1-phosphate also inhibited galactose transport, indicating that glucose 1-phosphate may be involved in the regulation of sugar transport in general. It is interesting to note that Dietzler, Leckie and Magnani (167) have reported that decreases in the level of glucose 6-phosphate in intact cells of *E. coli* can be quantitatively correlated with increases in the rates of glucose utilization, indicating that glucose 6-phosphate is an inhibitor of glucose utilization. The fall in glucose 6-phosphate levels could be correlated with a decrease in ATP concentration, suggesting that ATP can exert control of the rate of glucose utilization by altering the cellular level of glucose 6-phosphate. These authors note that the effect of glucose 6-phosphate on glucose utilization is consistent with the role of glucose 6-phosphate as an inhibitor of glucose transport.

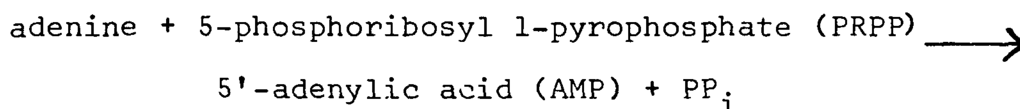
Sheu, Konings and Freese (168) have reported that acetate and other short chain fatty acids noncompetitively inhibit the uptake of serine and other amino acids, but not the uptake of α-methylglucoside or fructose in whole cells and membrane vesicles of *B. subtilis*. These authors suggest that the fatty acids uncouple the amino acid carrier proteins from the electron transport systems which

they require to energize active transport. Morgan and Kornberg (169) have reported that pyruvate inhibits the accumulation of certain sugars by *E. coli*, and suggest that the inhibitory agent is acetyl-coenzyme A. Anraku has suggested that carrier proteins that comprise permeases have multi-binding sites and that negative cooperativity may provide cells with the ability to regulate the intracellular concentration of amino acids (170). Halpern has suggested such a model in *E. coli* for glutamate transport (171).

Ion effects. In addition to the possible role that salt gradients may play in providing energy for active transport of some compounds (172,173) several investigators have reported that sodium ion or other cations can affect transport activity. MacLeod and his co-workers have studied the uptake of the amino acid analog α-aminoisobutyrate (AIB) in a marine pseudomonad which requires sodium for growth. They found that sodium decreases the K_m for the transport of AIB via a facilitated diffusion mechanism (174,175). Potassium has also been shown to be required for AIB transport; however, it appears to act intracellularly, causing accumulation of AIB against a concentration gradient after the sodium-dependent uptake process (175,176). The energy required for the accumulation of AIB is not derived from the potential energy of the Na^+-K^+ gradients present (177). Instead, it has

been shown that transport is coupled to the oxidation of specific electron donors, e.g., ethanol (178). Halpern *et al.* (179) have described a similar sodium-and potassium-dependent transport system for glutamate in *E. coli*. Stock and Roseman (180) have reported a sodium-dependent sugar co-transport system in *S. typhimurium* in which sodium increases the affinity of the transport system for its substrate. Ariel and Grossowicz (181) have recently described a similar effect of sodium on the uptake of succinate, glucose, and valine in *Micrococcus lysodeikticus*.

Controls of purine transport. The regulation of purine uptake in *E. coli* has been studied by Hochstadt-Ozer and Stadtman (182-184) and appears to involve controls of both enzyme activity and enzyme formation. These workers provided evidence that adenine is taken up by a group translocation mechanism involving adenine phosphoribosyltransferase (182). This enzyme catalyzes the reaction:



adenine + 5-phosphoribosyl 1-pyrophosphate (PRPP) $\longrightarrow$ 5'-adenylic acid (AMP) + PP_i

In studies of adenine uptake in membrane vesicles, it was observed that adenine phosphoribosyltransferase activity associated with the membrane vesicles and the uptake system for adenine were similarly affected by variations in pH, substrates, and inhibitors. Removal of the enzyme from vesicles resulted in a concomitant loss of uptake activity. Adenine uptake by membrane vesicles was

accompanied by its conversion to AMP, and was stimulated by PRPP. The adenine phosphoribosyltransferase was derepressed under conditions which precluded *de novo* synthesis of purines and all uncyclized nuceloside 5'-phosphate esters inhibited activity, indicating that the total nucleotide pool is involved in the regulation of adenine utilization via the uptake system (183). Hochstadt-Ozer and Cashel (184) also reported that purine phosphoribosyltransferase activity and purine uptake in membrane vesicles were both inhibited by guanosine tetraphosphate (ppGpp) and suggested that ppGpp inhibition might account for the amino acid dependence of the uptake of purines and pyrimidines observed in rel^{+} strains.

V. Multiple transport systems.

Amino and transport. Early studies of bacterial transport indicated that the synthesis of amino acid permeases often is relatively unaffected by the provision of their substrates in the growth medium, especially when the amino acids are utilized by the organism only as a supply for protein synthesis (185,186). On the other hand, in cases where the amino acids can be degraded as a source of nitrogen or carbon, permeases often are inducible. Classic examples of the latter case in *E. coli* have been reported by Halpern and his co-workers for glutamate permease (187) and by Boezi and DeMoss for the tryptophan permease (188). Early studies of regulation in *E. coli*

by Vogel (189) revealed that arginine represses the synthesis of the permease for acetylornithine, the precursor of arginine. Inui and Akedo (190), in a study of the effect of cycloleucine on amino acid uptake in *E. coli* indicated that an excess of a single amino acid can interfere with the subsequent uptake of the same and structurally related amino acids.

The study of amino acid permeases has revealed the presence of multiple transport systems for these substrates which are characterized by differences in regulation and in substrate specificities and affinities. In several cases, these appear to involve a general permease with several structurally related substrates in addition to more specific permeases. The existence of general permeases is not unreasonable in light of the studies of Konings and Freese (191). In studies of amino acid transport in membrane vesicles of *Bacillus subtilis*, these workers have demonstrated mutual inhibition of the uptkae of several amino acids, indicating that the amino acids are transported by the same carrier.

Rosen (192,193) has described three distinct systems for the transport of basic amino acids in *E. coli*: an arginine-specific system, a lysine-specific system, and the lysine-arginine-ornithine (LAO) system. Rosen has reported that although arginine, its precursors citrulline and ornithine, and its analog canavanine, are substrates

of the LAO system, arginine appears to be merely a competitive inhibitor of that system, its transport being accomplished primarily via the arginine-specific system (192). In addition to these three transport systems, Celis, Rosenfeld and Maas (194) have reported a fourth permease, specific for ornithine. In contrast to Rosen (192), Celis *et al.* found that arginine was transported not only by its specific permease, but also by the LAO system. Kinetic studies revealed that the LAO system has a higher affinity for each of its substrates than does any of the specific permeases. Repression studies showed that arginine and ornithine repress their own and each other's specific transport systems, whereas the lysine specific system and the LAO system are repressed only by lysine. Both Rosen (195) and Celis *et al.* (194) have described a pleiotropic mutant with a reduction in the activity of both the LAO and the specific arginine permeases. Rosen (195) has proposed that the LAO and the arginine-specific carrier proteins interact with a common energy coupling mechanism, and that it is this coupling mechanism that is the source of the pleiotropic mutation.

Multiple transport systems for basic amino acids have also been reported by Rodwell and his co-workers (196) in *Pseudomonas putida.* In this organism, lysine-grown cells contain two inducible permeases, one specific for lysine and ornithine (the diamino acid system), and the other able

to transport lysine, ornithine, and arginine (the general system). In addition, while cells grown on arginine lack the general basic amino acid transport system, they are induced for a permease specific for arginine. In addition to the differences in regulation for basic amino acid permeases in *E. coli* and *P. putida*, it is interesting to note that whereas in the former organism, the general permease has a higher affinity for its substrates than the specific permeases, in *P. putida*, the transport substrates have a lower K_m for the diamino acids system and the arginine-specific system than for the general system.

Multiple systems involving a general permease and more specific permeases have also been identified by Ames (197) for aromatic amino acids, and by Oxender and his co-workers (198) and Iaccarino and his co-workers (199,200) for branched chain amino acids.

Other multiple transport systems. In addition to amino acid permeases, multiple transport systems have been identified for sugars (e.g., galactose and galactosides), nucleosides (201) and for di- and tricarboxylic acids (202-205). Guardiola *et al.* (200) have pointed out that *E. coli* uses a substantial fraction of its genome for transport. The usefulness of multiplicity, from an evolutionary point of view, is not easy to understand since most other genes do not appear to be duplicated. Guardiola *et al.* (200) have therefore suggested that different transport systems may

serve different functions. It is interesting to point out, in this regard, that Kay (202,203) has described two transport systems for aspartate in *E. coli*, each with a separate physiological function. The low affinity system, *dct*, is inducible and is nonspecific toward a wide variety of four carbon dicarboxylic acids. This system was shown to be absolutely necessary for dicarboxylic acid catabolism (203) since mutation of the *dct* gene resulted in an inability to transport and grow on aspartate, fumarate, malate and succinate as sole carbon sources. Kay observed, however, that aspartate was still utilized as a sole source of nitrogen in strains harboring the *dct* mutation. The other permease for aspartate, *ast*, was studied by Kay (202) and found to be specific for aspartate and to have a higher affinity for the substrate than the *dct* system. Mutants of the *ast* system can grow normally on the four carbon dicarboxylic acids as carbon sources, but grow slowly on aspartate when the latter is present as the nitrogen source in the medium. In addition to its role in supplying aspartate as a nitrogen source, Kay (202) also demonstrated that the *ast* system could act as a supply route for four carbon acids required for anapleurosis.

INTRODUCTION

Most work on sugar transport and the induction of enzymes for sugar catabolism has been done on bacteria for which sugars, especially glucose, are the preferred source of carbon for growth. The ability of bacteria to grow most efficiently on glucose is not universal, however. Cook and Fewson (206) have reported, for example, that a strain of *Acinetobacter calcoaceticus* is impermeable to extracellular sugars and sugar phosphates. Other organisms can utilize glucose, but do not do so preferentially in the presence of other energy sources. The glucose permease of *Thiobacillus intermedius* (207), for example, is both repressed and inhibited in the presence of the preferred energy source thiosulfate.

The study of glucose transport in several species of *Pseudomonas* and *Arthrobacter* has revealed that glucose permeases in these obligately aerobic bacteria are inducible and are in fact repressed by other carbon sources. In 1959, Hamilton and Dawes (208) reported the first case of "reverse diauxie." Cells of *Pseudomonas aeruginosa* incubated in the presence of both glucose and an organic acid such as citrate were shown to utilize the organic acid in preference to the hexose. Subsequent studies revealed that citrate represses both the formation of enzymes responsible for the metabolism of glucose (209) and a glucose permease (210, 211). Mukkada, Long, and Romano (211) reported that even

in the presence of excess glucose, the glucose permease is strongly repressed by acetate, citrate, succinate, fumarate, and malate. Similarly, Midgley and Dawes (210) have shown that citrate, or its metabolic products, inhibits the glucose permease once it is induced. Multiple transport systems for glucose, all inducible, have been described in both *P. aeruginosa* and *P. putida* (212-214).

Krulwich and Ensign (215) have reported that *Arthrobacter crystallopoietes* exhibits "reverse diauxie" in a medium containing glucose and limiting succinate, and Sokol and Klein (216) have reported a similar diauxie in a different species of *Arthrobacter*. In *A. crystallopoietes* the cells deplete all the dicarboxylic acid and then show a lag before commencing growth on the sugar (215). During growth on succinate, an inducible transport system for glucose is repressed. Transport of glucose is inhibited by succinate. Despite the fact that glucose is not a preferred carbon source in *A. crystallopoietes*, Krulwich and her co-workers have reported that glucose, as well as succinate, can inhibit histidine transport (217). Both succinate and glucose can repress histidase, presumably in part by inducer exclusion.

Initial metabolic studies of *Arthrobacter pyridinolis* revealed that it is deficient in its ability to accumulate intracellular dicarboxylic acids such as malate during metabolism (218). The organism also appeared to be severely

restricted in its ability to utilize sugars. Preliminary evidence indicated that hexose utilization was influenced markedly by the composition of the medium, specifically by the presence of dicarboxylic acids. In order to further elucidate regulatory mechanisms of sugar utilization in organisms which do not preferentially use such compounds, a detailed study was initiated of the transport systems for glucose and fructose in *A. pyridinolis*.

MATERIALS AND METHODS

Bacteria and growth conditions. *A. pyridinolis* (obtained from J. C. Ensign, University of Wisconsin) was used for all studies and was grown in a defined mineral salts medium (MS) containing (w/v) 0.1% $(NH_4)_2SO_4$ and 0.01% $MgSO_4 \cdot 7H_2O$ and (v/v) 1.0% trace salts of Hegeman (219) in 0.025 M potassium phosphate buffer (pH 7.0). Carbon sources (employed at 0.05 M unless otherwise indicated) and trace salts were added to the rest of the medium after autoclaving from separate sterile solutions. For routine maintenance, cells were grown in PYE medium containing (w/v) 0.2% peptone, 0.1% yeast extract, and 0.02% $MgSO_4 \cdot 7H_2O$. Cultures were grown, with shaking at 200 rev/min in a New Brunswick G25 shaker at 30°C. For growth studies, 19 ml of medium in 300 ml side-arm flasks (Bellco Glass, Inc.) were inoculated with 1.0 ml of a logarithmic phase culture grown in PYE medium and were incubated at 30°C with shaking. Cell densities were determined by use of a Klett-Summerson colorimeter with a no. 42 filter blanked against a side-arm flask containing uninoculated medium. Under these conditions, 50 Klett units are equivalent to a cell concentration of 2.5×10^8 cells/ml.

Chemicals. (U-^{14}C)-L-Asparagine, (2,3-^{14}C)-succinate, and α-(U-methyl-^{14}C)-glucopyranoside were purchased from Amersham-Searle. (U-^{14}C)-D-Glucose, (1-^{14}C)-2-deoxyglucose

and (U-^{3}H)-L-rhamnose were purchased fron New England Nuclear. (4-^{14}C)-L-Malate and (U-^{14}C)-D-fructose were obtained from Biochemical and Nuclear Corp.

The D-isomers of all sugars were used except that the L-isomers of rhamnose, fucose, and arabinose were employed whenever these compounds are specified. The L-isomers of all amino acids, malate, and fumarate were used.

Reduced nicotinamide adenine dinucleotide (NADH), oxidized nicotinamide adenine dinucleotide (NAD), their phosphates (NADPH, NADP), flavin adenine dinucleotide (FAD), adenosine 5'-triphosphate (ATP), lysozyme, phosphohexose-isomerase, and glucose 6-phosphate dehydrogenase were obtained from Boehringer-Mannheim Corp. Fructose 1,6-diphosphate, fructose 1-phosphate, fructose 6-phosphate, 2-deoxyglucose, chloramphenicol, phosphoenolpyruvate (PEP), penicillin-G, DL-isocitric acid, carbonylcyanide *m*-chlorophenylhydrazone (CCP), 2,4-dinitrophenol (DNP), 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO), N-ethylmaleimide (NEM), dimethylsulfoxide, 2,6-dichlorophenolindophenol, alkaline phosphatase, cytidine 5'-triphosphate (CTP), uridine 5'-triphosphate (UTP), and guanosine 5'-triphosphate (GTP) were purchased from Sigma Chemical Co. Deoxyribonuclease (DNase) and ribonuclease (RNase) were obtained from Worthington Biochemicals and ethylmethanesulfonate (EMS) was purchased from Eastman Chemicals. All other chemicals were purchased commercially at the highest purity available.

Preparation of membrane vesicles. Spheroplasts were prepared from wild type or mutant cells grown to the logarithmic phase (20-22 h) in MS plus the appropriate carbon source(s). After centrifugation of the culture at 16,000 x *g* for 10 min, the pellet was washed with 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.0) and was resuspended in 30 mM Tris (pH 8.0) containing 20 mM magnesium sulfate and 10% mannitol. The cells were then incubated at 30°C and lysozyme was added to a final concentration of 250 μg/ml for 2 min. Spheroplasts were then isolated by centrifugation at 16,000 x *g* for 10 min and membrane vesicles were prepared by a procedure adapted from the hypo-osmotic lysis method of Kaback (6). The isolated spheroplasts were resuspended by mild homogenization in a small volume of 100 mM potassium phosphate buffer (pH 6.6) containing 10% mannitol and 1.7 mg/ml each of DNase and RNase. The resuspended spheroplasts were then diluted to 150 times their volume in 50 mM potassium phosphate buffer (pH 6.6), and incubated with gentle shaking at 30°C. After 15 min, EDTA was added to a final concentration of 10 mM, followed in 15 min by the addition of magnesium sulfate to a final concentration of 20 mM. After another 15 min, the preparation was centrifuged at 45,000 x *g* until the supernatant was clear. The pellet was resuspended with mild homogenization in 100 mM potassium phosphate buffer (pH 6.6), containing 10 mM EDTA and 20 mM

magnesium sulfate. After addition of DNase and RNase to give final concentrations of 100 μg/ml each, the preparation was incubated at 30°C for 30 min and then centrifuged at 45,000 x *g* for 30 min. The pellet was resuspended in 100 mM potassium phosphate buffer (pH 6.6) containing 10 mM EDTA and then centrifuged at 800 x *g* to remove whole cells and spheroplasts. The supernatant was then carefully decanted and centrifuged at 45,000 x *g*. The high speed pellet was washed several times by resuspension and vigorous homogenization in 100 mM potassium phosphate (pH 6.6) containing 10 mM EDTA. The membrane vesicles, at a concentration of 1.0 mg protein/ml, were finally resuspended in 100 mM potassium phosphate buffer (pH 6.6) and were frozen and stored in liquid nitrogen. Electron micrographs of vesicles were prepared by Dr. J. Schwartz as described by Schwartz and Roizman (220).

