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REGULATION OF AEROBIC/ANAEROBIC METABOLISM IN ARTHROBACTER PYRIDINOLIS

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REGULATION OF AEROBIC/ANAEROBIC METABOLISM IN *ARTHROBACTER PYRIDINOLIS*

by

NICHOLAS J. PELLICCIONE

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.

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ABSTRACT

REGULATION OF AEROBIC/ANAEROBIC METABOLISM IN ARTHROBACTER PYRIDINOLIS

by

Nicholas J. Pelliccione

Advisor: Dr. Terry Ann Krulwich

It had been well established that Arthrobacter pyridinolis possessed two transport systems for both D-fructose and L-rhamnose. These were shown to be a respiration-coupled transport system, which is energized by the oxidation of Krebs cycle intermediates, and a phosphoenolpyruvate: hexose phosphotransferase system (PTS). The PTS was an unusual activity for an aerobic bacterium, and was usually associated with fermentative metabolism. The induction of the PTS in A. pyridinolis was shown to be dependent on respiration-coupled transport of free D-fructose.

A study was undertaken to elucidate the roles and the regulatory properties associated with the cell's ability to utilize two different modes of transport for a single substrate. Studies of D-fructose uptake by A. pyridinolis, in the presence and absence of cyanide, demonstrated that respiration-coupled transport represented about 90% of D-fructose transport during the first half of logarithmic growth, and thereafter about 15-20%. Activity of the D-fructose PTS, on the other hand, was low during log phase, peaked in late log and then slowly declined. The relationship between the respiratory activity of the cell and the two transport activities was then investigated. Experiments were conducted with a mutant of A. pyridinolis (BJ200), which required δ-aminolevulinic

acid (ALA) for growth. The growth rate, cell cytochrome content and respiration-coupled D-fructose transport of the mutant increased with increasing ALA up to 50 µg ALA/ml. By contrast, PTS activity peaked in cells grown on D-fructose with a concentration of ALA which was sub-optimal for growth and cytochrome content. L-malate, which stimulates respiration-coupled transport, repressed the PTS.

Several indicators of aerobic metabolism were studied over the course of growth of A. pyridinolis on D-fructose. These parameters were examined to ascertain whether the time of appearance of PTS activity (mid-logarithmic phase) was a time at which metabolism became generally more fermentative. Spectral studies revealed an increase in b-type cytochromes as cultures got older, and the presence of a cyanide-resistant oxidase, cytochrome d, only after cells entered the late-log phase of growth. Measurement of oxygen consumption and CO_2-released from D-fructose grown cells was consistently low throughout the growth cycle, showing no significant decrease in the later stages of growth. Isocitrate lyase activity was then examined as a function of growth on D-fructose. This enzyme is part of the glyoxylate pathway, which is required for aerobic growth on D-fructose by A. pyridinolis. The cells had higher isocitrate lyase activity during the early stages of growth, while activity declined to undetectable levels in cells harvested from the mid-log phase and later. It appears then, that there is no gross physiological change and that D-fructose metabolism might be somewhat fermentative throughout. However, during the middle of log phase a more anaerobic metabolic mode took over.

A metabolic signal which could coordinate the aerobic/anaerobic metabolic changes was sought. Studies utilizing a citrate synthase-deficient strain of A. pyridinolis (JF3) indicated a positive regulatory

role of acetyl CoA in glyoxylate pathway activity. This mutant exhibited the following properties: (1) constitutive levels of glyoxylate pathway enzymes on various substrates, while such levels were found in the wild type only when grown on acetate; and (2) acetyl CoA levels almost 20 times higher than in the wild type on several substrates, whereas other metabolite levels were similar in the two strains. Consistently, a mutant that was deficient in acetyl CoA synthetase showed no induction of the glyoxylate pathway, even in the presence of acetate, although acetate transport was normal. Moreover, further studies with the citrate synthase mutant suggest a possible negative regulatory role for acetyl CoA in PTS activity. The mutant, containing high cellular acetyl CoA levels, under conditions for PTS induction, produced no more than 50% of the wild type level of the PTS.

The results indicate that the PTS functions under conditions of relative anaerobiosis in the obligately aerobic A. pyridinolis. Some of the relevant metabolic activities are effected by acetyl CoA. This metabolite may, in part, coordinately control aerobic/anaerobic metabolic modes.

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To the two people on whom I relied most heavily I give the greatest thanks: Dr. Krulwich and Loretta, my wife. Dr. Krulwich gave me the push and direction I often needed, while immeasurably enriching my graduate education. Loretta graciously endured the weekends in the lab, and provided much needed support in the clutch. I must also thank my parents and friends for their cheerful support throughout my studies.

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I. The Bacterial Phosphoenolpyruvate:Hexose Phosphotransferase System

The phosphoenolpyruvate (PEP):hexose phosphotransferase system (PTS) catalyzes the energy-dependent transport of sugars generally classified as group translocation processes. Group translocation was a term used by Mitchell to describe certain classes of transport mechanisms (1,2). In this early work, Mitchell proposed several generalized transport mechanisms. Group translocation specifically involved the transfer of a chemical group from donor to acceptor, concomitant with the vectorial translocation of acceptor across a plasma membrane. In 1964 Kundig, Ghosh and Roseman (3) first described such a series of reactions in Escherichia coli. The PTS required a series of phosphoryl transfers from PEP to a hexose substrate, forming the phosphate ester of the hexose. This reaction sequence involves several proteins acting together as a multi-enzyme complex to effect the translocation of substrate across the bacterial cell membrane. Since the first description of the PTS by Roseman's group, a tremendous body of work has been published. The major findings have come from studies using E. coli, Salmonella typhimurium and Staphylococcus aureus, with much of the important work coming from the laboratory of Roseman and his early coworkers, e.g., Saier, Kundig, Hays and others (4-9). In this section I will discuss some of the general features of the PTS, citing some of the important specific references. Many excellent reviews of the PTS also exist (10-14).

In Figure 1, a scheme for PTS activity is presented, showing the involvement of both soluble and membrane bound proteins.

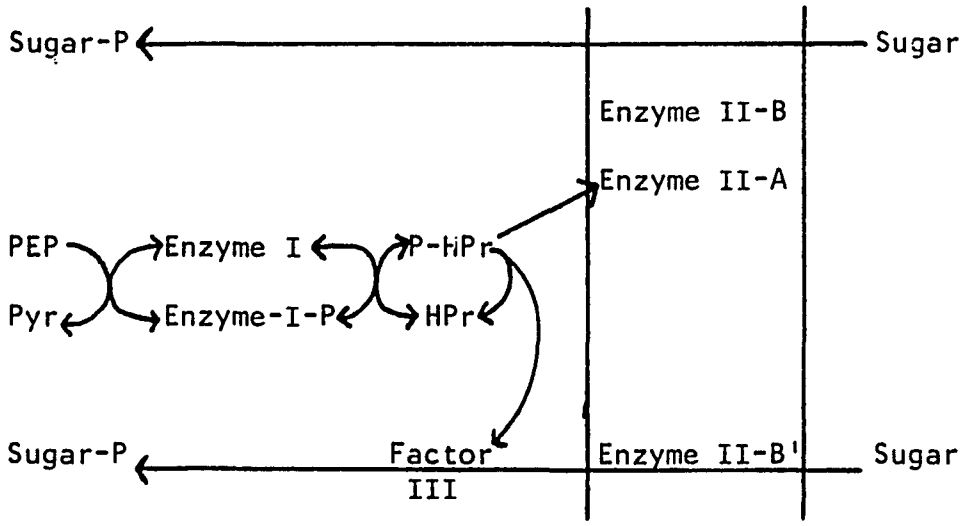

FIG. 1 Scheme for the phosphotransferase system.

The sum of all the reactions is:

$$\text{PEP} + \text{Sugar} \xrightarrow[Mg^{++}]{\text{PTS}} \text{Sugar-P} + \text{Pyruvate}.$$

The PTS contains two proteins that function in the phosphorylation of all the sugar substrates, Enzyme I and HPr (histidine-containing protein), and two sugar-specific proteins, Factor III (or Enzyme IIa) and Enzyme II (or IIb). HPr and Enzyme I are relatively soluble, while Enzyme II is an integral membrane protein.

The first step in the PTS is the catalytic conversion of HPr to P-HPr (phospho-HPr) by Enzyme I, with PEP the phosphate donor. In the process of transferring the phosphate group to HPr, a phosphoryl-Enzyme I is formed, with the phosphate group apparently bound to a histidine residue. This was shown using two highly purified, but not homogenous, preparations of Enzymes I from S. typhimurium (15) and S. aureus (16).

The HPr protein is the second phosphoryl carrier in the PTS sequence of reactions. It has been purified from several sources, as a small, stable single polypeptide chain: the HPr from S. typhimurium and E. coli are apparently indistinguishable (mol. wt. 9500), S. aureus, mol. wt. 8700

and Mycoplasma capricolum, mol. wt. 9500 (17,18,19). All except M. capricolum were dhown to form phosphoprotein linkages at an N-1 of a histidine residue.

In vitro complementation experiments indicate that HPrs from E. coli and S. typhimurium are freely interchangeable; however, they are not functional with S. aureus' Enzymes II and III, and vice versa (10,13). Enzyme I of E. coli will inefficiently phosphorylate S. aureus HPr, and the converse is also true. When P-HPr was generated from M. capricolum E + HPr + PEP, it catalyzed the phosphorylation of α-MG with an E. coli membrane fraction as efficiently as an analogous E. coli system. The use of E. coli P-HPr, however, was only 1/10 as efficient with M. capricolum as was the M. capricolum P-HPr (19).

The transfer of the phosphoryl group from P-HPr to the sugar substrate involves two more protein components. It is with these two componenets, the sugar specific components, that the PTS takes on a diverse and complex nature. These two components are referred to as Enzyme IIa/IIb if they are both integral proteins, or Factor III and Enzyme IIb if only Enzyme IIb is an integral protein. Some examples of the spectrum of PTS activities which exist are as follows. S. aureus contains a lactose PTS which utilizes a soluble Factor III^{lac} and membrane-bound Enzyme II^{lac} (18,20,21) to accumulate lactose phosphate (6-phosphogalactosyl-β,1→4-glucose). E. coli and S. typhimurium both have two PTSs for glucose, one designated $EIII^{glc'}/EII^{glc'}$ and the other EIIa/EIIb (10,13). The former represents a high affinity, glucose specific transport system which requires both a soluble $EIII^{glc'}$ and the membrane bound $EII^{glc'}$ (11), while the latter system has a broader substrate specificity and represents the only documented system with two integral sugar-specific proteins (4,5).