Enzyme assays. For preparation of cell extracts, cells were harvested by centrifugation at 16,000 x *g* for 10 min. The cells were washed by resuspending the pellets in 25 ml of the buffer to be used in the assay, followed by a second centrifugation. The cells were suspended in 5 to 10 ml of the same buffer and broken by sonic disruption for 2 min in a Heat Systems-Ultrasonics Inc. model W185D Sonifier. During sonic treatment, the cells were kept below 10°C. Finally, unbroken cells and large debris were removed from the extracts by centrifugation at 16,000

x *g* for 10 min. Before they were used for enzyme assays, the extracts were dialyzed against the same buffer in which they were prepared.

For assays of enzymatic activities in the membrane preparations, a sample of vesicles was first diluted to twice the original volume in the buffer to be used in the assay. The vesicles were then subjected to sonic disruption for 30 sec.

All spectrophotometric assays were conducted using a Gilford model 240 spectrophotometer at 25°C. The following enzymes were assayed by following the reduction of NADP. Glucokinase (EC 2.7.1.2) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were determined by the method of Patni and Alexander (221). Fructokinase (EC 2.7.1.4, ATP: fructose 6-phosphotransferase) was assayed by a procedure adapted from the method of Sapico *et al.* (222), using a reaction mixture containing 0.25 mM NADP, 5.0 mM magnesium chloride, 3.5 mM ATP, excess phosphohexoseisomerase and glucose 6-phosphate dehydrogenase, and 25.0 mM fructose in 0.05 M Tris buffer (pH 7.5). Fructose 1,6-diphosphatase (fructose-1,6-biphosphate 1-phosphohydrolase, FDPase, EC 3.1.3.11) was assayed by the method of Fraenkel and Horecker (223). Isocitric acid dehydrogenase (EC 1.1.1.42) was determined by the method of Hanson and Cox (224). The oxidation of NADH was followed in the assays listed below. Corrections were made for the small amount of NADH

oxidation that occurred in the absence of substrate. Fructose 1-phosphate kinase (1-phosphofructokinase, EC 2.7.1.56) and fructose 6-phosphate kinase (6-phosphofructokinase, EC 2.7.1.11) were assayed by the method of Sapico *et al.* (222); ATP-dependent formation of fructose 1-phosphate versus fructose 6-phosphate was also detected by their method. Fructose 1,6-diphosphate aldolase (EC 4.1.2.13) was determined by the method of Groves, Calder and Rutter (225), pyruvate kinase (EC 2.7.1.40) by the method of Valentine and Tanaka (226), and 6-phosphogluconic dehydrogenase (EC 1.1.1.44) by the method of Pontremoli and Grazi (227). NAD-linked malate dehydrogenase (EC 1.1.1.37) was assayed by the procedure of Kitto (228). The FAD-linked malate dehydrogenase was determined by following the reduction of ferricyanide at 400 mμ as described by Benziman (229). Glucose dehydrogenase (EC 1.1.1.47) was assayed by following the reduction of 2,6-dichlorophenolindophenol at 600 mμ as described by Hauge (230). All assays were linear with both enzyme concentration and time in the ranges employed.

PEP: hexose phosphotransferase (PEP: protein phosphotransferase, EC 2.7.3.9) activity for fructose or glucose was determined by the radiochemical assay of Tanaka, Lerner, and Lin (70). Unless otherwise indicated, reaction mixtures contained 2 mM hexose (radioactively labeled), 5 mM PEP, 0.05 mM magnesium chloride, and 50 uliters of

cell extract in a total volume of 0.4 ml of 50 mM Tris (pH 7.6). Reactions were terminated by diluting the radioactive substrate with 0.6 ml of 0.2 M fructose. Unless otherwise indicated, assays were conducted at room temperature. Samples from the reaction mixture were spotted on diethylaminoethylcellulose (DEAE) discs (DE81 filters, Whatman Co., reference 231). The discs were washed exhaustively with water and then dried. The radioactivity was measured by scintillation counting in either Bray's solution (232) or a mixture containing 6.0 g PPO and 0.1 g POPOP in one liter of toluene. The assay was linear with both enzyme concentration and time in the ranges employed. For *in vitro* complementation assays of PEP: fructose phosphotransferase activity, dialyzed extracts were separated into soluble and membrane fractions by centrifugation for 120 min at 150,000 x *g*; the pellet was resuspended in 50 mM Tris (pH 7.6). Reaction mixtures in complementation assays contained 50 uliters each of soluble and membrane fractions.

Enzyme specific activities are expressed as micromoles or nanomoles of substrate consumed or product formed per minute per mg of extract protein. Protein was determined by the method of Lowry *et al.* (233) using lysozyme as a standard.

Uptake assays in whole cells. Cells were washed and resuspended in MS. The cell concentration was adjusted

to either 100 Klett units for assays of 2-deoxyglucose and α-methylglucopyranoside uptake or to 50 Klett units for determination of fructose uptake. Cells were then incubated with shaking at 30°C for 30 min with 40 μg of chloramphenicol/ml. At room temperature, 4 ml aliquots of cells were transferred to 25 ml Erlenmeyer flasks and agitated with magnetic stirring bars to ensure adequate aeration. To start the reaction, 1 ml of the desired concentration of radioactive substrate was added to the flask. At intervals 1 ml samples were filtered through 0.45 μm filters (Matheson-Higgins Co.). Filters were immediately washed with 10 ml of of cold MS, dried, and the radioactivity was measured by scintillation counting as described above. During inhibition studies, cells were incubated with the indicated inhibitor for 10 min prior to the start of the reaction. In the study of competitive inhibition of uptake of 2-deoxyglucose, the inhibitor was added at the start of the reaction.

Transport assays in membrane vesicles. Transport of fructose by isolated membrane preparations via the PEP: fructose phosphotransferase was assayed by the method of Kaback (6) using a filtration assay with a standard reaction mixture containing vesicles, 0.05 M potassium phosphate buffer (pH 6.6), 10 mM magnesium sulfate, 0.3 M lithium chloride, 100 mM PEP, and 40 μM (U-^{14}C)-fructose. Vesicles were incubated in the reaction medium at the

desired temperature for 15 min before the reaction was started by addition of fructose. The total reaction mixture was either 50 or 100 µl. Reactions were terminated, usually after 10 min, by a 25-fold dilution with 0.5 M lithium chloride and the entire reaction mixture was filtered through 0.45 µm filters (Matheson-Higgins Co.). Filters were immediately washed with 2.5 ml of 0.5 M lithium chloride and dried. The radioactivity of the filters was measured by scintillation counting. Malate was substituted for PEP in the reaction mixture during studies of malate-dependent transport of fructose.

Substrate incorporation studies. Cells grown in PYE medium were inoculated into MS medium containing radioactively labeled substrates in varying combinations with nonradioactive compounds. In each study, the specific radioactivity of all labeled substrates was the same. At intervals, 0.1 ml samples were filtered through 0.45 µm filters (Matheson-Higgins Co.). The filters were washed with 10 ml of cold 10% trichloroacetic acid and dried. The radioactivity was measured by scintillation counting in Bray's solution (232).

Isolation of mutants. Mutants were prepared from washed cells using ethylmethane sulfonate (EMS) as described by Wolfson and Krulwich (218). For isolation of mutants which were negative for growth on fructose, cells were washed after treatment with EMS and incubated overnight in MS plus fructose and 100 µg of penicillin G/ml. After this

incubation, the cells were washed and plated on PYE plates. The colonies formed were replicated onto MS plates containing fructose as sole carbon source; fructose-negative colonies were identified and picked. For further characterization, extracts were prepared from mutant and wild type strains grown in glutamate-glucose medium, in glutamate-fructose medium, or malate-fructose medium and enzyme assays were conducted to determine the lesions in the mutant strains.

Mutants identified as PEP: fructose phosphotransferase-negative have been further characterized and described by Wolfson and Krulwich (98,234,235). AP100 and AP133 are, respectively, deficient in the soluble fructose-specific factor III and the membrane-bound fructose-specific enzyme II of the phosphotransferase system. AP243 is deficient in the constitutive, soluble enzyme I of this system. AP253 is deficient in a different constitutive soluble component, the phosphocarrier protein (HPr). AP437 was derived from AP243 and is a double mutant lacking the fructose-specific component of the respiration-coupled transport system as well as the phosphotransferase component that is missing in its parental strain.

RESULTS

Growth properties of A. pyridinolis: crypticity toward glucose. A series of growth studies were performed to establish which carbohydrates could be used to support the growth of *A. pyridinolis*. The organism did not grow when either glucose, sucrose, maltose, galactose, mannose, sorbitol, mannitol, fucose, lactose, xylose, ribose, or arabinose was present as sole carbon source. As shown in Figure 1, however, cells of *A. pyridinolis* grew readily on fructose. The organism also grew on rhamnose as sole carbon source, with a lag period similar to that shown for fructose.

Although *A. pyridinolis* did not grow on glucose as sole carbon source, glucokinase as well as other enzymes of the Embden-Meyerhof and pentose phosphate pathways are detectable in cell extracts (Table 1). In addition, the organism has a complete tricarboxylic acid cycle as shown by assays (data not shown) and by the ability of the species to grow on intermediates of that pathway when they are present as sole source of carbon (Table 2).

Abolition of glucose crypticity. During preliminary growth experiments there were indications that glucose, while not used when present as sole carbon source, was utilized in the presence of at least certain other compounds. Growth studies were conducted to determine in what way crypticity toward glucose might indeed be abolished. Var-

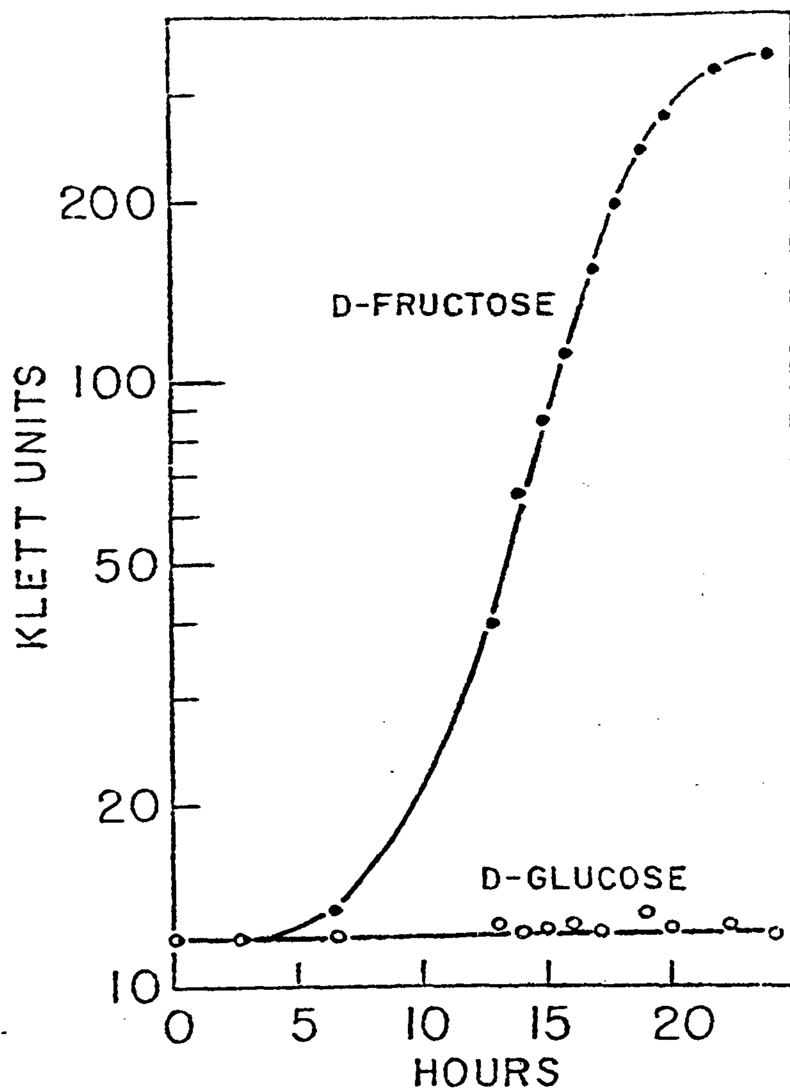


FIG. 1. Growth of *A. pyridinolis* on fructose or glucose. Cells were inoculated from a PYE culture into MS containing 0.05 M of the carbon source indicated.

TABLE 1

SURVEY OF SOME ENZYMES FOR GLUCOSE METABOLISM IN CELL EXTRACTS OF *A. PYRIDINOLIS*[1]

Enzyme	Specific activity[2]
Glucokinase	0.076
Glucose 6-phosphate dehydrogenase	0.025
6-Phosphogluconic dehydrogenase	0.224
Fructose 6-phosphate kinase	0.021
Fructose 1,6-diphosphate aldolase	0.039
Pyruvate kinase	0.036
Fructose 1,6-diphosphatase(FDPase)	0.082

[1]Extracts were prepared from washed cells grown in PYE by sonic disruption in 40 mM glycylglycine buffer, pH 7.4. Assays were conducted as described in Materials and Methods.

[2]Specific activities are expressed as micromoles of substrate consumed or product produced per minute per milligram of protein.

ious compounds were screened for their ability to facilitate utilization of glucose. Comparisons were made between the turbidities attained in 30 h by cultures grown on the compound alone and cultures grown on the compound plus glucose. Glucose utilization seemed to occur in cultures containing citrate, succinate, fumarate, or malate in addition to glucose (Table 2). Oxaloacetate had a much smaller effect, and other carbon sources, such as asparagine, which support as rapid growth as does malate, did not confer the ability to utilize glucose. Fructose and rhamnose (the only hexoses able to support growth of *A. pyridinolis* when present as sole carbon source) also did not abolish crypticity toward glucose.

The pattern of growth of *A. pyridinolis* on limiting (0.015 M) malate plus 0.05 M glucose is shown in Figure 2. Initially, the culture grew at a rate that was characteristic of that observed on malate alone. Then, at the time when growth of cells on 0.015 M malate alone ceased, the cells on malate plus glucose exhibited a lag of 4 to 5 h. After the lag, a second phase of exponential growth occurred. Extracts of cells from the two exponential phases contained the same specific activities of glucokinase. Neither extract contained detectable glucose dehydrogenase activity. Growth curves of *A. pyridinolis* in 0.015 M citrate, succinate, or fumarate plus 0.05 M glucose show a pattern similar to that shown in Figure 2 for malate plus

TABLE 2

ABILITY OF VARIOUS ADDITIONAL CARBON SOURCES TO ENHANCE THE UTILIZATION OF GLUCOSE FOR GROWTH BY *A. PYRIDINOLIS*[1]

Supplement	Klett units after 30 h in		ΔKlett units
	Glucose plus supplement	Supplement alone	
None	21	16	5
Asparagine	185	167	18
Aspartate	80	38	42
Citrate	338	220	118
Fructose	370	375	-5
Fumarate	378	204	174
Glutamate	144	82	62
Glycerol	16	17	-1
Malate	383	213	170
Oxaloacetate	300	220	80
Rhamnose	390	340	50
Succinate	385	238	147

[1]Cells of *A. pyridinolis* were inoculated from PYE cultures into MS plus the indicated supplements (at 0.015 M) and those supplements (0.015 M) plus 0.05 M glucose. After 30 h, the turbidities of the cultures were determined. The values in the last column are the Klett units for the glucose-containing cultures minus the Klett units for the cultures containing the corresponding supplement alone. The first line represents the difference between cultures containing glucose and those containing no carbon source.