Among the organisms known to require three soluble and one membrane bound components is A. pyridinolis, which has a PTS for D-fructose and L-rhamnose (22,23); the D-fructose PTS of this species is the subject of many of the studies reported here. In a different organism, Aerobacter aerogenes, Anderson et al. (24,25) have described two fructose PTSs, a high-affinity (assayed around 0.1 mM fructose) and a low-affinity (assayed at 90 mM fructose) system. A novel feature associated with the high-affinity PTS is that a soluble, inducible protein designated "K_m factor" replaces the HPr in this organism and requires an induced Enzyme II for D-fructose. HPr was not required for activity with the induble Enzyme II, but was essential for the low affinity system which was constitutive. The photosynthetic bacteria, Rhodospirillum rubrum and Rhodopseudomonas sphaeroides, possess a novel PEP-dependent PTS for the formation of ructose-1-phosphate (26). This system contains only two components: a firmly membrane bound enzyme and a soluble protein, which is loosely associated with the membrane, and found not to correspond to other known EI, HPr or EIII proteins. The inducible hexitol PTS in E. coli, A. aerogenes, S. mutans and S. aureus are also somewhat specialized. They have been studied by Lengeler (27,28), Lin and coworkers (29,30), and others (31,32). It was shown that the hexitol PTS had a somewhat broader substrate specificity than most PTS. Moreover, neither a sugar specific component, i.e., an EIII, nor an equivalent sugar-specific membrane-bound component (IIa) was found. Thus far, of the hexitol PTSs, only the mannitol PTS in S. aureus has been shown to contain both soluble and membrane-bound hexitol-specific components (20).

In general, Enzyme I and HPr are constitutive proteins; however, it has been shown that the level of these proteins can very up to three-fold depending on the carbon source used for growth (6). The inducibility of

the specific Enzyme II complexes is complicated (10). Many of these complexes are clearly inducible, e.g., those for fructose, mannitol and sorbitol. However, the totally membrane-bound IIA/IIB systems for glucose and other PTS substrates are not as clearly understood. This system has been measured in membrane preparations regardless of the carbon source used for growth.

The use of mutants greatly facilitated studies of the role of the PTS in bacterial physiology. Lin and coworkers, using both E. coli (33) and A. aerogenes (30,34) found that mutants which lacked either Enzyme I or HPr showed pleiotropic characteristics, i.e., they wouldn't grow on several PTS substrates. If an HPr or Enzyme I mutant was allowed to revert spontaneously on a single carbon source, all other pleiotropic deficiencies were corrected. A different mutant of A. aerogenes, that was only unable to use mannitol, was shown to be a defect in Enzyme II. A mutant of S. typhimurium isolated by Simoni et al. (35) was particularly important in showing that the PTS was indeed a major system for the transport of sugars in bacteria. The mutant lacked Enzyme I of the PTS, and was unable to utilize nine carbohydrates that were used by the wild type. The inability to utilize the carbohydrates was shown to be due to the mutant's inability to transport the sugars into the cells. Critical evidence that phosphorylation and translocation by the PTS are concomitant came from the work of Kaback (36). Using isolated membrane vesicles, Kaback demonstrated that PEP was required for the accumulation of certain sugars as their phosphate esters; an Enzyme I mutant was unable to take up appreciable quantities of α-methylglucoside (α-MG); and if ^{32}P-labelled PEP was used there was a stoichiometric relation between ^{32}P lost from PEP to its appearance in α-MG-phosphate. A key experiment showed that when ^{3}H-glucose was added to the incubation medium containing ^{14}C-glucose loaded vesicles, the

added ^{3}H-glucose was phosohprylated at a more rapid rate than the intravesicular ^{14}C-glucose. This provided rather convincing evidence that the PTS was indeed involved with the transport of sugars across a membrane.

II. Distribution of the PTS Among Bacteria

Although the PTS exists in many of the bacteria commonly used for study, it is not a universal system. In a recent review by Saier (12), a complete listing of organisms known to utilize the PTS, along with representative organisms known not to use the PTS, was presented. A rather diverse group of bacteria utilize the PTS, ranging from enteric organisms to marine bacteria and several species of *Mycoplasma*. Notably, with the exception of several organisms, PTS-containing bacteria are generally strict or facultative anaerobes. Romano and coworkers (37,38) had originally reported that the PTS was completely absent from obligate aerobes, on the basis of a survey in which the glucose-specific PTS was measured. This generalization is no longer true, however, as noted by Saier (12). A different type of generalization may be made: Those organisms which are capable of metabolizing sugars via anaerobic glycolysis usually have the ability to transport sugars via the PEP-dependent PTS. Organisms which utilize the Entner-Doudoroff pathway to metabolize sugars aerobically usually utilize active transport for sugar accumulation. The evolution of the PTS has not been widely studied, but some consideration of this issue has been presented by Saier (12) and by Andrews and Lin (39).

The work of Krulwich *et al.* (22,23) first demonstrated that the PTS existed in strict aerobes. *Arthrobacter pyridinolis* was shown to have a PTS for D-fructose and L-rhamnose. Since that time several different aerobes have been shown to contain a PTS. A PTS for D-fructose has been characterized in *Bacillus subtilis*. Gay and Delobbe and coworkers

reported, in a series of papers (40,41,42), that sorbitol and D-fructose metabolism were dependent on a PTS. Exogenous D-fructose or D-fructose formed from sorbitol could be phosphorylated by the PTS or a different but unknown mechanism of transport. The induction of subsequent metabolic enzymes depended on the presence of Enzyme I of the PTS. Another group reported the purification of HPr from B. subtilis Marburg 168 and found it to be similar to that of E. coli and S. aureus (43). A survey of several Pseudomonas spp. was undertaken by Sawyer, Baumann and coworkers (44,45). They showed that a PEP-dependent D-fructose PTS was present in these aerobic cells, producing fructose-1-phosphate, which was shown to be metabolized further largely by the Entner-Doudoroff pathway.

III. Hexose Transport in A. pyridinolis

The finding of a PTS in A. pyridinolis, a strict aerobe, was unique at the time, and still represents one of a few such documentations in aerobes. A. pyridinolis grows on Krebs cycle intermediates such as L-malate in preference to hexoses; the only hexoses it has been shown to grow on as sole carbon sources are D-fructose, L-rhamnose, and the hexose derivative D-gluconate.

Sobel, Wolfson and Krulwich showed that D-glucose would not support growth of A. pyridinolis when the substrate was present as sole carbon source (46). However, it was shown that a diauxic growth pattern was observed if L-malate, succinate, citrate or fumarate were included in the growth medium, such that the D-glucose was utilized during the second phase of growth. Activity of the D-glucose transport system, which was repressed by high levels of Krebs cycle intermediates, actually required the presence of some L-malate or L-malate precursor. Transport could be inhibited by the respiratory inhibitors cyanide and 2,4-dinitrophenol

(DNP), and was shown to be active transport, not a PEP-dependent:D-glucose PTS. The L-malate was the energy source, and was oxidized to OAA during D-glucose utilization. These findings suggested that D-fructose and L-rhamnose, which could serve as sole carbon source, might be transported by a different mechanism.

Sobel and Krulwich first reported the existence of a PEP-dependent: D-fructose PTS in A. pyridinolis in 1973, and showed that fructose-1-phosphate was the product of this PTS (47). While studying a PTS-deficient mutant of A. pyridinolis, it was found that both a respiration-coupled (RC) system and PTS existed for D-fructose transport (48). (Figure 2.) The RC system required the addition of exogenous L-malate and had several properties similar to the D-glucose transport system. The need for exogenous L-malate for the RC transport system relates to the relative inability of A. pyridinolis to generate intracellular L-malate, as discussed later. Further studies carried out on the D-fructose transport system in this organism, utilizing whole cell studies, membrane vesicles and various mutants, revealed that: (1) the PTS contained three soluble components, one of which was inducible (Factor III) and an inducible D-fructose-specific membrane bound component (Enzyme II); (2) there was a D-fructose-specific component for the respiration-coupled system which was inducible; (3) inhibitors of electron transport such as DNP markedly inhibited L-malate-dependent D-fructose transport while not affecting PEP-dependent PTS activity; and (4) that a flavin adenine dinucleotide-linked L-malic dehydrogenase was associated with the membrane fraction of these cells and appeared to be a constitutive enzyme that might be involved in the malate oxidation (49,50). Using a series of mutants deficient in the various RC and PTS fructose transport components, Wolfson and Krulwich

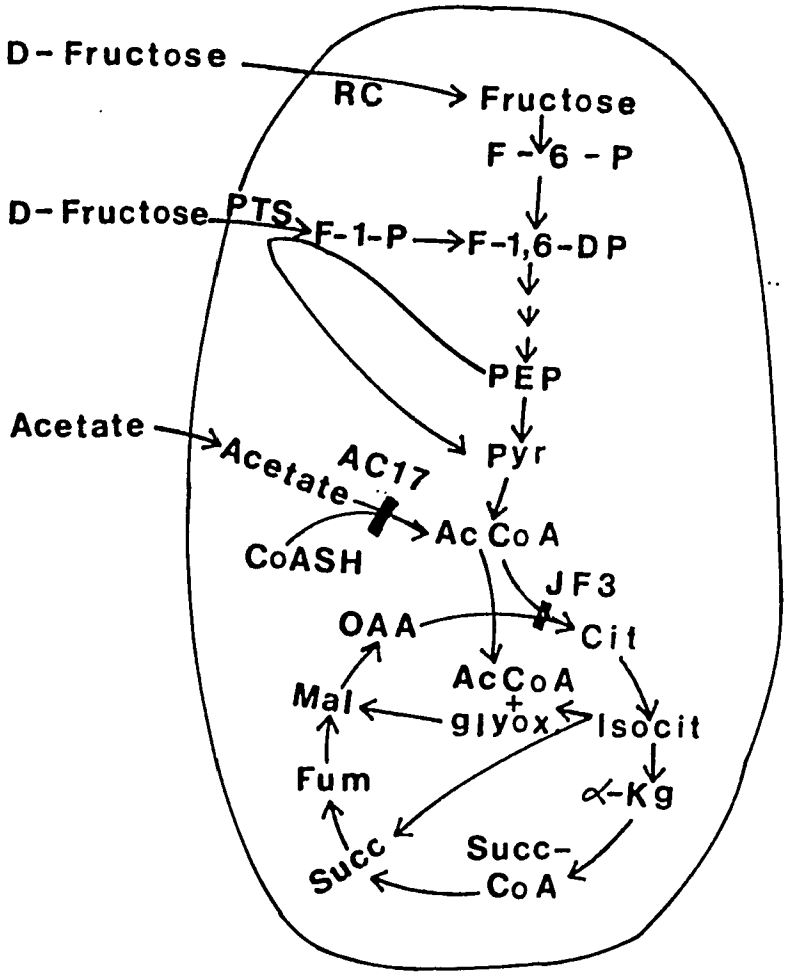

Fig. 2. Metabolic pathways in A. pyridinolis

showed that a functional RC-D-fructose transport system was required for induction of the D-fructose PTS (22). These results suggested that free D-fructose must be transported by the RC system to sufficient intracellular levels in order for induction of the PTS to occur.

Although much of the work conducted on the alternate transport systems in A. pyridinolis centered on the D-fructose transport systems, some work has also been done on L-rhamnose transport and metabolism. The finding that L-rhamnose was the only other hexose which could be used as sole carbon source by A. pyridinolis led to the subsequent finding that L-rhamnose, like D-fructose, could be transported by both a RC and PTS transport system (23). The L-rhamnose transported, unchanged, by the RC transport system is metabolized via the following pathway: L-rhamnose → L-rhamnulose → L-rhamnulose 1-phosphate → dihydroxyacetonephosphate + L-lactaldehyde (51). The L-rhamnose 1-phosphate produced via the PTS was postulated to be dephosphorylated by a specific L-rhamnose 1-phosphate phosphatase. Mutants apparently lacking this activity accumulate a toxic metabolite (probably L-rhamnose 1-phosphate) when grown in the presence of L-rhamnose. Introduction of a mutation in Enzyme I of the PTS alleviates this growth inhibition.