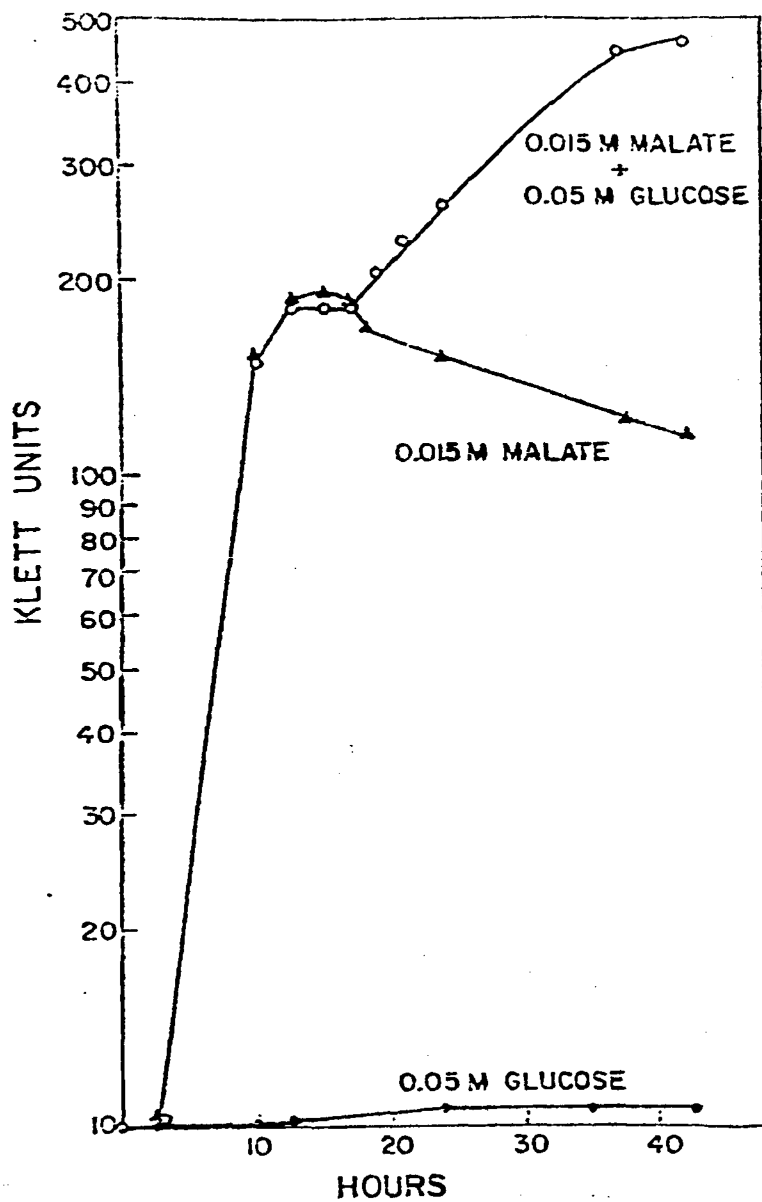


FIG. 2. Growth of *A. pyridinolis* on glucose, malate, and malate plus glucose. Cells were inoculated from PYE cultures into MS containing the indicated carbon sources.

glucose.

To determine whether, in fact, glucose is used during the second period of the diauxic growth curve, and is used only during that period, an experiment was conducted with radioactive growth substrates. Incorporation of label into cold trichloroacetic acid-insoluble material was followed as described in Materials and Methods. Cells grown on 0.015 M radioactive succinate, either with or without 0.045 M glucose, incorporated label for 9 to 10 h (Figure 3A). Incorporation of label from succinate then markedly decreased. Cells incubated with radioactive glucose alone incorporated very little label. Cells incubated in 0.015 M succinate plus 0.045 M radioactive glucose incorporated only small amounts of label until 15 h after inoculation. Incorporation of glucose was then observed, and continued at the rate shown until approximately 40,000 counts per min per ml was incorporated. Thus significant amounts of label from glucose are incorporated into trichloroacetic acid-insoluble cell material only after the rate of incorporation of succinate has slowed and a lag in growth has occurred.

The same incorporation experiment was conducted with the substitution of asparagine for succinate (Figure 3B). Label from asparagine was incorporated for about 10 h either in the presence or absence of glucose; after this time the amount of label in trichloroacetic acid-insoluble

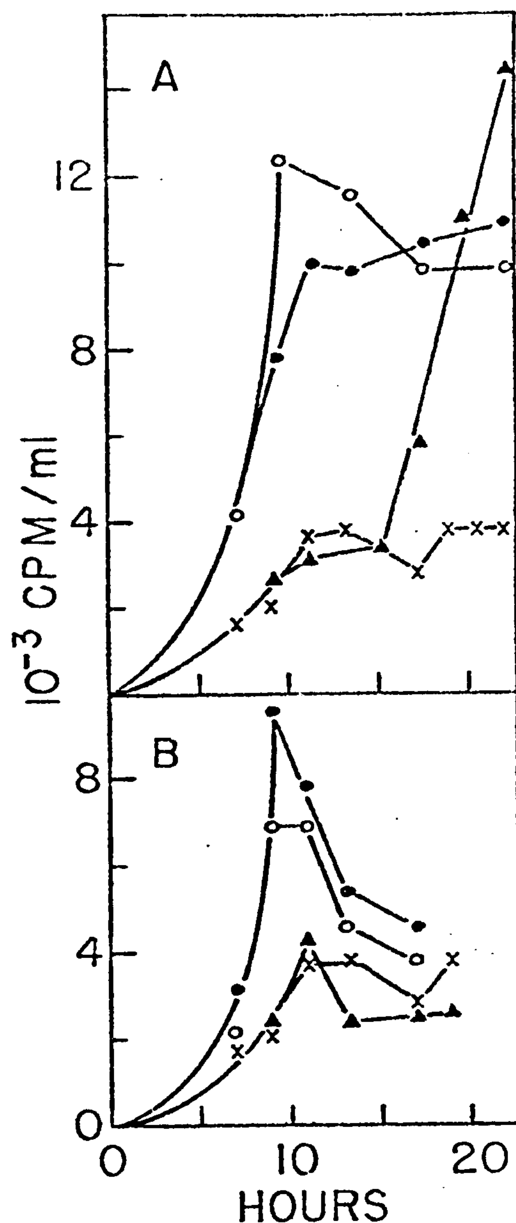


FIG. 3. Incorporation of label from radioactive growth substrates into trichloroacetic acid-insoluble cell material. A: Cells were grown in MS plus 0.015 M (2,3-^{14}C)-succinate (O); 0.015 M (2,3-^{14}C)-succinate plus 0.045 M glucose (●); 0.045 M (U-^{14}C)-glucose (X); and 0.015 M succinate plus 0.045 M (U-^{14}C)-glucose (▲). B: Cells were grown in MS plus 0.015 M (U-^{14}C)-asparagine (O); 0.015 M (U-^{14}C)-asparagine plus 0.045 M glucose (●); 0.045 M (U-^{14}C)-glucose (X); and 0.015 M asparagine plus 0.045 M (U-^{14}C)-glucose (▲). The specific radioactivity of all labeled substrates was 2 x 10^{-3} Ci/mol. Samples (0.1 ml) were taken at intervals, treated with cold trichloroacetic acid and counted as described in Materials and Methods.

material began to decrease. As expected from the growth data, no significant incorporation of label from radioactive glucose occurred, even after utilization of asparagine.

Many carbohydrates other than glucose fail to support growth of *A. pyridinolis* when present as sole carbon source. The possibility that tricarboxylic acid cycle intermediates might facilitate the utilization of these carbohydrates was investigated. Maltose and sucrose could be utilized when malate but not asparagine was also present in the medium (Table 3). Growth curves of *A. pyridinolis* in 0.015 M malate plus 0.05 M maltose or sucrose were identical to that shown in Figure 2 for glucose. Utilization of a variety of other carbohydrates tested was not enhanced by malate.

Transport of glucose. The possibility was examined that the crypticity of *A. pyridinolis* toward glucose and the abolition of this crypticity are related to transport of the hexose. The uptake of 2-deoxyglucose, which is nonmetabolizable in *A. pyridinolis*, was used to monitor the transport system for glucose. Uptake of radioactive 2-deoxyglucose was measured in cells grown in asparagine, asparagine plus glucose, malate, and malate plus glucose for 9, 13, or 20 h. Only malate-glucose-grown cells which had been growing for 20 h and were in the phase of exponential growth on glucose took up 2-deoxyglucose appre-

TABLE 3

ENHANCED UTILIZATION OF VARIOUS GROWTH SUBSTRATES IN THE PRESENCE OF ADDITIONAL COMPOUNDS[1]

Growth substrate	Growth increment (ΔKlett units) supported by	
	Asparagine	Malate
Glucose (21)	18	149
Galactose (12)	5	35
Ribose (16)	-8	1
Maltose (26)	40	215
Sucrose (34)	25	224
Mannitol (19)	-24	8
Lactose (27)	-33	-21

[1]Asparagine and malate (0.015 M) were tested for ability to enhance the growth of *A. pyridinolis* on the carbon sources listed in the first column. The values are the ΔKlett units between cultures containing 0.05 M of the carbohydrate growth substrate plus 0.015 M of the supplement and the cultures containing 0.015 M supplement alone. The numbers in parentheses next to the growth substrates indicate the Klett units attained in 30 h in cultures with that substrate present alone at 0.05 M. The experiment was conducted as described in Table 2.

ciably. The data for the 20 h cultures on each of the media are shown in Figure 4.

An experiment was conducted to determine whether the failure of the 9 and 13 h cultures in malate-glucose to take up 2-deoxyglucose might be due to an insufficiently long period of exposure to glucose. Cells were incubated in 0.015 M malate for 12 h. Glucose was then added to a final concentration of 0.05 M, and the cells were incubated for an additional 8 h. These cells took up 2-deoxyglucose as well as did the cells incubated in 0.015 M malate plus 0.05 M glucose for the entire 20 h.

An experiment was then conducted to determine whether protein synthesis is required for development of the capacity to transport glucose. Two cultures were grown in 0.015 M malate for 14 h. Chloramphenicol was added to one of the cultures (to a final concentration of 40 µg/ml). After 15 min glucose (0.05 M) was added to both cultures. The cells were incubated for an additional 5 h, after which they were washed and assayed for viability and for the capacity to transport 2-deoxyglucose. Incubation with chloramphenicol caused no loss of viability, but prevented development of transport activity. Cells already capable of transport activity, which were then treated with chloramphenicol for 5 h in the presence of glucose, maintained their capacity to take up 2-deoxyglucose (data not shown).

Cells grown for 21 h in 0.015 M malate plus 0.05 M

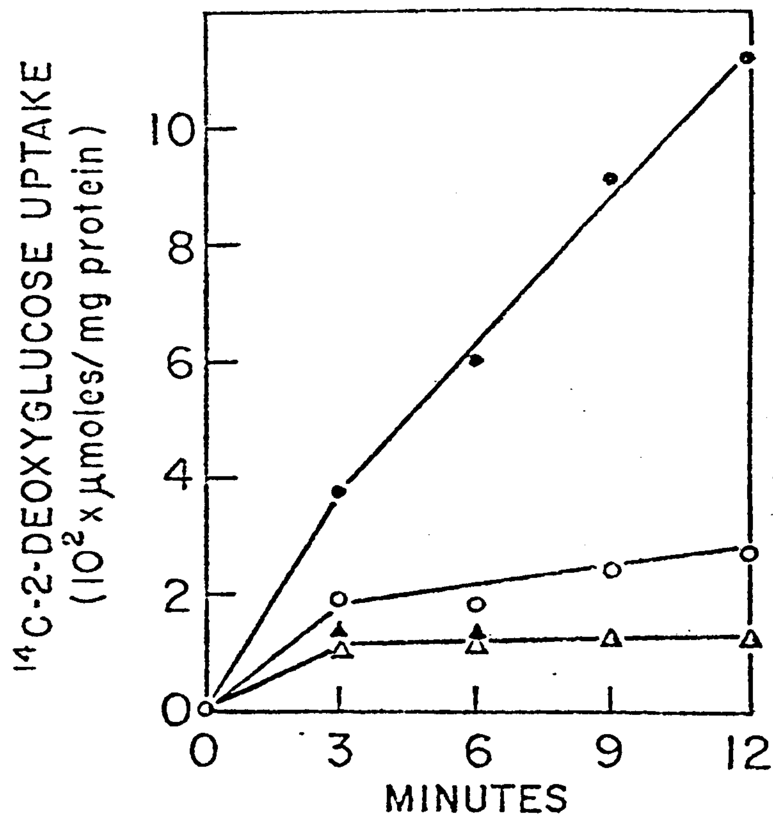


FIG. 4. Uptake of 2-deoxyglucose by *A. pyridinolis*. The cells were grown for 20 h in MS containing 0.015 M malate (O); 0.015 M malate plus 0.05 M glucose (●); 0.015 M asparagine (Δ); 0.015 M asparagine plus 0.05 M glucose (▲). Uptake was determined as described in Materials and Methods by using 1 mM (1-^{14}C)-2-deoxyglucose (0.025 Ci/mol).

maltose took up 2-deoxyglucose as well as did cells grown for the same period of time in malate plus glucose. Cells grown for 21 h in 0.015 M asparagine plus 0.05 M maltose, however, did not take up 2-deoxyglucose.

Cells grown for 21 h in 0.015 M malate or asparagine, with and without 0.05 M glucose or maltose, were also tested for uptake of α-[U-methyl-^{14}C]-glucopyranoside. In all cases, uptake of α-methylglucopyranoside was essentially identical to the uptake of 2-deoxyglucose observed under the same conditions.

The effect of 2-deoxyglucose concentration on the initial rate of its uptake is shown in Figure 5. The K_m for uptake was 2.9 x 10^{-4}M. This $K_m$value as well as the rates of uptake observed are in the same range as the values found for glucose uptake in *A. crystallopoietes* (215) and are consistent with the doubling times of cultures growing on glucose. The uptake of 2-deoxyglucose is competitively inhibited by 0.5 mM glucose, α-methylglucopyranoside, and maltose (Figure 6).

No PEP-dependent phosphorylation of glucose or 2-deoxyglucose could be detected in extracts from cells grown in 0.015 M malate plus 0.05 M glucose for 20 h. To ascertain whether 2-deoxyglucose is accumulated as a phosphorylated derivative, washed cells were incubated in MS containing 1 mM radioactive 2-deoxyglucose for 10 min as described for uptake experiments in Materials and Methods.

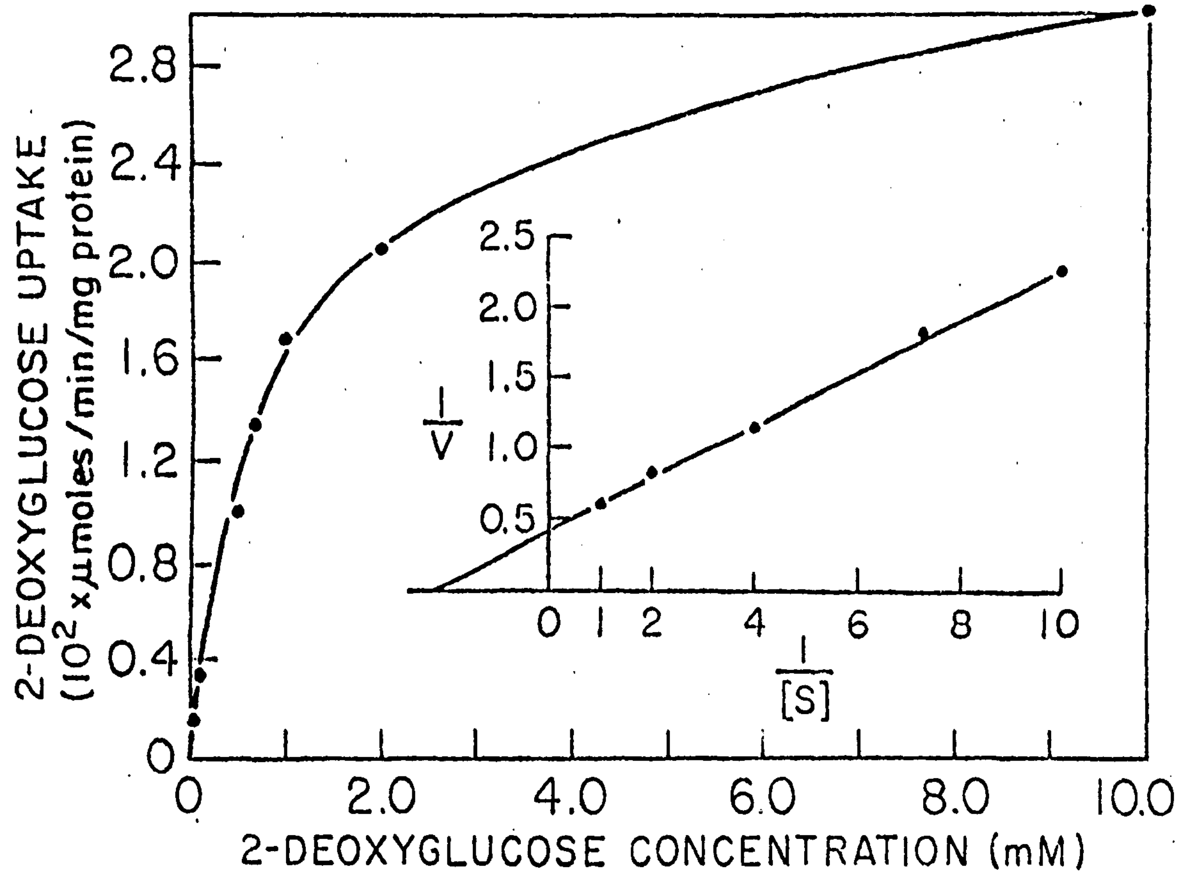


FIG. 5. Effect of 2-deoxyglucose concentration on the initial rate of its uptake into malate-glucose-grown cells. Uptake was determined as described in Materials and Methods by using cells grown for 25 h in MS containing 0.015 M malate plus 0.05 M glucose. Inset: Lineweaver-Burk plot of the data.