IV. Cellular Regulation Of and By the PTS

Although the primary function of the PTS is probably to provide the bacterial cell with sufficient growth substrate, the PTS also functions in the regulation of various cellular processes. Adler and Epstein (52) have shown that the proteins of the PTS function in chemoreception, allowing the bacterium to swim up a concentration gradient of PTS sugars. Another suggested target of PTS-mediated control is regulation of flagellar protein synthesis and flagellar motion itself (12). However, the regulatory

function which has received more attention is the PTS's effects on several transport systems for non-PTS sugars, and its effects on adenylate cyclase activity. Soon after the discovery of the PTS, it was noted that Enzyme I and HPr mutants of E. coli could not induce β-galactosidase (53). Pastan and Perlman (54) showed that such mutants were hypersensitive to repression of β-galactosidase by glucose, and suggested that they had particularly low cellular levels of cyclic AMP. Since that time much work has been done, with E. coli and S. typhimurium, to develop some cohesive mechanism for explaining these phenomena. Saier, first in collaboration with Roseman and then with others, has studied how the PTS might coordinately regulate permease function (6,8,9,55) and adenylate cyclase activity (56, 57,58). These studies have clearly shown that the presence of a PTS sugar (or non-metabolizable PTS substrate analog) will rapidly inhibit adenylate cyclase activity and thus lower cyclic AMP, while also directly inhibiting a number of permeases. This in turn affects those operons, e.g., the lac operon, which are controlled by inducer exclusion and cyclic AMP (12). Saier (12) has proposed that a central regulatory protein, RPr, provides an allosteric regulatory mechanism over both adenylate cyclase and the various carbohydrate permeases. A series of phosphorylation/dephosphorylation steps is involved in positive and negative control functions, with the phosphorylation of RPr being dependent upon the phospho-HPr of the PTS. Over the past few years, Peterkofsky et al. have also developed a mechanism for the regulation of adenylate cyclase by the PTS (59,60,61, 62,63). Their proposals are similar to those of Saier's group, with a phosphoprotein interaction with adenylate cyclase possibly involving Enzyme I (12). A recent publication by this group (63) also suggests that the protonmotive force regulates the adenylate cyclase complex of

E. coli.

Thus, a large number of studies have been done on the ability of the PTS to regulate other cellular functions. By contrast, relatively little has been done on the regulation of the PTS itself (10,14). However, a growing body of evidence indicates that the protonmotive force inhibits PTS activity.

In the course of studying the glucose permease of E. coli and S. typhimurium, using α-methyl glucoside (α-MG, a non-metabolizable analog of glucose), Hoffee et al. (64,65) and others (66,67,68,69) made these initial observations: (1) addition of fructose or another metabolizable carbon source to cells which have accumulated α-MG (a PTS substrate) to steady state caused an immediate and several-fold decrease in this level; (2) addition of azide or DNP to cells had no effect on α-MG accumulation alone, but increased its steady state level of accumulation several-fold in the presence of exogenous oxidizable substrate; (3) a change to anaerobiosis increases the steady state level of α-MG in the presence of the carbon source on which it was aerobically grown; and (4) showed that azide effected α-MG uptake by decreasing the K_m for uptake three-fold, without affecting its V_{max}.

More recent studies, all conducted with E. coli using α-MG as a substrate, show that the glucose-specific transport protein (Enzyme II^{glc}) of the PTS may be sensitive to the transmembrane electrical potential (14). Hernandez-Asensio et al. (70) first showed that substrates which stimulate respiration inhibited α-MG uptake; the higher the rate of respiration the more severely α-MG uptake was inhibited. They also demonstrated that azide and CCCP, an uncoupler, relieved the inhibition of α-MG transport by the added respiratory substrate. In agreement with Gachelin (68), they showed

that the K_m for transport was affected. A subsequent paper by this group (71) utilized E. coli mutants with a defective Ca^{2+},Mg^{2+}-ATP'ase to rule out any inhibitory effects that ATP per se might have caused. In such mutants, oxidizable substrates produced an even more severe inhibition of α-MG uptake than in the wild-type, and CCCP reversed this inhibition. Direct measurements of cellular ATP levels ruled out any effect of ATP on the rate of α-MG uptake. Additional support for the idea that the energized membrane state inhibits the PTS comes from work with colicins. As early as 1969, Fields and Luria (72) showed that colicins E1 and K, as well as azide, completely inhibited β-galactoside uptake, while α-MG uptake was highly resistant to inhibition under the same conditions. Subsequent work by Luria (73), Jetten (74) and Gilchrist and Konisky (75,76) further showed that various colicins (col E1,K,1a) could be used to inhibit the formation of an energized membrane state and thus inhibit processes requiring the energized membrane state, such as proline and TMG transport. At the same time, it was demonstrated that PTS-mediated α-MG uptake was not effected, or increased under these conditions. The conclusion drawn by all of these groups was that the energized state of the membrane served to inhibit PTS-mediated α-MG transport. The most direct treatment of this topic comes from a recent report by Reider et al. (77). Using both whole cells and membrane vesicles of wild type and ATP'ase deficient strains of E. coli, they showed that energization of the cell membrane inhibits PTS-mediated transport. Only substrates which donated electrons directly to the respiratory chain could inhibit α-MG uptake. In membrane vesicles prepared from a cytochrome-deficient mutant, which could not be energized by D-lactate or ascorbate, α-MG transport was not inhibited by these substrates. The work of Reider et al. (77) strongly supports the theory

that the protonmotive force controls the activity of the PTS. An even more definitive conclusion will be justified when measurements of the protonmotive force and transport are made simultaneously.

V. Aerobic vs. Anaerobic Bacterial Physiology

For fermentative organisms, the PTS makes direct use of PEP derived from the glycolytic catabolism of its sugar substrates. The primary substrate for many of these organisms is D-glucose; some of the regulatory roles of the PTS, noted above, may essentially focus cellular metabolism on D-glucose dissimilation. What about those organisms such as A. pyridinolis and Pseudomonas aeruginosa which are strict aerobes? Interestingly, in many aerobic organisms, hexoses and D-glucose in particular, are not preferentially utilized. Instead, intermediates of the Krebs cycle such as citrate and succinate are found to be the preferred carbon sources. Dawes and coworkers (78,79,80,81) and others (82) studying P. aeruginosa have shown that cells grown on succinate contain low levels of D-glucose catabolizing enzymes and that citrate represses these enzymes. It was later established that this organism contained an inducible active transport system for glucose (83,84,85,86) which was not PTS-mediated and was repressible by Krebs cycle intermediates. Krulwich and Ensign (87) also showed that the glucose transport system of Arthrobacter crystallopoietes, a strict aerobe, was also repressed by succinate, and A. pyridinolis, the bacterium studied in this thesis, will utilize D-glucose only after prior growth on Krebs cycle intermediates (46). As pointed out by Mukkada et al. (84), the regulation and transport of various nutrients by aerobes such as the arthrobacters and pseudomonads suggest a point of evolutionary divergence of these organisms from the fermentative type of metabolism and transport.

However, aerobic organisms do exist which utilize a PTS for sugar transport (i.e., 42,45,47), as indicated in Section II. Could there be some conditions under which these aerobic organisms utilize an essentially anaerobic mode of metabolism? It is possible that these bacteria, mostly soil organisms, are subjected to periods of low oxygen availability. Perhaps the PTS activity in such organisms is part of a survival mechanism to adapt to such environmental conditions. Certainly, arthrobacter and coryneform bacteria are able to survive long periods of starvation and can utilize many unusual compounds as nutrients (88).

Although much has been written on what defines an anaerobe (e.g., reviewed in 89), little is known about the transition from an aerobic to an anaerobic condition. Most of the relevant information comes from studies of facultative organisms, such as E. coli, or of organisms such as P. aeruginosa, which can grow without oxygen using nitrate as a terminal electron acceptor.

In 1966, Gray et al. (90,91) studied the structural and functional changes in E. coli that occurred as a result of shifting from aerobic to anaerobic growth. Aerobic E. coli have high levels of Krebs cycle enzymes and terminal respiratory enzymes. During anaerobic growth of E. coli, some respiratory components remain membrane associated. This may allow rapid changes to take place under conditions of changing oxygen tension (90). Krebs cycle enzymes were shown to be controlled by several separate mechanisms (91). Anaerobiosis resulted in the lowest levels of enzymes, but D-glucose produced significant repressive effects under several nutritional conditions. Thomas et al. (92) documented the changes of several important functions in E. coli during an aerobic to anaerobic shift. Their findings indicated that, as dissolved oxygen tension approached zero, several respiratory enzymes and cytochromes first increased and then decreased

precipitously. Metabolic adjustments to fermentative metabolism were initiated well before the dissolved oxygen tension reached zero. This was shown by the increase of several glycolytic enzymes. Ruch and Lin (93,94) demonstrated that Klebsiella aerogenes can dissimilate glycerol aerobically or anaerobically by two separate pathways. Both pathways are induced by growth on glycerol, but their relative levels depend on the degree of aeration of the culture.

Several investigations using a variety of organisms characterized the changes in the cytochrome content of bacterial cells as a function of available oxygen. Sinclair and White (95) showed that in Haemophilus parainfluenzae the concentration of cytochromes was higher in O_2-limited cultures than in freely aerobic cultures. The type of electron acceptor used in anaerobic growth, i.e., nitrate or fumarate, affected the amount of cytochromes formed under these conditions, e.g., the presence of nitrate repressed the formation of cytochrome d. In a study of propionic acid bacteria, de Vries et al. (96) showed that these aerotolerant anaerobes exhibited several cytochromes, including cytochromes o, a or a_1, and d under anaerobic conditions; synthesis of these particular cytochromes was severely repressed by the presence of oxygen. As reviewed recently (97), E. coli has a branched respiratory chain at the level of terminal respiratory components; this is a common finding in bacteria. What appears to occur in E. coli is that cytochrome o is used exclusively during log phase of growth. Under conditions of low oxygen tension, as in stationary phase, cytochrome d is induced. This effect has been documented by Doelle and Hollywood (98) who also correlated their findings with respect to biomass and the utilization of aerobic fermentation of glucose. Sweet and Peterson (99) found that P. putida, when grown aerobically, demonstrated

fluctuations in its cytochrome content over the course of growth. When grown through log phase, c, b and o-type cytochromes were observed. However, in late-log to stationary phase, as the O_2-tension in the medium dropped, cytochrome d appeared. The latter is a cyanide resistant cytochrome.

VI. The Glyoxylate Pathway and Anaplerotic Reactions

Anaplerotic enzymes are vital to all aerobic organisms which rely on hexoses or glycolytic intermediates for growth (100). Although the Krebs cycle serves as a primary central pathway for energy production in cells, it is also a major source of precursors for the biosynthesis of cellular macromolecules, i.e., proteins and DNA. In order to provide for biosynthesis but also to retain its cyclic nature for energy production, a means of replenishing the OAA and other intermediates which are removed must exist. Such pathways have been named anaplerotic reactions by Kornberg (101). In microorganisms these pathways are represented by the enzymes pyruvate carboxylase and PEP carboxylase, which form OAA from pyruvate and PEP respectively (102,106), and by the glyoxylate pathway. The glyoxylate pathway has been clearly implicated in serving an anaplerotic role for cells growing on acetate (100). This pathway bypasses the two CO_2-liberating reactions of the Krebs cycle so that a portion of the acetate may provide net synthesis of dicarboxylic acids, while the rest of the acetate is dissimilated to CO_2 during energy production. Isocitrate lyase and malate synthase are the two enzymes responsible for this bypass, which proceeds as follows: Isocitrate undergoes an aldol cleavage to yield glyoxylate plus succinate (which can be used directly in the cycle) in the isocitrate lyase reaction. In the second reaction, malate synthase catalyzes the condensation of glyoxylate plus acetyl CoA to produce malate