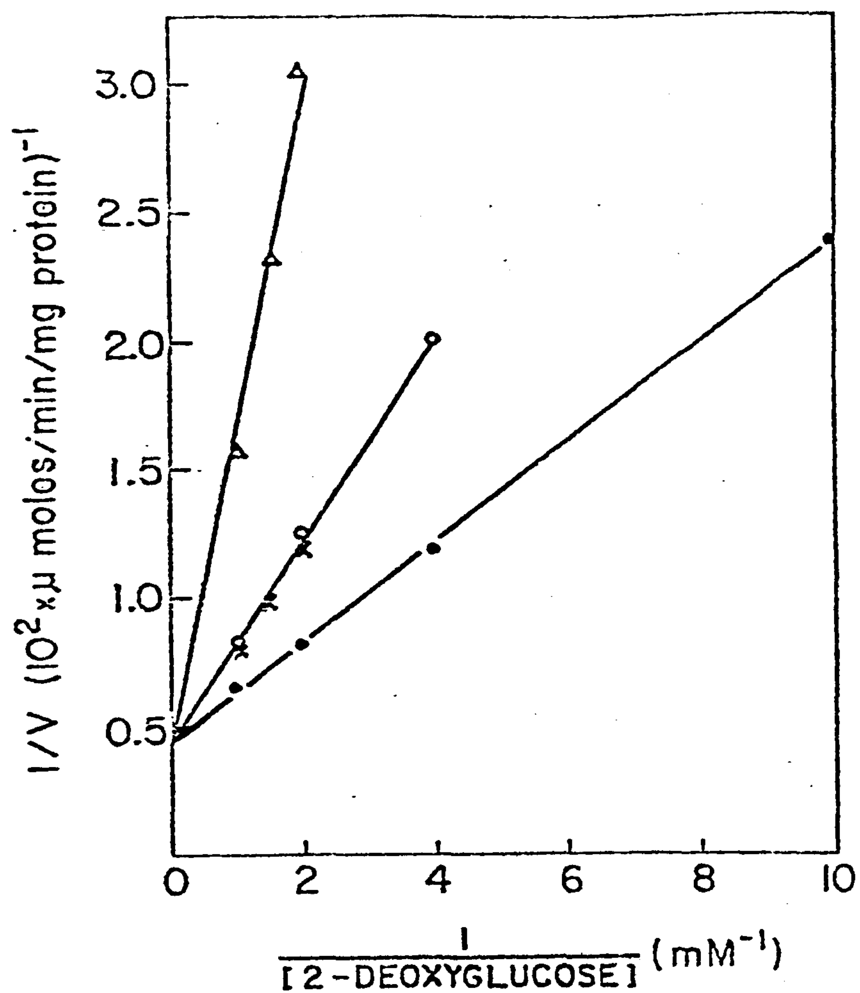


FIG. 6. Inhibition of 2-deoxyglucose uptake by maltose, glucose, and α-methylglucopyranoside. The cells and procedure used for uptake were as described in Fig. 5. Maltose (X), glucose (Δ), or α-methylglucopyranoside (O) were added at 0.5 mM; the kinetics of uptake in the absence of additives other than substrate is designated by closed circles.

The cells were then harvested by centrifugation and resuspended in 0.05 M Tris buffer, pH 7.0. Extracts were prepared and were found to contain 6,160 counts per min per ml. Approximately 4% of this radioactivity was retained by cationic DEAE filter disks after the disks were washed with water. A sample of the extract was adjusted to pH 9 and incubated with alkaline phosphatase for 10 min at 37°C. After neutralization, the treated extract was spotted on DEAE discs. Again, 4% of the total counts was retained by the disks after they had been washed with water. The same results were obtained when radioactive α-methylglucopyranoside was substituted for 2-deoxyglucose in the experiment.

The possibility remained that phosphatase activity in the cell rendered PEP-dependent phosphorylation of glucose or 2-deoxyglucose undetectable. If this were true, however, mutants lacking enzyme I of the Roseman phosphotransferase system would not be expected to transport and use glucose as well as the wild type strain. Such mutants have been isolated as fructose-negative strains and have been characterized by Wolfson and Krulwich (98). When tested for growth on malate plus glucose, they exhibit a growth pattern that is identical to that of the wild type (e.g., see Figure 14).

The effects of several inhibitors on 2-deoxyglucose uptake were determined. *N*-ethylmaleimide inhibited up-

take almost totally (Table 4). Both cyanide and 2,4-dinitrophenol also inhibited strongly. Azide caused less inhibition, and fluoride and malonate had only a slightly inhibitory effect. The possibility that malate might inhibit or stimulate 2-deoxyglucose uptake was also tested. Concentrations of malate ranging from 0.5 to 50 mM stimulated 2-deoxyglucose uptake up to 50% over that observed in the control.

It thus appeared that when certain tricarboxylic acid cycle intermediates are present in the growth medium in addition to glucose, a transport system for glucose and α-glucosides could be induced and that this transport system did not involve phosphorylation of the substrate but was sensitive to uncouplers and inhibitors of electron transport. In order to determine whether the presence of the tricarboxylic acid cycle intermediate was still required after the transport system had been induced, cells which had been grown in malate (0.015 M) plus glucose (0.05 M) for 21 h were washed with MS and inoculated into medium containing 0.05 M glucose alone, 0.05 M glucose plus 0.015 M malate, or 0.015 M malate alone. Only the cells inoculated into medium containing both glucose and malate grew on the glucose; these cells grew to a much greater density than did cells inoculated into 0.015 M malate alone. Thus the glucose transport system appeared to be an inducible, respiration-coupled system whose activity was dependent

TABLE 4

EFFECTS OF VARIOUS INHIBITORS ON 2-DEOXYGLUCOSE UPTAKE BY A. PYRIDINOLIS[1]

Inhibitor	2-Deoxyglucose uptake	
	Amt of uptake ($x10^2$ μmol/mg of protein)	Inhibition(%)
None	6.3	0
N-ethylmaleimide	0.1	99
Potassium cyanide	1.1	83
Dinitrophenol	1.9	71
Sodium azide	4.3	32
Sodium fluoride	5.6	11
Malonate	5.8	8

[1]Cells grown for 22 h (to the glucose logarithmic phase) in 0.015 M malate plus 0.05 M glucose were washed with MS and incubated in MS plus chloramphenicol as described in Materials and Methods. The inhibitor indicated was added to a final concentration of 10 mM. After 10 min of incubation with the inhibitor, [*1*-^{14}*C*]-2-deoxyglucose was added to a final concentration of 0.5 mM, 0.025μCi/ml. The uptake of radioactive substrate was determined after 9 min as described in Materials and Methods.

upon the presence of malate or a precursor thereof.

PEP: fructose phosphotransferase activity. Since growth on fructose as sole carbon source occurred without any special prior growth conditions, it appeared likely that transport of this hexose might occur via some mechanism different from that for glucose transport. The possibility that fructose was transported via a phosphotransferase system was investigated. Cells of *A. pyridinolis* grown on fructose but not on other carbon sources, such as glutamate, contained PEP: fructose phosphotransferase activity. The PEP dependence of the reaction could be demonstrated in crude cell extracts only after the extracts had been dialyzed for 3 h against 0.05 M Tris buffer (*p*H 7.6). In such dialyzed extracts, the specific activity of the PEP: fructose phosphotransferase was 2.3 nmoles per min per mg of protein. When ATP was substituted for PEP in the reaction, less than 1% of the activity was observed. To determine what the phosphorylated product of the PEP-dependent reaction is, a reaction was carried out in which a high concentration of extract protein was used as described by Patni and Alexander (221). After the reaction mixture was deproteinized, it was assayed for fructose 1-phosphate and fructose 6-phosphate. The assay and controls for fructose 1-phosphate were those used for the determination of fructose 1-phosphate kinase with the addition of extract from fructose-grown cells

(a source of fructose 1-phosphate kinase) and the omission of fructose 1-phosphate (221). The assay for fructose 6-phosphate was that used for fructokinase in which the deproteinized extract is substituted for the fructose and ATP. Only fructose 1-phosphate was detected, and the amount formed was 2.1 nmoles per min per mg of protein.

A fractionation experiment was then conducted to determine whether both membrane-associated and soluble cell fractions are required for PEP: fructose phosphotransferase activity. Indications were also sought as to whether the inducible component(s) of the activity is membrane-associated or soluble, or both. Accordingly, 3.0-ml volumes of extracts from fructose-grown and glutamate-grown cells were fractionated by centrifugation at 150,000 x g. The supernatant fluids were collected and the pellets were suspended in 3.0 ml of 0.05 M Tris buffer (*p*H 7.6). Assays for PEP: fructose phosphotransferase were conducted on samples of the whole extracts and on various combinations of fractions. As shown in Table 5, whole extract from glutamate-grown cells (G) contains very little activity in comparison with the extract from fructose-grown cells (F). Neither the supernatant fluid nor the pellet from the F extract contained appreciable activity by itself. A combination of the F supernatant fluid and F pellet, however, restored essentially all the activity of the whole F extract. Combinations of F supernatant fluid with G pellet or G

TABLE 5

FRACTIONATION OF THE PEP:FRUCTOSE PHOSPHOTRANSFERASE ACTIVITY OF *A. PYRIDINOLIS* INTO SUPERNATANT AND PELLET COMPONENTS[1]

Fractions assayed[2]	Fructose 1-phosphate formed[3]
Whole F extract	1.42
F supernatant	0.05
F pellet	0.03
F supernatant + F pellet	1.44
Whole G extract	0.07
G supernatant	0.01
G pellet	0.02
G supernatant + G pellet	0.04
F supernatant + G pellet	0.21
G supernatant + F pellet	0.25

[1]Extracts were prepared from washed fructose-grown (F) and glutamate-grown (G) cells of *A. pyridinolis* by sonic disruption in 0.05 M Tris buffer, pH 7.6. The F and G extracts contained 12.6 and 16.5 mg of protein/ml, respectively. A portion (3 ml) of each extract was fractionated into supernatant and pellet fractions after centrifugation at 150,000 x g for 2 hr. The pellets were resuspended in 3.0 ml of 0.05 M Tris buffer, pH 7.6. The protein concentrations of the fractions were: F supernatant, 6.8 mg/ml; F pellet, 6.4 mg/ml; G supernatant, 11.0 mg/ml; G pellet, 10.0 mg/ml. The whole extracts and indicated combinations of fractions were assayed for PEP: fructose phosphotransferase activity as described in Materials and Methods.

[2]The amount of each fraction assayed was 50 μliters.

[3]Nanomoles per minute per reaction mixture.

supernatant fluid with F pellet resulted in partial restoration of activity, but the values were always less than 20% of the activity of whole F extract.

Mutants of the PEP: fructose phosphotransferase were isolated on the basis of their inability to grow on fructose as sole carbon source. Using *in vitro* complementation assays, Wolfson and Krulwich (234) divided these mutants into four types and characterized the PEP: fructose phosphotransferase as a four component system. Both enzyme I and phosphocarrier protein are constitutive, soluble proteins. Enzyme II and factor III are both inducible and sugar-specific proteins; enzyme II is membrane bound while factor III is soluble (98). Thus *A. pyridinolis* indeed has a phosphotransferase system which resembles that found in other species. It was also found that mutants which are deficient in either enzyme I or phosphocarrier protein are unable to grow on rhamnose as sole carbon source, indicating that the phosphotransferase system is for rhamnose transport as well as fructose transport (100).

Properties of membrane vesicles prepared from fructose-grown wild type cells. In order to study further details of the transport of fructose in *A. pyridinolis*, membrane vesicles were prepared from fructose-grown wild type cells. The preparations of membrane vesicles contained no whole cells, and fewer than one unlysed spheroplast per oil immersion field (800-fold magnification) as observed

with the phase-contrast microscope. The structures seen in electron micrographs of thin sections of vesicles (Figure 7) supported the view that the vesicles were closed membranous sacs. Sonicated vesicle preparations were assayed for isocitrate dehydrogenase and fructose 1, 6-diphosphatase. While crude extracts of whole cells contained high levels of these cytoplasmic enzymes (0.72 and 0.3 μmoles/min/mg protein, respectively), the vesicles contained no detectable activity of either enzyme. In addition, the fact that membrane vesicles could not use 2-phosphoglycerate as a substitute for PEP in support of fructose transport (see Table 6), indicated that the membrane preparations had no enolase activity.

PEP-dependent fructose transport in membrane vesicles. In the presence of PEP at 35°C, membrane vesicles took up 0.35 nmole of fructose/min per mg of protein. In the absence of PEP or when nucleotide triphosphates or 2-phosphoglycerate are substituted for PEP, no more than 0.05 nmoles of fructose were taken up per min per mg of protein. In Figure 8, the relationship between PEP concentration and the initial rate of fructose uptake is shown. The uptake of fructose by the vesicles increased rapidly with increasing concentrations of PEP from 0 to 20 mM, and then continued to increase less rapidly up to 100 mM. In all subsequent experiments PEP was employed at a concentration of 100 mM.

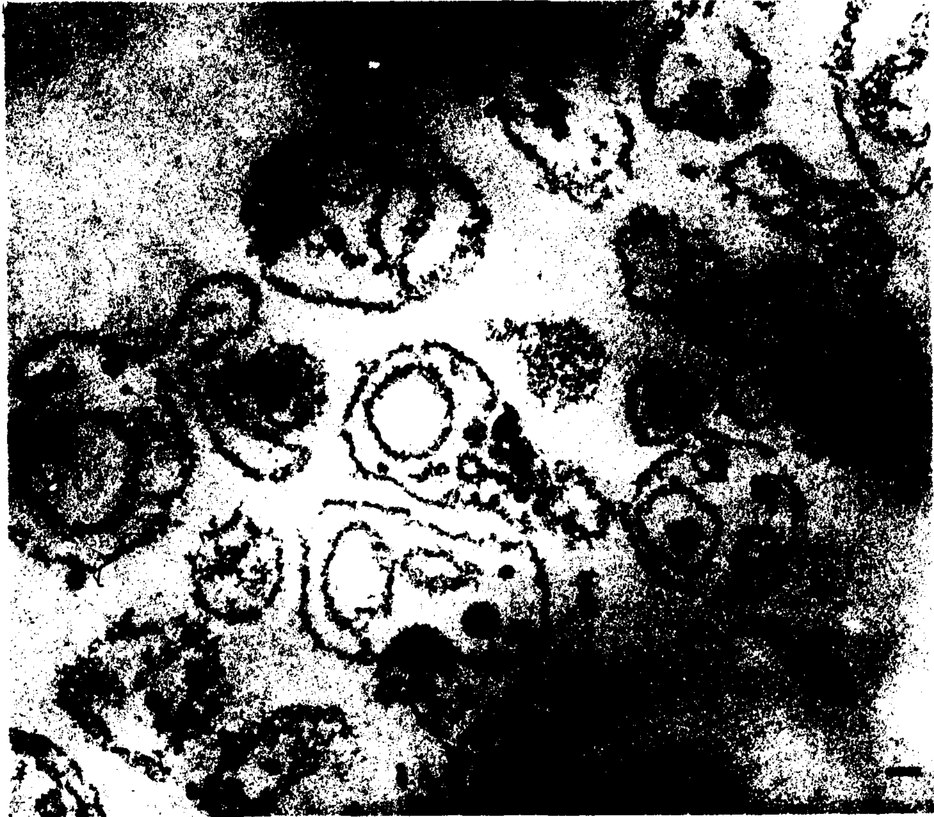


FIG. 7. Electronmicrograph of membrane vesicles prepared from fructose-grown wild type cells of *A. pyridinolis*. The bar is 0.1 μm.

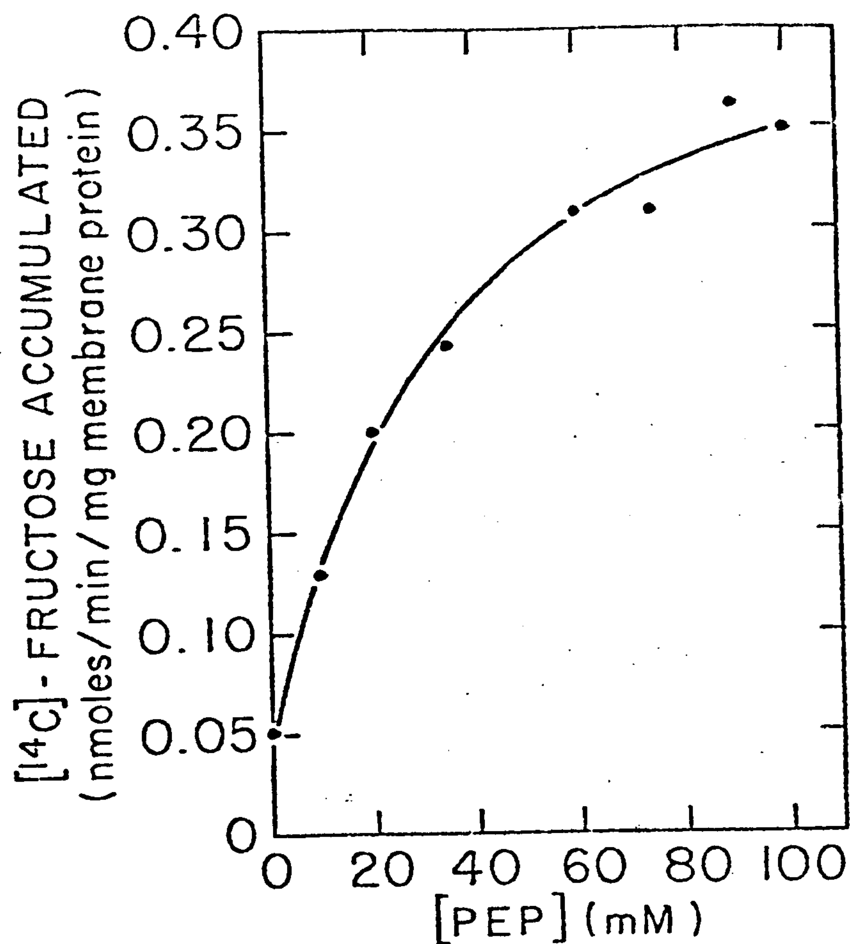


FIG. 8. Effect of PEP concentration on the initial rate of fructose uptake into vesicles. Transport was assayed at 38°C as described in Materials and Methods, using varying concentrations of PEP. The reactions were terminated 10 min after the addition of (U-^{14}C)-fructose.

The effect of temperature on the time course of accumulation of fructose by the vesicles in the presence of PEP is shown in Figures 9A and 9B. A slow steady accumulation was observed at 0°C, and increasingly rapid rates of accumulation were found at 22°C and 30°C over a 15-min period. At higher temperatures, 35°-45°C, fructose was accumulated for 10 min, after which there was an apparent loss of fructose from the vesicles. No accumulation was observed at 50°C. As depicted in Figure 9B, the smaller the time period used to measure fructose accumulation (over a 5 to 20-min range), the higher the temperature optimum for uptake.

The effect of fructose concentration on the initial rate of its uptake by vesicles was then determined at 32°C in the presence of 100 mM PEP (Figure 10). Uptake showed Michaelis-Menten kinetics; a K_m of 1.5×10^{-5} M was calculated from a Lineweaver-Burk plot of the data in Figure 10.

Malate-dependent fructose transport in vesicles. The effect of various compounds on fructose uptake was then studied to see if sources of energy other than PEP could be used to support transport of fructose. The results, summarized in Table 6, indicated that: (1) malate caused an almost three-fold stimulation of fructose accumulation over that observed in the absence of additions to a level representing 65% of that seen in the presence of PEP; (2) all the other compounds tested caused much less stimulation,

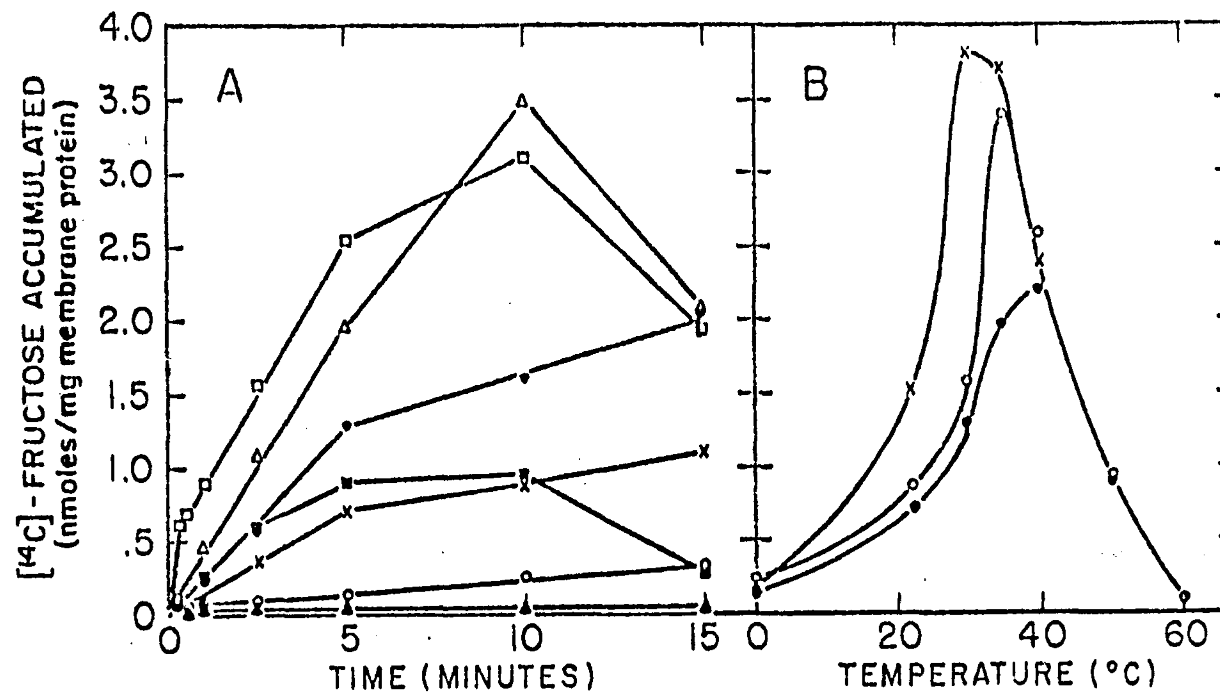


FIG. 9. Effect of temperature on the time course of PEP-dependent fructose uptake by vesicles. Transport was assayed at different temperatures as described in Materials and Methods.
A: Time course of fructose uptake at 0°C (O), 22°C (X), 30°C (●), 35°C (Δ), 40°C (□), 45°C (■), or 50°C (▲). B: Fructose accumulated as a function of temperature after 5 min (●), 10 min (O), or 20 min (X).

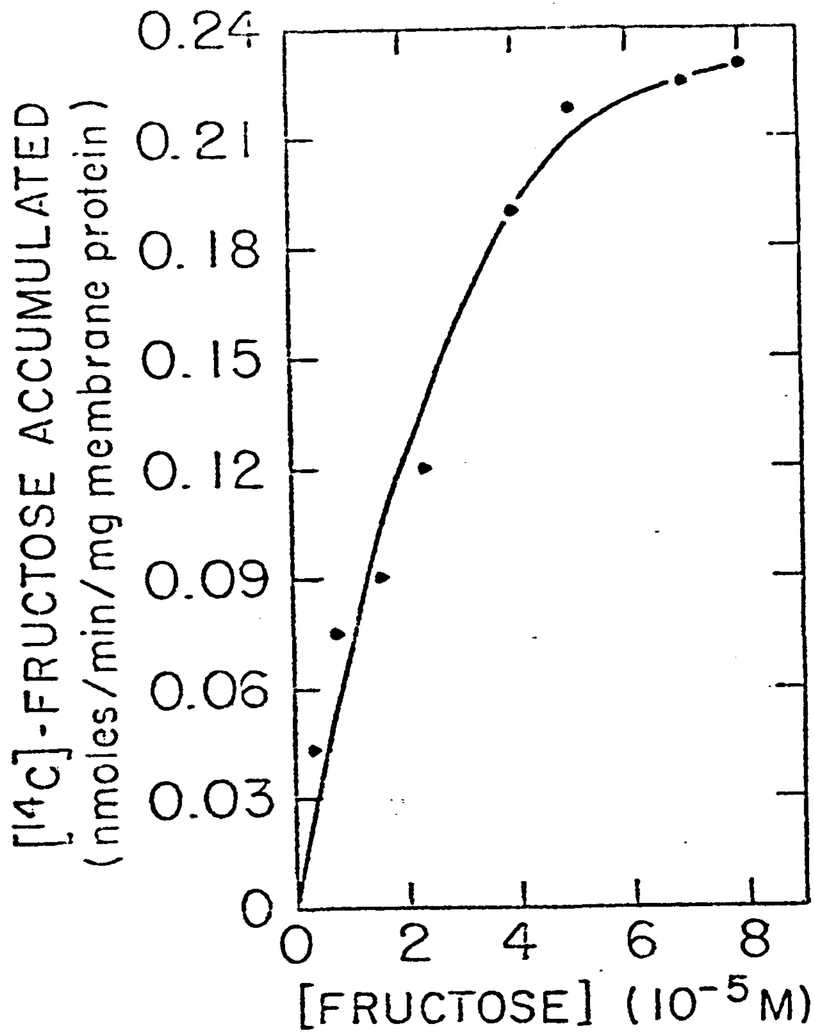


FIG.10. Kinetics of PEP-dependent fructose uptake by isolated membrane preparations. Vesicles were assayed for PEP: fructose phosphotransferase activity as described in Materials and Methods at 32°C with varying concentrations of (U-^{14}C)-fructose. Incubations wereterminated 10 min after addition of the sugar substrate.

TABLE 6

THE EFFECT OF VARIOUS COMPOUNDS ON FRUCTOSE UPTAKE BY ISOLATED MEMBRANE VESICLES[1]

Addition	Relative fructose uptake	Addition	Relative fructose uptake
None	0.22	Malate	0.65
PEP	1.00	Lactate (D-isomer)	0.34
ATP	0.10	Pyruvate	0.22
CTP	0.10	α-Glycerolphosphate	0.31
GTP	0.16	α-Hydroxybutyrate	0.28
UTP	0.11	Oxaloacetate	0.28
NAD	0.15	2-Phosphoglycerate	0.22
NADH	0.33	3-Phosphoglycerate	0.33
NADP	0.12	Malate + PEP	1.01
NADPH	0.33	Malate + 2,4-Dinitrophenol (10^{-3}M)	0.09
FAD	0.15	Malate + KCN (10^{-2}M)	0.14
Citrate	0.25	PEP + 2,4-Dinitrophenol (10^{-3}M)	1.08
Fumarate	0.25	PEP + KCN (10^{-2}M)	1.06
Succinate	0.36		

[1]Uptake of (U-^{14}C)-fructose by isolated membrane vesicles from fructose-grown *A. pyridinolis* was determined using 10 minute incubations at 35° C, in the presence of the additions shown. Unless indicated, the additions were present at 0.1 M. The values are expressed relative to the uptake of fructose in the presence of PEP (0.2 nmoles/min/mg vesicle protein).

caused no stimulation, or even appeared to depress accumulation below that seen in the absence of additions; and (3) 2,4-dinitrophenol or potassium cyanide completely abolished malate-stimulated accumulation of fructose while causing no inhibition of PEP-stimulated accumulation. These data suggested that there existed an alternate transport system to the phosphotransferase system for transport of fructose, i.e., a malate-dependent, respiration-coupled system. This suggestion was supported by the observation that phosphotransferase-negative mutants, which cannot grow on fructose alone, grew to a much greater density in medium containing malate plus fructose than could be accounted for on the basis of the malate content of the medium (see Figures 13 and 14). Malate-dependent transport of fructose was therefore examined in greater detail.

In Figure 11, the rate of fructose uptake by vesicles is shown as a function of the malate concentration. At optimal concentrations, the presence of malate typically caused a 2- to 2.5-fold increase in the rate of fructose uptake. Addition of FAD, over a range of 0.05 - 100 mM, had no effect on malate-dependent fructose uptake. Using 5 mM malate, the dependence of the rate of fructose uptake on the concentration of fructose was determined. As shown in Figure 12, fructose uptake exhibited Michaelis-Menten kinetics; a K_m of 5.6×10^{-7} M for fructose was calculated from the reciprocal plot.

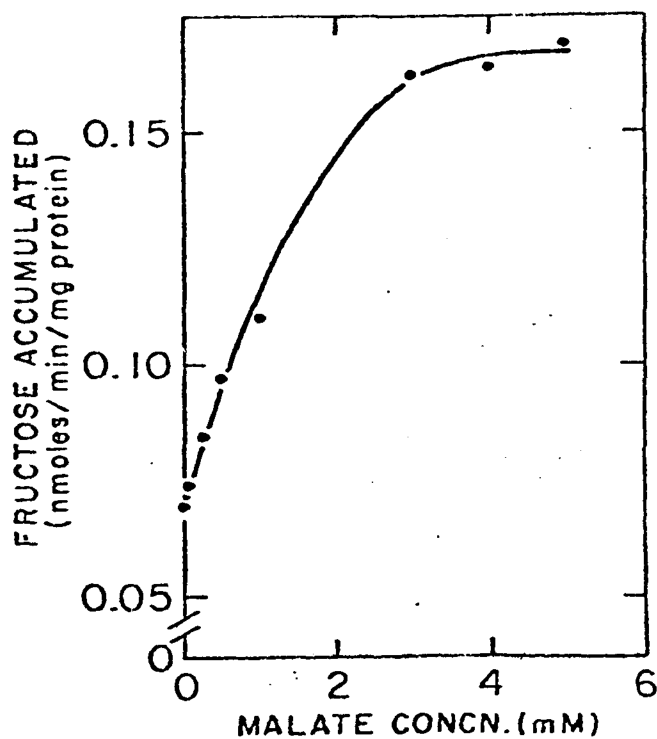


FIG. 11. Effect of varying malate concentrations on fructose uptake by membrane vesicles. Transport was assayed as described in Materials and Methods, except that PEP was deleted from the standard reaction mixture. Malate, at the concentrations indicated, was added at the start of the 10 min period prior to the addition of radioactive fructose. The incubations were terminated after 10 min.

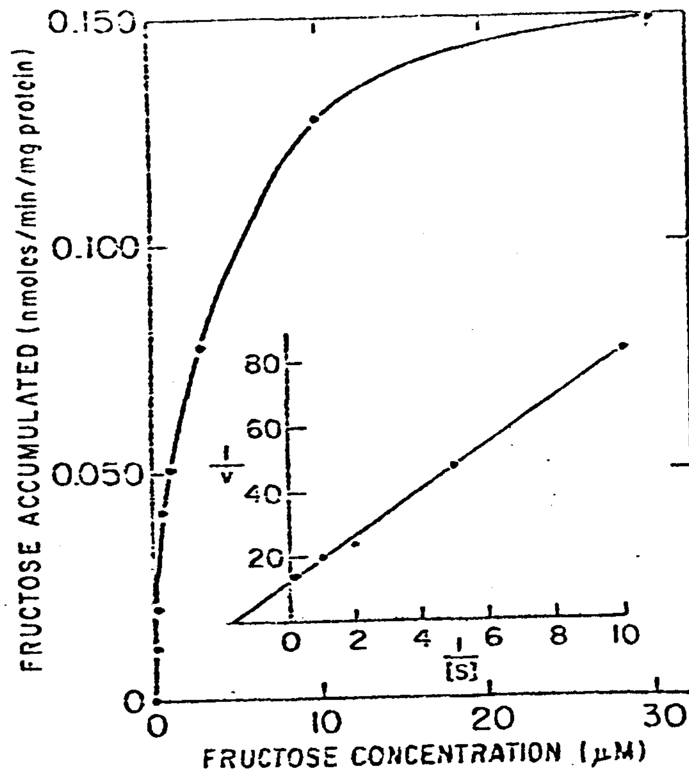


FIG. 12. Effect of fructose concentration on the rate of malate-dependent fructose uptake by vesicles. Transport was assayed as described in Fig. 11, using 5 mM malate and the indicated concentrations of fructose. Inset: Lineweaver-Burk plot of the data.