(which also enters the cycle) and free coenzyme A. The details of this pathway have been elucidated by Kornberg (100,103) and McFadden (104, 105) working with E. coli and various Pseudomonas spp. It was recognized early in this work that the glyoxylate cycle was fully induced only when cells were grown on acetate (104,107). Kornberg demonstrated that even small amounts of C-3 or C-4 compounds in the growth media could repress the pathway (107). Kornberg and coworkers (107,108,109) continued to study the control of the glyoxylate pathway using metabolic mutants. Phosphoenolpyruvate was implicated as both a repressor of isocitrate lyase and as an inhibitor, providing a fine control mechanism. Kornberg (100) notes that such a role for PEP is consonent with its central role in gluconeogenesis, cell wall biosynthesis, amino acid biosynthesis, and several important metabolic pathways in the cell. A study of purified isocitrate lyase from P. indigofera (110) showed that this enzyme was also inhibited by PEP as well as several other metabolites. Isocitrate lyase was again suggested as the primary control point of the glyoxylate pathway.

A more general role of the glyoxylate pathway in the aerobic metabolism of A. pyridinolis underlies its use as an indicator of such metabolism in the studies described here. The glyoxylate cycle has been shown to be the only anaplerotic route available to A. pyridinolis and another species, Arthrobacter atrocyaneous (111,112). It was first demonstrated that the growth of these two strains on certain carbon sources, e.g., acetate or D-glucose, was subject to inhibition by pyruvate or pyruvate precursors (111). Pyruvate was then shown to inhibit isocitrate lyase in cell free extracts; PEP had no effect. A variety of observations suggested that A. pyridinolis and A. atrocyaneous were deficient in the ability to convert pyruvate to C-4 acids. It was postulated that such a

deficiency could underlie the requirement for exogenously supplied L-malate to energize respiration coupled transport systems in A. pyridinolis (47,113). Subsequently, Krulwich et al. (112) demonstrated that, indeed, A pyridinolis lacked pyruvate carboxylase and PEP carboxylase, and contained the glyoxylate cycle as its only anaplerotic route. Nevertheless, the glyoxylate cycle was fully induced only on acetate in A. pyridinolis. A related species, A. crystallopoietes, contained PEP carboxylase as well as a glyoxylate pathway. This explained its insensitivity to pyruvate and its ability to grow on D-glucose without added L-malate. Consistently, a PEP carboxylase-deficient mutant of A. crystallopoietes was found to require L-malate for growth on D-glucose, and was thus effectively mutagenized to an A. pyridinolis-like strain. Interestingly, mutants of A. pyridinolis which lack isocitrate lyase lose the ability to grow on D-fructose and L-rhamnose alone, as well as on acetate. This requirement for the glyoxylate pathway for growth on hexoses is consistent with its unique role in replenishing dicarboxylic acids.

INTRODUCTION

Although bacterial phosphoenolpyruvate:hexose phosphotransferase systems are commonly found in strict or facultative anaerobes (10,14), they are less commonly found in aerobic bacteria (37). These activities translocate sugar substrates concomitantly with their phosphorylation. Krulwich and coworkers (23,47) were among the first to document a D-fructose and L-rhamnose PTS in Arthrobacter pyridinolis, a strictly aerobic bacterium. A respiration-coupled active transport system for these two sugars was also described (48). Moreover, transport of the free hexose by active transport provided necessary intracellular inducer for the PTS (22). Because two mechanistically different transport systems existed for the same substrates in the same cell, it was of interest to learn how the alternate systems were regulated.

As a starting point for studies of the roles and regulation of the two D-fructose transport systems, it was notable that the PTS is generally associated with fermentative metabolism, whereas the active transport system of D-fructose requires respiration in A. pyridinolis. Could the PTS function in sugar transport under conditions of relative anaerobiosis? If so, what regulatory signals might be involved? It was already known that A. pyridinolis was not only unusual in possessing PTS activity, but also has an unusual paucity of anaplerotic enzymes for replenishing the Krebs cycle (112). As a result, the glyoxylate pathway, which facilitates net synthesis of C-4 dicarboxylic acids, is essential for maintenance of Krebs cycle activity during growth on hexoses. Studies of the transport systems, in connection with aerobic and relatively anaerobic metabolic modes, were undertaken with the expectation that glyoxylate pathway activity as well as respiration might be a useful indicator of aerobic

metabolism. The results described in this thesis suggest that the PTS indeed functions under conditions of relative anaerobiosis, that acetyl CoA is the inducer of glyoxylate pathway activity, and that it may also have a repressive effect on the PTS.

MATERIALS AND METHODS

Bacteria and Growth Conditions

Arthrobacter pyridinolis and mutants derived from it were used in all studies. The organism was routinely grown in PYE medium containing (w/v) 0.2% peptone, 0.1% yeast extract and 0.12% $MgSO_4 \cdot 7\ H_2O$. For most experiments, cells were grown in a defined mineral salts medium (MS) containing (w/v) 0.1% $(NH_2)_2SO_4$ and 0.01% $MgSO_4 \cdot 7\ H_2O$ and (v/v) 1.0% trace salts of Hegeman (114) in 0.025 M potassium phosphate buffer pH 7.0. Carbon sources were added to a final concentration of 0.05 M unless otherwise indicated. Cultures were grown at 30°C in a New Brunswick Scientific G25 incubator shaker at 200 rpm. Growth studies were performed in 300 ml sidearm flasks using a total of 25 mls of medium, carbon sources and inoculum, by measuring cell densities using a Klett-Summerson colorimeter with a no. 42 filter.