The effects of several inhibitors on malate- and PEP-dependent fructose uptake were examined using vesicles from fructose-grown wild type cells of *A. pyridinolis*. As shown in Table 7, CCP, DNP, HQNO, KCN, and NEM all caused significant inhibition of malate-dependent uptake while causing much less or no inhibition of PEP-dependent uptake. It thus appeared that like the malate-dependent transport system for glucose in *A. pyridinolis*, malate-dependent transport of fructose is indeed respiration-coupled.

A similar malate-dependent transport system for rhamnose was subsequently found in *A. pyridinolis* by Levinson and Krulwich (100). It thus became of interest to determine the specificity of the putative carriers for the three sugars whose transport could be coupled to respiration. Therefore, vesicles from fructose-grown cells were tested for the ability to transport glucose and rhamnose. As shown in Table 8, neither malate nor PEP supported transport of either glucose or rhamnose. These results suggested that there was a fructose-specific component associated with the respiration-coupled system in addition to the two known fructose-specific components involved in the PEP: fructose phosphotransferase system. Since mutants lacking either fructose-specific component of the phosphotransferase system could still grow on fructose in the presence of malate (see Figures 13 and 14), the sugar-specific component of the respiration-coupled system could not be identical to

TABLE 7

EFFECT OF INHIBITORS ON FRUCTOSE UPTAKE BY VESICLES[1]

Inhibitors	Concentration (M)	% Malate- or PEP-dependent fructose uptake	
		Malate	PEP
None	-	100	100
CCP[2]	10^{-6}	0.0	71.9
DNP	10^{-4}	17.3	79.9
HQNO[2]	10^{-4}	14.5	133.3
KCN	10^{-2}	44.2	101.5
NEM	10^{-3}	10.5	62.8

[1]Uptake was determined as described in the legend to Table 6. Inhibitors, at the concentrations indicated, were present during the 10 min before radioactive fructose was added as well as during the uptake period.

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

TABLE 8

PEP- AND MALATE-DEPENDENT UPTAKE OF SEVERAL SUGARS BY VESICLES FROM FRUCTOSE-GROWN *A. PYRIDINOLIS*[1]

Sugar	Energy source	Uptake of sugar (nmoles/min/mg protein)
Fructose	None	0.049
Fructose	Malate	0.152
Fructose	PEP	0.218
Glucose	None	0.021
Glucose	Malate	0.021
Glucose	PEP	0.025
Rhamnose	None	0.010
Rhamnose	Malate	0.011
Rhamnose	PEP	0.011

[1]Vesicles were incubated in a standard incubation mixture with no addition, 5 mM malate, or 100 mM PEP for 10 min. Radioactive fructose, glucose, or rhamnose (20μm) was then added. Uptake was terminated after 10 min.

either of these phosphotransferase components.

Vesicles were prepared from glutamate-grown cells of *A. pyridinolis* and from fructose plus glutamate-grown cells of strain AP100, a strain lacking the soluble fructose-specific component of the phosphotransferase system. These vesicle preparations were tested for PEP- and malate-dependent fructose transport. As expected, neither one of these vesicle preparations showed appreciable PEP-dependent fructose transport as compared to that observed with vesicles from fructose-grown wild type cells (Table 9). On the other hand, vesicles from glutamate-grown wild type cells and from fructose-glutamate-grown cells of AP100 both exhibited considerable malate-dependent fructose uptake. Malate-dependent uptake of fructose by the vesicles from AP100 was expected; this strain is PEP: fructose phosphotransferase negative but should have an intact respiration-coupled system for fructose transport as long as the missing phosphotransferase component is not a component of the respiration-coupled system.

The finding of malate-dependent uptake of fructose by vesicles from glutamate-grown wild type cells suggested that the fructose-specific component of the respiration-coupled system may be constitutive. Data from studies of vesicles prepared from rhamnose-grown cells support this conclusion. In these studies, Levinson and Krulwich (100) showed that vesicles prepared from rhamnose-grown cells contained malate-dependent transport systems for rhamnose

TABLE 9

PEP- AND MALATE-DEPENDENT FRUCTOSE UPTAKE IN VARIOUS VESICLE PREPARATIONS[1]

Vesicle preparation from	Nmoles fructose taken up per min per mg protein in the presence of		
	No add	PEP	Malate
Wild type, fructose-grown	0.049	0.218	0.152
Wild type, glutamate-grown	0.021	0.038	0.078
AP100, glutamate + fructose-grown	0.039	0.048	0.085

[1]Uptake was determined as described in the legend to Table 8, except that the vesicles used were as indicated above.

as well as for fructose, but not for glucose. In contrast to the malate-dependent transport system for fructose, the sugar-specific components of the malate-dependent respiration-coupled system for rhamnose and glucose appear to be inducible (see above).

Malate dehydrogenase activity in cells and vesicles. Vesicles from fructose-grown wild type cells were found to have activity of an FAD-linked malate dehydrogenase (0.66 μmoles of malate oxidized/min/mg of vesicle protein). This activity was also detected in sonic extracts of whole cells where it was present at a range of specific activities from 0.31 to 0.43 μmoles/min/mg protein in fructose-, asparagine-, malate-, and glutamate-grown cells. When sonic extracts of fructose-grown cells were fractionated by centrifugation for 1 h at 100,000 x g, over 90% of the FAD-linked malate dehydrogenase activity was associated with the pellet. No NAD-linked malate dehydrogenase was detected in either vesicles or the pellet fractions from whole cell extracts.

Metabolism of fructose. Assays of enzymes which might be involved in fructose metabolism were conducted in crude cell extracts to ascertain how the fructose 1-phosphate produced in the phosphotransferase reaction and how the fructose transported by the malate-dependent system enter into the major catabolic pathways. Extracts from fructose-grown cells contained appreciable levels of fructokinase, fructose 6-phosphate kinase, and fructose 1-phos-

phate kinase in addition to PEP: fructose phosphotransferase (Table 10). Such extracts also contained very high levels of FDPase; the specific activity of FDPase in extracts of fructose-grown cells was about five times higher than in extracts of malate- or PYE-grown cells and 24 times higher than in extracts of glucose-glutamate-grown cells. Mixed extract experiments eliminated the possibility that any of the dialyzed extracts contained an inhibitor (or activator) which was responsible for the apparent difference in FDPase activity. Extracts of glucose-glutamate-grown cells contained approximately five times higher levels of glucokinase and somewhat lower levels of fructose 1-phosphate kinase than did extracts from fructose-grown cells. Levels of fructokinase were similar in these two types of extracts. In extracts prepared from cells grown on fructose or limiting (0.015 M) malate plus either fructose, glucose, or sucrose, fructokinase activity is approximately two-fold higher than that found in extracts of cells grown on malate as sole carbon source (data not shown). The fructokinase assay, conducted as described in Materials and Methods, followed the disappearance of NADPH by coupling the fructose 6-phosphate formed in the fructokinase reaction to phosphohexoseisomerase and glucose 6-phosphate dehydrogenase. It was possible that the product of the fructokinase reaction was fructose 1-phosphate and that crude extracts of *A. pyridinolis* contain a phosphofructomutase which can convert the fructose 1-phosphate to fructose 6-phosphate. To rule

TABLE 10

SURVEY OF SOME ENZYMES FOR FRUCTOSE METABOLISM IN FRUCTOSE- AND GLUCOSE-GLUTAMATE-GROWN CELLS OF *A. PYRIDINOLIS*[1]

Enzyme	Specific activity in extracts from cells grown on	
	Glucose-glutamate	Fructose
PEP: fructose phosphotransferase	<0.0001	0.002
Fructose 1-phosphate kinase	0.012	0.023
Fructokinase	0.031	0.025
Fructose 6-phosphate kinase	0.022	0.023
Fructose 1,6-diphosphatase	0.013	0.310
Glucokinase	0.056	0.013

[1]Extracts were prepared from washed cells grown in MS plus 0.05 M fructose or MS plus 0.015 M glutamate plus 0.05 M glucose. Extracts were prepared and assays were conducted as described in Materials and Methods; specific activities are expressed as micromoles of substrate consumed or product formed per minute per mg. of protein.

out this possibility, the fructokinase assay was modified by substituting fructose 1-phosphate, in concentrations varying from 1 to 20 mM, for fructose and ATP. Extracts prepared from either fructose-grown or malate plus fructose-grown cells did not contain phosphofructomutase activity.

The results above suggested that fructose that is transported via PEP: fructose phosphotransferase is phosphorylated at carbon-1 by that system and is subsequently phosphorylated by fructose 1-phosphate kinase to form fructose 1,6-diphosphate. Fructose that is transported into the cell as free fructose by the malate-dependent respiration-coupled system is converted by fructokinase to fructose 6-phosphate. The fructose 6-phosphate can then be phosphorylated by fructose 6-phosphate kinase to form fructose 1,6-diphosphate. Alternatively, the fructose 6-phosphate can be isomerized to glucose 6-phosphate. If these pathways were correct, it was anticipated that mutants deficient in either PEP: fructose phosphotransferase or fructose 1-phosphate kinase would be unable to grow on fructose as sole carbon source while retaining a normal ability to utilize glucose (when the latter sugar is present in addition to a compound such as malate). Mutants deficient in FDPase would also be expected to have lost the ability to grow on fructose as sole carbon source; without FDPase, cells growing on fructose alone would presumably not produce fructose 6-phosphate and hence could not generate glucose 6-phosphate for cell wall synthesis. On the other

hand, mutants deficient in any of these enzymes would be expected to utilize fructose when the growth medium is supplemented with malate since the malate-dependent respiration-coupled system and the enzymes for metabolism of fructose to fructose 6-phosphate and its glucose isomer should still be intact. The enzyme contents of extracts from several mutants having a reduced ability to grow on fructose as sole carbon source were examined (Table 11). The growth of these strains on fructose (0.05 M), malate (0.015M), and malate (0.015 M) plus either glucose (0.05 M) or fructose (0.05 M) is shown in Figure 13. AP9 is a mutant strain which produces about 10% of the wild type levels of FDPase during growth on glutamate plus fructose. AP9 was completely incapable of growth on fructose as sole carbon source, but was capable, after a lag, of limited growth on malate at a rate less than one-half the rate of growth of the wild type strain on malate. The growth data depicted in Figure 13 suggest that AP9 is able to utilize glucose and fructose when malate is present in the medium.

Strain AP104 is typical of many isolates with reduced levels of both fructose 1-phosphate kinase and PEP: fructose phosphotransferase. In some of these strains, one of the two activities was reduced to a greater extent than the other. AP104 and the other strains with low levels of PEP: fructose phosphotransferase and fructose 1-phosphate kinase activities were all unable to grow on fructose as sole carbon source. Extracts prepared from cells of AP104 grown

TABLE 11

ENZYMATIC ACTIVITIES IN EXTRACTS OF MUTANTS OF *A. PYRIDINOLIS* WHICH SHOW REDUCED GROWTH IN FRUCTOSE[1]

Strain	Specific activity of			
	PEP: fructose phospho-trans-ferase[2]	Fruc-tose 1-phosphate-kinase[3]	Fruc-tose 6-phosphate-kinase[3]	Fruc-tose 1,6 diphos-phatase[3]
Wild type.....	2.3	0.038	0.026	0.310
AP9...........	1.7	0.026	0.020	0.028
AP104.........	0.5	0.009	0.023	0.250

[1]Extracts were prepared from cells grown in MS plus 0.05 M glutamate plus 0.05 M fructose and were assayed as described in Materials and Methods.

[2]Nanomoles of fructose 1-phosphate formed per minute per milligram of protein.

[3]Micromoles of product formed per minute per milligram of protein.

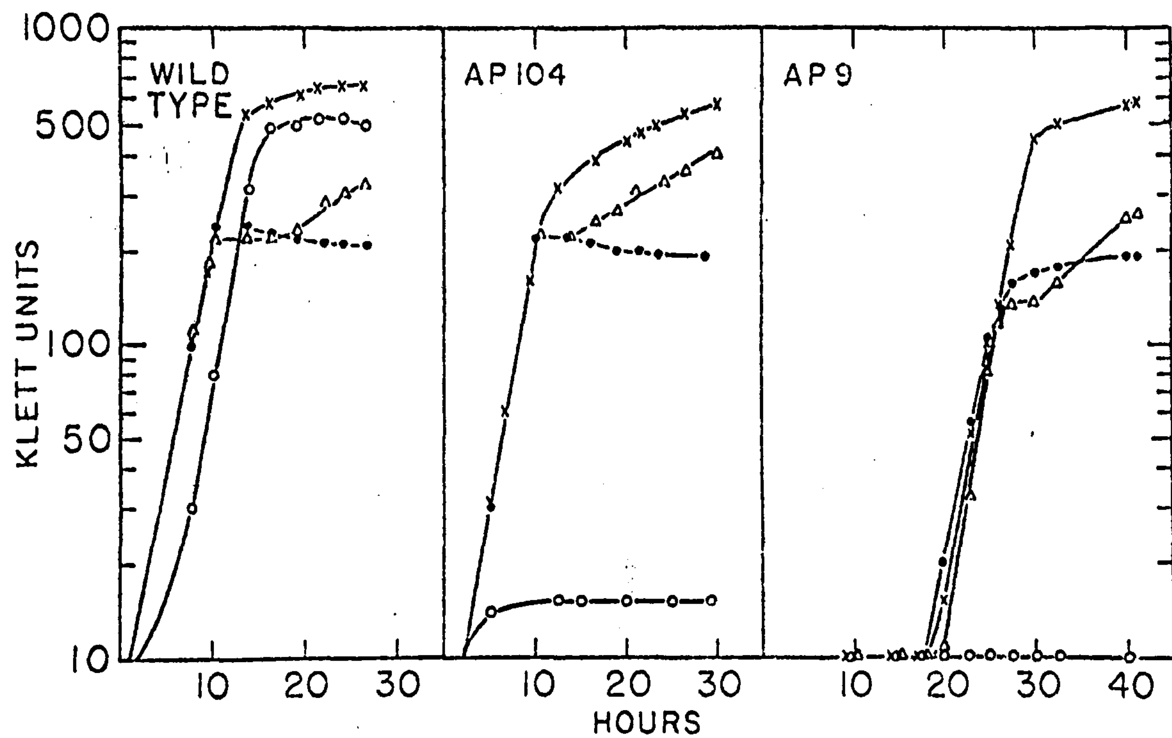


FIG. 13. Growth of wild type and mutant strains of *A. pyridinolis* on fructose, malate, and malate plus fructose or glucose. Cells of the strain indicated were grown in MS containing 0.05 M fructose (O), 0.015 M malate (●), 0.015 M malate plus 0.05 M glucose (Δ), and 0.015 M malate plus 0.05 M fructose (X).

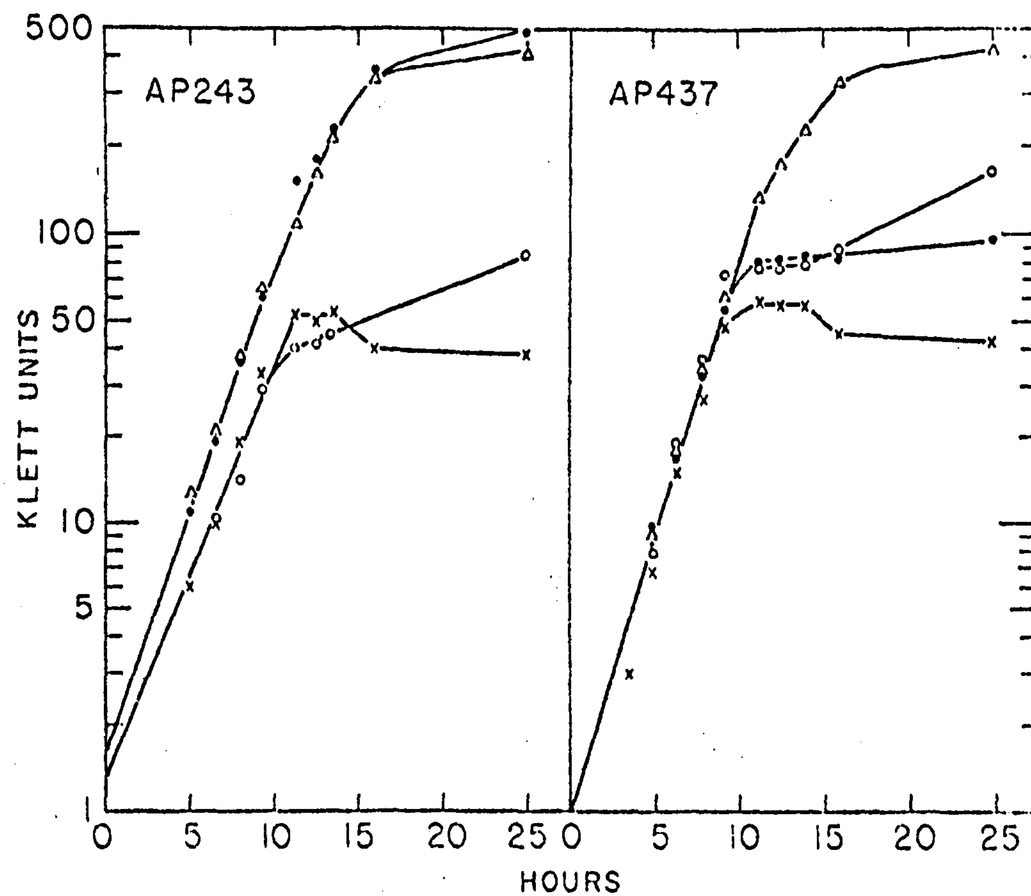


FIG. 14. Growth of *A. pyridinolis* strain AP243 and its derivative, AP437, on limiting malate plus various sugars. Cells were grown in MS containing 0.005 M malate plus: no add (X), 0.05 M glucose (O), 0.05 M fructose (●), or 0.05 M rhamnose (Δ).

on limiting (0.015 M) malate plus fructose contained wild type levels of fructokinase. These strains retained a normal ability to grow on malate and to utilize glucose after a period of growth on malate. They could also utilize fructose when malate was present in the growth medium. Subculturing of cells after 20 h indicated that the apparent utilization of fructose by this mutant in the presence of malate was not due to reversion.

The growth properties of mutant strains AP243 (deficient in enzyme I of the phosphotransferase) and AP437 (deficient in both enzyme I and the fructose-specific factor of the malate-dependent respiration-coupled system) are demonstrated in Figure 14. Neither AP437 nor AP243 could use fructose or rhamnose as sole carbon source because of the mutation in the phosphotransferase system. AP437 grew normally on malate and grew on rhamnose and glucose in the presence of malate. However, this strain used fructose to only a small extent even when malate was provided. AP243, on the other hand, like AP104, grew normally on fructose, glucose, and also rhamnose in the presence of malate. Both AP243 and AP437 contained fructokinase activity.

Effect of tricarboxylic acid cycle intermediates on the level of PEP: fructose phosphotransferase activity. For most of the studies of the PEP: fructose phosphotransferase system described above, wild type and phosphotransferase-negative strains were grown on 0.05 M fructose plus

0.05 M glutamate. When, on occasion, 0.05 M malate was substituted for glutamate in the growth medium, it was observed that levels of phosphotransferase activity were markedly depressed in wild type cells. Previous findings (see above) had shown that growth on high concentrations of malate resulted in a failure to induce the respiration-coupled transport system for glucose. Also, Wolfson and Krulwich have demonstrated that a functional respiration-coupled transport system for fructose is required for induction of PEP: fructose phosphotransferase (98), i.e., that some free fructose had to enter via the former transport system in order to induce the inducible components of the latter transport system. The experiments described below were conducted to investigate the possibility that the lowered levels of phosphotransferase activity observed in the presence of malate might be a secondary effect of lowering the activity of the respiration-coupled system.

PEP: fructose phosphotransferase activity was measured in extracts prepared from wild type cells grown in the presence of different substrates. As shown in Table 12, wild type cells grown in the presence of fructose plus either glutamate or malate had markedly lower levels of phosphotransferase activity than did cells grown on fructose as sole carbon source. As the concentration of malate was increased in the growth medium this effect became more pronounced. The possibility that an inhibitor was present in the extracts prepared from cells grown in the presence of

TABLE 12

EFFECT OF GROWTH ON TRICARBOXYLIC ACID CYCLE INTERMEDIATES ON THE LEVEL OF PEP: FRUCTOSE PHOSPHOTRANSFERASE ACTIVITY[1]

Cells grown on:	Specific activity[2]
0.05 M Fructose	12.60
0.05 M Glutamate	0.28
0.05 M Glutamate + 0.05 M fructose	5.68
0.005 M Malate + 0.05 M fructose	4.51
0.015 M Malate + 0.05 M fructose	2.80
0.05 M Malate + 0.05 M fructose	2.26

[1]Cells were grown on the carbon sources indicated for 20 h. Extracts were prepared and dialyzed as described in Materials and Methods. PEP: fructose phosphotransferase was assayed as described in Materials and Methods except that the assay was conducted at 30°C and 0.25 mM (U-^{14}C)-fructose was used.

[2]Specific activity is expressed as nanomoles of fructose 1-phosphate formed per min per mg. protein.

malate was excluded by mixed extract experiments. Wild type cells growing on either fructose alone, low (0.005 M) concentrations of malate plus fructose, or high (0.05 M) concentrations of malate plus fructose were then assayed for PEP: fructose phosphotransferase at intervals to see if the activity was present during various phases of growth on malate-containing media. As shown in Table 13, at 10 h of growth while cells were utilizing malate (see Figure 16), even at a relatively low concentration (0.005 M), no significant activity was detectable. Eventually, cells grown in the presence of fructose plus 0.005 M malate were able to induce full levels of activity as compared to cells grown on fructose as sole carbon source. However, cells grown in the presence of fructose plus 0.05 M malate did not appear to ever attain significant levels of PEP: fructose phosphotransferase. It was also interesting to note that, regardless of the nature of the growth medium, as the growth period increased past 15 h, PEP: fructose phosphotransferase activity decreased.

In order to determine whether the effect of malate on the phosphotransferase system was due to a failure to form the inducible components of the PEP: fructose phosphotransferase system (soluble factor III and membrane-bound enzyme II), extracts were fractionated into supernatant and pellet fractions as described in Materials and Methods. The results of complementation studies between fractionated extracts prepared from fructose-grown cells (F) and cells

TABLE 13

INDUCTION OF PEP: FRUCTOSE PHOSPHOTRANSFERASE IN CELLS OF *A. PYRIDINOLIS* GROWN IN THE ABSENCE AND PRESENCE OF MALATE[1]

Time period of growth	Specific activity[2] of cells grown in 0.05 M fructose plus:		
	No add	0.005 M malate	0.05 M malate
10 hours	N.D.[3]	0.8	1.1
15 hours	12.6	12.3	4.6
20 hours	9.3	6.2	2.4
25 hours	8.0	5.8	2.5

[1]Extracts were prepared as described in Materials and Methods and assays were conducted as described in Table 12. Cells were grown for either 10, 15, 20 or 25 hours in fructose plus the additional carbon source noted before harvesting.

[2]Nanomoles fructose 1-phosphate formed per min per mg. protein.

[3]Not done due to insufficient growth of cells.

grown on fructose plus 0.05 M malate (MF) are summarized in Table 14; they suggest that there are lower levels of both inducible components of PEP: fructose phosphotransferase in the latter cells. Combinations of F supernatant with MF pellet, or MF supernatant with F pellet resulted in only partial restoration of activity. However, in the presence of F pellet, the soluble cell fraction from MF cells could complement soluble fractions from AP243 (enzyme I-deficient) and AP253 (phosphocarrier protein-deficient) cells grown on glutamate plus fructose (Table 15), indicating that the malate-fructose grown cells have normal levels of the constitutive phosphotransferase components.

To determine whether the observed effect of growth on malate on PEP: fructose phosphotransferase activity was physiologically significant, fructose uptake in whole cells was followed. Since a nonmetabolizable analog of fructose was not available, uptake experiments were conducted at short time intervals of 30 sec; in this way, the effects of metabolism on uptake were hopefully minimized. Fructose uptake, as a function of fructose concentration, was compared in fructose-grown wild type cells and glutamate-fructose grown cells of phosphotransferase-negative strain AP243; in the latter cells, all uptake was presumed to be catalyzed by the respiration-coupled system. Wild type cells grown on fructose in the absence of malate or in the presence of 0.005 M malate showed similar patterns of fructose uptake over a relatively wide range of fructose concentrations

TABLE 14

EFFECT OF GROWTH ON TRICARBOXYLIC ACID CYCLE INTERMEDIATES ON THE LEVEL OF PEP: FRUCTOSE PHOSPHOTRANSFERASE ACTIVITY IN FRACTIONATED EXTRACTS OF *A. PYRIDINOLIS*[1]

Fractions assayed[2]	Fructose 1-phosphate formed[3]
F supernatant	33.3
F pellet	4.0
F supernatant + F pellet	157.0
MF supernatant	11.4
MF pellet	1.3
MF supernatant + MF pellet	38.5
F supernatant + MF pellet	94.0
MF supernatant + F pellet	62.0

[1]PEP: fructose phosphotransferase activity was assayed as described in Table 12.

[2]Fractionated extracts were prepared from washed fructose (0.05 M)-grown cells (F) and malate (0.05 M) plus fructose (0.05 M)-grown cells (MF) as described in Materials and Methods. The protein concentrations of the fractions were: F supernatant, 9.2 mg/ml; F pellet, 4.2 mg/ml; MF supernatant, 7.9 mg/ml; MF pellet, 5.0 mg/ml. Fifty µl of each fraction was employed per assay.

[3]Nanomoles per min per reaction mixture.

TABLE 15

COMPLEMENTATION OF PEP: FRUCTOSE PHOSPHOTRANSFERASE ACTIVITY BETWEEN WILD TYPE CELLS GROWN ON 0.05 M MALATE PLUS FRUCTOSE AND STRAINS DEFICIENT IN CONSTITUTIVE COMPONENTS OF THE PHOSPHOTRANSFERASE[1]

Source of soluble fractions[2]	Fructose 1-phosphate formed[3]
F	54.1
MF	19.1
AP243	35.5
AP253	31.4
MF + AP243	63.6
MF + AP253	63.0
AP243 + AP253	67.4

[1]PEP: fructose phosphotransferase was assayed as described in Table 12.

[2]Extracts were prepared from washed fructose-grown (F) and malate-fructose-grown (MF) cells of wild type *A. pyridinolis* as described in Table 14, or from glutamate (0.05 M)-plus fructose (0.05 M)-grown cells of phosphotransferase-negative strains AP243 and AP253. The protein concentrations of the fractions were: F pellet, 4.2 mg/ml; F supernatant, 9.2 mg/ml; MF supernatant, 7.9 mg/ml; AP243 supernatant, 8.9 mg/ml; AP253 supernatant, 6.0 mg/ml. Each reaction mixture contained 50 µl F pellet, plus 25 µl of each supernatant fraction noted.

[3]Nanomoles per min per reaction mixture. The data are corrected for the activity of each of the fractions when assayed alone.

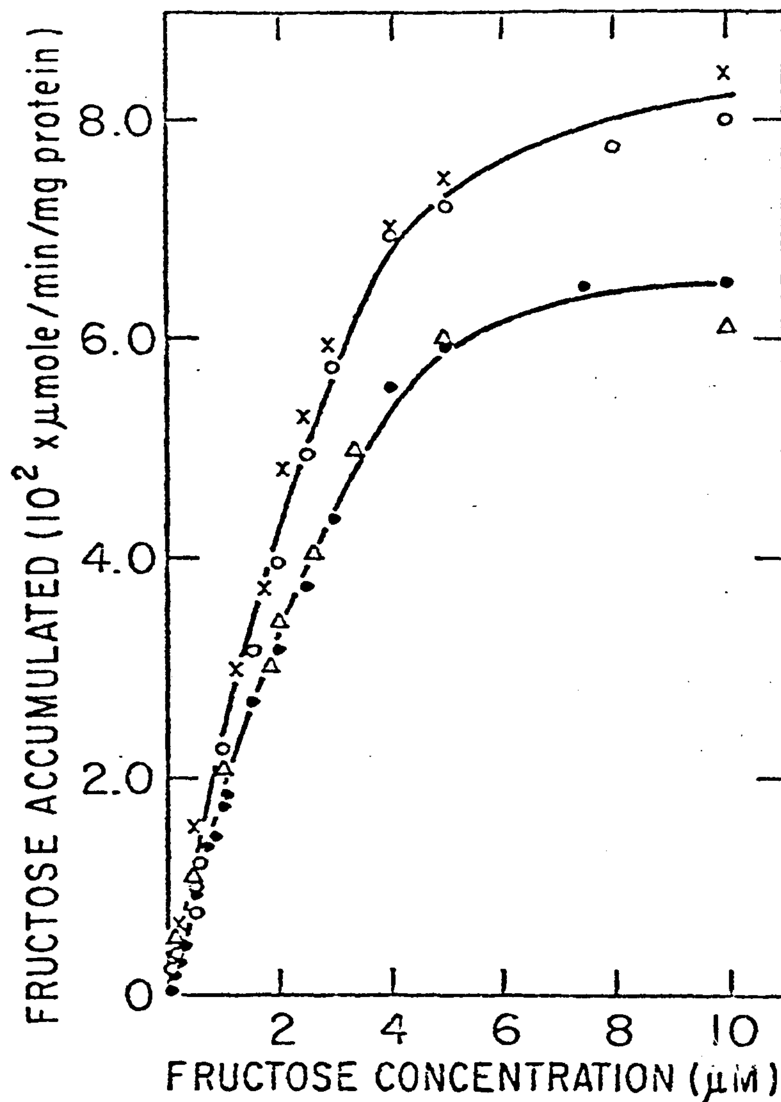


FIG. 15. Kinetics of fructose uptake by whole cells of wild type *A. pyridinolis* and mutant strain AP243 grown under varying conditions. Wild type cells were grown in MS containing 0.05 M fructose plus: no add (O), 0.005 M malate (X), or 0.05 M malate (●). Cells of strain AP243 were grown in MS containing 0.05 M fructose plus 0.05 M glutamate (Δ). Uptake was determined as described in Materials and Methods by using varying concentrations of (U-^{14}C)-fructose. Reactions were terminated after 30 sec.

(Figure 15). On the other hand, wild type cells grown on fructose in the presence of 0.05 M malate showed a pattern of uptake identical to that shown by cells of strain AP243, reaching a lower level of accumulation of fructose than did wild type cells grown on fructose alone. These results indicated that uptake of fructose in whole cells via the phosphotransferase system could be distinguished from fructose uptake via the respiration-coupled system and suggested that the effect of malate on formation of PEP: fructose phosphotransferase was indeed physiologically important.

Since a functional respiration-coupled transport system for fructose is required for induction of factor III and enzyme II of the PEP: fructose phosphotransferase, attempts were made to determine whether the effect of growth on malate on phosphotransferase activity was a primary effect, or was secondary to an effect of high concentrations of malate on the respiration-coupled system. This was first investigated by studying the patterns of incorporation of label from radioactive growth substrates into cold trichloroacetic acid-insoluble material, as described in Materials and Methods, by the wild type strain and by phosphotransferase-negative strain AP243 (Figure 16). When grown on medium containing fructose plus (4-^{14}C)-malate, cells of both strains incorporated malate to the same extent. When the concentration of malate was increased from 0.005 M to 0.05 M, five times as much malate was incorporated by each

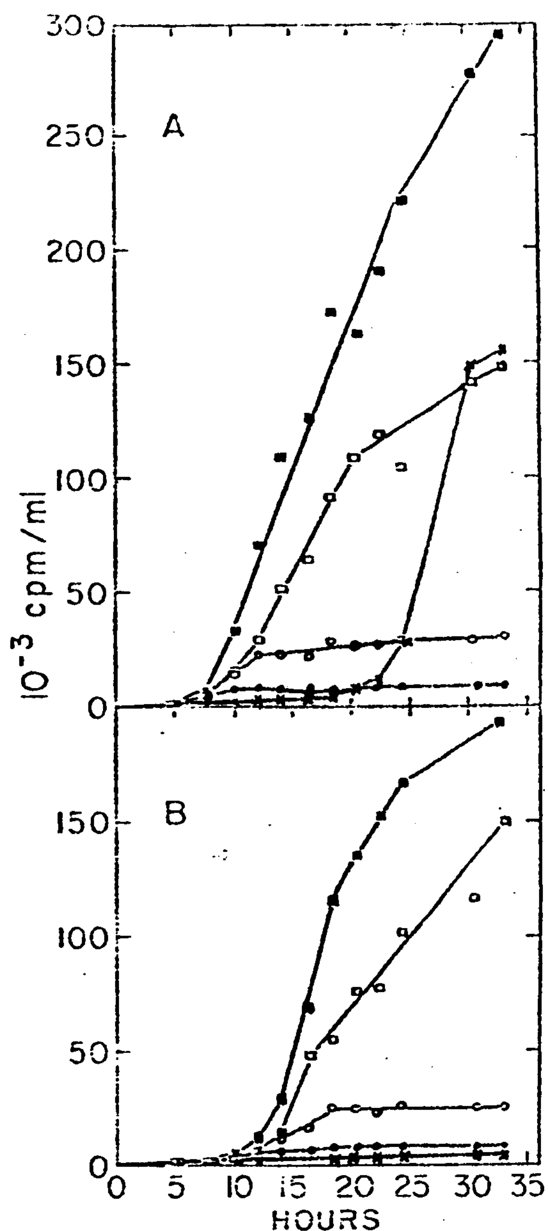


FIG. 16. Incorporation of label from radioactive fructose and malate into trichloroacetic acid-insoluble cell material. Cells of either wild type (A) or AP243 (B) strains were grown in MS plus 0.05 M (U-^{14}C)-fructose (X), 0.005 M (4-^{14}C)-malate plus 0.05 M fructose (●), 0.05 M (4-^{14}C)-malate plus 0.05 M fructose (O); 0.005 M malate plus 0.05 M (U-^{14}C)-fructose (■); and 0.05 M malate plus 0.05 M (U-^{14}C)-fructose (□). The specific radioactivity of all labeled substrates was 0.01 Ci/mol. 0.1 ml samples were taken at intervals, diluted to 1.0 ml with water and filtered. Filters were then treated with cold trichloroacetic acid and counted as described in Materials and Methods.

strain. Incorporation of malate was completed well before fructose incorporation ceased. The lower levels of (4-^{14}C)-malate incorporation, relative to the incorporation of label from (U-^{14}C)-fructose, was most likely due to the loss of label from malate as $^{14}CO_2$. When the incorporation of fructose was followed, it was evident that in both the wild type strain and in AP243, utilization of the hexose commenced during the phase of malate incorporation,whether malate was present at 0.005 M or 0.05 M. Since wild type cells did not contain PEP: fructose phosphotransferase activity during growth on malate (see Table 13), this incorporation of fructose was due to the activity of the respiration-coupled fructose transport system. This distinguished the respiration-coupled transport system for fructose from that for glucose, since the latter sugar was not utilized for several hours after incorporation of the tricarboxylic acid cycle intermediate ceased (see Figure 3). Despite the fact that utilization of fructose began before the malate was depleted, it is evident from Figure 16 that cells grown in the presence of 0.05 M malate incorporated less fructose than did cells grown in the presence of 0.005 M malate. This effect, although somewhat more pronounced in wild type cells, was seen also in cells of strain AP243, and continued even after incorporation of malate ceased. The results of the incorporation experiments suggested that although malate-fructose utilization is not diauxic, utilization of fructose that is transported via the respiration-coupled system is depressed in the presence of high concen-

TABLE 16

EFFECT OF MALATE ON FRUCTOSE UPTAKE
IN STRAIN AP243[1]

Cells grown on 0.05 M fructose plus:	(U-^{14}C)-fructose accumulated[2] in the presence of:	
	No add	0.05 M malate
0.05 M glutamate	0.076	0.095
0.015 M malate	0.074	0.085
0.05 M malate	0.078	0.091

[1]Cells were prepared and uptake assayed as described in Materials and Methods, using 10 μM (U-^{14}C)-fructose. Reactions were terminated after 30 sec.

[2]μmoles/min/mg protein.

trations of malate. It was not clear from these experiments, however, whether the depressed rate of fructose utilization in the presence of 0.05 M malate was due to an effect on transport of the hexose via the respiration-coupled system or due to an effect on the metabolism of fructose once it entered the cell (or both).

To investigate this question, experiments were conducted in which the uptake of fructose by cells of phosphotransferase-negative AP243 was measured. These cells presumably transport fructose solely by the respiration-coupled system. As shown in Table 16, 30 sec uptake of fructose by cells of AP243 grown under varying conditions was not inhibited, but was instead stimulated approximately 20%, when 0.05 M malate was added to the assay mixture 10 min prior to the start of the reaction. This effect was seen in the presence or absence of chloramphenicol. Thus, respiration-coupled transport of fructose, like respiration-coupled transport of glucose, is stimulated by the presence of malate. It thus appeared that the depressed rate of fructose utilization in the presence of 0.05 M malate seen in the incorporation experiments (Figure 16) was due to an effect on the metabolism of fructose once it entered the cell, and not due to an effect on transport via the respiration-coupled system. Therefore, the failure of wild type cells to induce PEP: fructose phosphotransferase when grown on high concentrations of malate was apparently due to a primary effect of the organic acid rather than a secondary

effect due to a depression of inducer transport via the respiration-coupled system.

DISCUSSION

A schematic representation of the pathways for fructose transport and metabolism in *A. pyridinolis* is presented in Figure 17. The respiration-coupled system is represented by two proteins proposed to be a constitutive malate dehydrogenase and a sugar-specific component which is also constitutive. The respiration-coupled system appears to be the same type of transport system that is known, through the work of Kaback and his collaborators (7), to be involved in sugar and amino acid transport in a wide variety of bacterial species. In *A. pyridinolis*, as with the transport systems for glucose in *Azotobacter vinelandii* (236-238) and for gluconate in *Pseudomonas aeruginosa* (239), malate is the optimal oxidizable substrate for support of fructose, glucose, rhamnose (100), and arginine (240) transport. Malate must be supplied exogenously for function of the respiration-coupled system, as evidenced by the growth characteristics of phosphotransferase-negative mutants and other strains deficient in fructose 1-phosphate kinase and FDPase, and by the abolition of crypticity toward glucose, maltose, and sucrose by the addition of malate to the growth medium. This requirement for exogenous oxidizable substrate for function of the respiration-coupled system is apparently due to failure of *A. pyridinolis* to accumulate intracellular dicarboxylic acids during metabolism (218). The finding of Wolfson and Krulwich (98) that function of the respiration-coupled

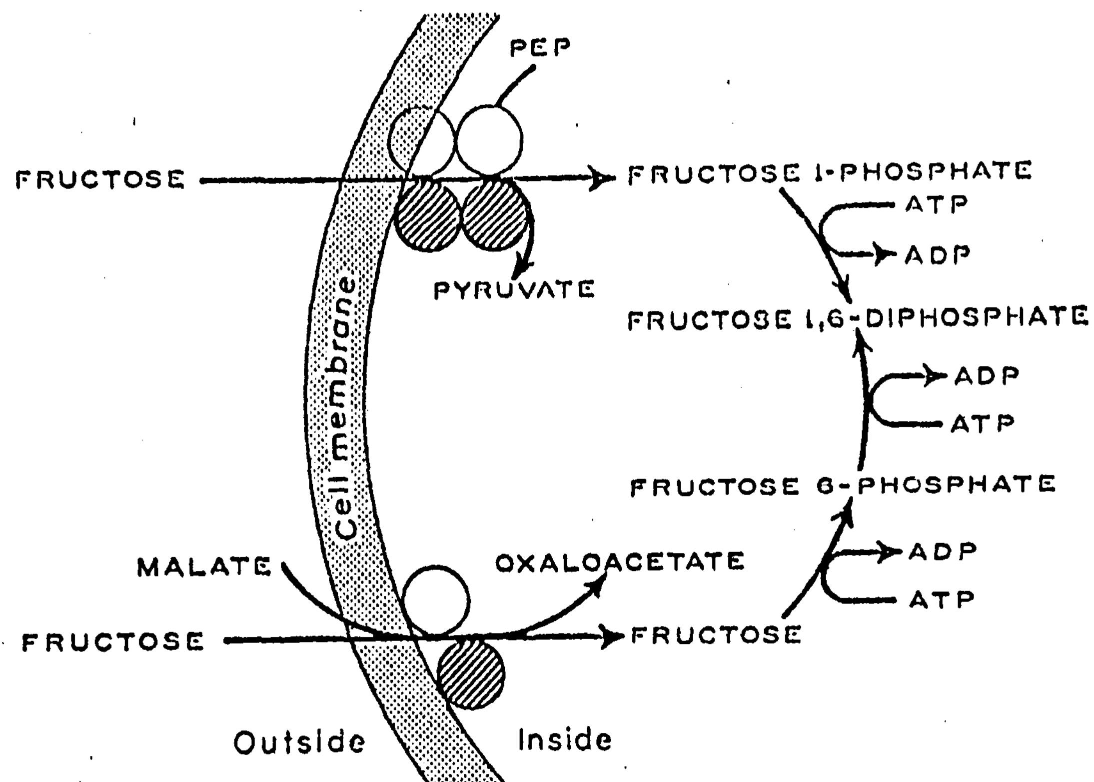


FIG. 17. Schematic representation of the routes of fructose uptake and metabolism in *A. pyridinolis*. The open and hatched circles represent proteins involved in transport.

system is required for induction of the fructose-specific components of the phosphotransferase is interesting in this regard. It implies that when cells of *A. pyridinolis* are inoculated into medium containing fructose as sole carbon source, there is sufficient intracellular malate to enable the requisite amount of fructose transport via the respiration-coupled system to allow induction of the phosphotransferase system. The requirement for malate may account for the long lag period exhibited by *A. pyridinolis* when it is inoculated into medium containing fructose as sole carbon source.

The PEP: fructose phosphotransferase in *A. pyridinolis* is a four component system, resembling other phosphotransferases, e.g., those of *Aerobacter aerogenes* (87,88) and *Staphylococcus aureus* (78,84). Using the nomenclature of Roseman (90), these components consist of two soluble constitutive proteins, enzyme I and the phosphocarrier protein HPr, a soluble sugar-specific protein (factor III), and the membrane-bound sugar-specific enzyme II B. The distinction between membrane-bound and soluble components is a relative one in view of the activity of this system in membrane vesicles. The properties of phosphotransferase activity in vesicles of *A. pyridinolis* are similar to those described by Kaback (80) in his studies of the phosphotransferase in *E. coli*. As in *E. coli*, high concentrations of PEP are required, perhaps reflecting a relative impermeability of the membranes to this compound. In *A. pyridinolis*, the concentration of fructose supporting a half-maximal rate of uptake is

approximately four times higher than in *E. coli*, which is consistent with the difference between the growth rates of the two organisms on fructose. The experiments on the effect of temperature suggest that while increasing temperature up to 40°C increases the initial rate of hexose uptake by the vesicles, as the temperature is raised above 30°C, the vesicles become leaky. This finding appears to be a general characteristic of membrane vesicles from other species (80, 166). Levinson and Krulwich (100) have demonstrated PEP: rhamnose phosphotransferase activity in membrane vesicles in *A. pyridinolis*; this activity is also temperature dependent and cannot be demonstrated clearly at temperatures below 30°C.

Whereas fructose transported by the malate-dependent respiration-coupled system is metabolized by conversion to fructose 6-phosphate and subsequent isomerization to glucose 6-phosphate or phosphorylation to form fructose 1,6-diphosphate, metabolism of fructose transported via the phosphotransferase occurs by the same sequence of reactions found in *A. aerogenes* (241), *E. coli* (242), *Clostridium thermocellum* (243) and several other species (244). The sugar is phosphorylated at carbon-1 by the phosphotransferase. The fructose 1-phosphate is then converted to fructose 1,6-diphosphate for entry into the Embden-Meyerhof pathway. The inability of mutants deficient either in PEP: fructose phosphotransferase, fructose 1-phosphate kinase, or FDPase to

grow on fructose as sole carbon source is consistent with this proposed catabolic route.

It is of interest that mutants such as AP9 which form reduced levels of FDPase retain some ability to grow on tricarboxylic acid cycle intermediates while losing all ability to grow on fructose. A requirement for higher levels of FDPase for growth on fructose than for growth on tricarboxylic acid cycle intermediates is also suggested by the presence of higher specific activities of FDPase in fructose-grown cells than in malate- or PYE-grown cells. A possible explanation for the production of high levels of enzyme during growth on fructose is that some early intermediate of fructose catabolism is an inhibitor of the FDPase reaction. Such an intermediate might be present in relatively great amounts during growth on fructose but not during growth on tricarboxylic acid cycle intermediates. High levels of FDPase would then be required to provide cells growing on fructose with sufficient enzyme activity under the inhibitory conditions. Fructose 1-phosphate and fructose 1,6-diphosphate would be reasonable candidates for the inhibitory compound; fructose 1,6-diphosphate at high concentrations is a known inhibitor of many FDPases (245). The fact that FDPase mutants cannot grow on fructose as sole carbon source implies that the organism utilizes the phosphotransferase system and not the malate-dependent respiration-coupled system during growth on fructose in the restricted medium. If the respira-

tion-coupled system and its associated metabolic pathway for fructose catabolism were utilized during growth on fructose as sole carbon source, fructose 6-phosphate would be available without FDPase. Apparently only minimal activity of the respiration-coupled system is required to induce the phosphotransferase, but this level of activity is not sufficient to sustain growth.

Although it is similarly clear that the presence of malate or a precursor therof is required for function of the glucose transport system, formation of this system appears to be repressed in the presence of high concentrations of tricarboxylic acid cycle intermediates. Activity of the glucose transport system when measured in the presence of chloramphenicol, however, is stimulated when malate, even at high concentrations, is added to the reaction mixture. High concentrations of tricarboxylic acid cycle intermediates also appear to repress formation of the inducible components of the PEP: fructose phosphotransferase, but stimulate uptake of fructose via the respiration-coupled system. Wolfson and Krulwich (98) have suggested that production of enzyme II and factor III may be coordinately controlled in *A. pyridinolis*. The observation that both of these proteins are present in significantly lower amounts in cells grown in the presence of high concentrations of malate supports this suggestion. Studies of the phosphotransferase of *S. typhimurium* have shown that

the cistrons for enzyme I and phosphocarrier protein HPr are geneticaly linked (91,92). Enzyme II and factor III may well be part of another unit of control. The mechanism of the malate effect on these components or on the production of the glucose transport system is still not clear.

Substrate incorporation studies in phosphotransferase-negative strain AP243 indicate that high concentrations of malate depress fructose utilization. Since malate stimulates the respiration-coupled fructose transport system, the depression of fructose utilization during growth on malate may well be due to an inhibition of enzymes of the Embden-Meyerhof pathway such as fructose 6-phosphate kinase (246), or due to an inhibition of glucose 6-phosphate dehydrogenase. In organisms utilizing the pentose phosphate or Entner-Doudoroff pathways for metabolism of glucose and fructose, the latter enzyme has been shown to be allosteric (247-250). The inhibition of glucose and fructose metabolism may be mediated directly by the tricarboxylic acid cycle intermediates or by the high levels of ATP, NADH, NADPH, or $FADH_2$ that would be expected during growth on such compounds.

ABBREVIATIONS

AIB	α-aminoisobutyrate
AMP	adenosine 5'-monophosphate (adenylic acid)
ATP	adenosine 5'-triphosphate
CCP	carbonylcyanide *m*-chlorophenylhydrazone
cpm	counts per minute
CTP	cytidine 5'-triphosphate
cyclic AMP	adenosine 3',5'-monophosphate
DEAE	diethylaminoethylcellulose
DNase	deoxyribonuclease
DNP	2,4-dinitrophenol
E	UDP-galactose 4-epimerase
EDTA	ethylenediaminetetraacetic acid
EMS	ethylmethanesulfonate
F	fructose-grown
FAD	flavin adenine dinucleotide (oxidized)
$FADH_2$	flavin adenine dinucleotide (reduced)
FDPase	fructose 1,6-diphosphatase
g	gravity
G	glutamate-grown
gal	galactose
G3P	L-α-glycerophosphate
GTP	guanosine 5'-triphosphate
h	hour(s)
HPr	phosphocarrier protein of PTS
HQNO	2-heptyl-4-hydroxyquinoline-N-oxide

i	regulatory gene of *lac* operon
IPTG	isopropyl-β-D-thiogalactoside
K	galactokinase
lac	lactose
LAO	lysine-arginine-ornithine
MF	malate-fructose-grown
M protein	membrane protein, product of *y* gene of *lac* operon
min	minute(s)
MS	mineral salts medium
NAD	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NEM	N-ethylmaleimide
ONPG	*o*-nitrophenyl-β-D-galactoside
PEP	phosphoenolpyruvate
POPOP	1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene
ppGpp	guanosine tetraphosphate
PP_i	pyrophosphate
PPO	2,5-diphenyloxazole
PRPP	5-phosphoribosyl 1-pyrophosphate
PTS	PEP: hexose phosphotransferase
PYE	peptone-yeast-extract
rel^+	gene for stringent response to amino acid starvation

rev	revolutions
RNase	ribonuclease
sec	second(s)
T	galactose 1-phosphate uridyltransferase
TDG	β-D-galactosyl 1-thio-β-D-galactoside
TMG	methyl-β-D-thiogalactoside
Tris	tris(hydroxymethyl)aminomethane
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate
y	gene for *lac* permease (M protein).

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