Isolation of Mutants

Mutant selection was conducted using the general procedure of Wolfson and Krulwich (111). A washed suspension of cells was treated with ethyl methane sulfonate (EMS) for 2 hrs. Cells were then washed thoroughly with MS, inoculated into a favorable growth medium for the mutant being sought and allowed to grow for 1-2 generations. An aliquot of these cells was then washed and incubated overnight in medium containing 100 μg/ml of penicillin G and a carbon source on which the mutant sought would not grow. Mutants utilized in this work were BJ200, which required δ-aminolevulinic acid (ALA) for growth; JF3, which required citrate for growth and was deficient in citrate synthase; and AC17, which does not grow on acetate.

Preparation of Cell Free Extracts

Cells were grown in 1 liter batches, usually to mid- to late-log phase. They were harvested at 10K rpm for 10 min., resuspended in the buffer to be used, at 4°C, and washed 2x by centrifuging at 14K rpm for 10 min. The pellet of washed cells typically contained 2 to 4 gms. wet weight of cells per liter for a culture harvested at 300 Klett units. This pellet was resuspended in a total volume of 8 to 10 ml in buffer. The suspension was then added to a stainless steel sonicating cup, containing roughly 3 ml of glass beads (5μm diam.), and placed in an ethanol-ice bath for sonication. The glass beads were essential for maximum breakage of cells. The cells were then sonicated in a Heat Systems Ultrasonics, Inc. Model W185D Sonifier, at a power setting of 7-8, which provided a power outpout of about 120 watts using 3x1 min bursts, with 15 sec in between. The extract was then centrifuged for 25 min at 14K rpm to remove large debris and the glass beads. The supernatant fluid was then carefully pipetted into dialysis tubing, and dialyzed against 4 liters of a suitable buffer for the assay to be conducted, overnight at 4°C.

Assay of Solute Uptake by Whole Cells

An aliquot of cells was washed twice with and resuspended in MS to a final concentration of about 70 Klett units. Cells were then incubated, for 10 min, in flasks with aeration, in MS containing chloramphenicol at 40 μg/ml of cells. The uptake reaction was started by adding radioactive substrate at the desired concentration; 1 ml aliquots of the cell suspension were filtered through 0.45 μm filters (Millipore) at intervals, and washed with 10 ml of MS. The filters were then dried and radioactivity counted by liquid scintillation counting. When inhibitors were used, they were added 10 min prior to the start of the assay.

PEP:Hexose Phosphotransferase Assay

Assays of phosphoenolpyruvate:hexose phosphotransferase (EC2.7.3.9) activity for D-fructose were conducted according to the radiochemical assay of Tanaka, Lerner and Lin (34). The assay was based on the formation of the sugar phosphate, which would be negatively charged at about neutral pH. The reaction mixture contained 0.25 mM ^{14}C-U-D-fructose (100 µCi/ml), 5.0 mM PEP, 0.05 mM $MgCl_2$ and 100 µl of extract protein (1-2 mg protein) in a total volume of 0.4 ml of 100 mM Tris pH 7.6 Reactions were carried out at 30°C for appropriate times, including a zero point to determine the degree of non-specific binding of radioactive D-fructose to the filter, and a minus-PEP control, to determine endogenous activity. Termination of the reaction was achieved by adding 0.6 ml of 0.2 M D-fructose. Typically, 0.025 ml of the reaction mixture were spotted on DE-81 filters (positively charged), washing under running water for 45 min to 1 hr and dried. The radioactivity retained on the filter was then measured by placing the dried filters in Betaflour (National Diagnostics) and counting in a liquid scintillation counter. The assay was linear with time and enzyme concentration over the range employed.

Spectral Studies

To study the cytochromes of A. pyridinolis, the cells were grown under the specific conditions described in the test. Cell free extracts were made as described above, except that the volumes of the cell suspensions to be sonicated were adjusted to give equal protein concentrations. The extracts used for the spectra were at a concentration of 5 mg protein/ml. Using a Cary Model 15 recording spectrophotometer, reduced vs. oxidized spectra were recorded. First a baseline was established with both reference and sample cuvettes being air oxidized. The sample cuvette was then reduced with excess sodium dithionite while the reference cuvette

was oxidized fully with potassium ferricyanide; spectra were recorded over the indicated ranges. To help clarify the cytochromes' patterns, carbon monoxide (CO):reduced vs. reduced spectra were obtained. This method provides a means of detecting terminal oxidases which bind CO when in the reduced state. To record these spectra a baseline was first established with both samples fully reduced. The fluid in the sample cuvette was then treated with CO by gently bubbling the gas through the sample for 1-2 min before recording the spectra.

Oxygen Consumption

A study of the rate of oxygen uptake by A. pyridinolis was conducted on L-malate and D-fructose grown cells. All measurements were made on cells harvested at various stages of growth, washed once in cold MS medium, and resuspended at cell densities of 50-100 Klett units. Aliquots of these suspensions (3 mls) were assayed in a Yellow Springs Instruments Oxygen Monitor (model J3) attached to a Beckman recorder. Substrates were added to a final concentration of 3 mM, and the suspension was stirred in the measuring chamber open to the air, for 5 min. Oxygen uptake was then monitored at 30°C for 5-10 min. When KCN was added (final concentration 3 mM) the suspension was again allowed to stir, open to the air, for 10 min. Then oxygen uptake was again monitored for 5-10 min. This preincubation period with aeration was found to provide consistent results both for oxygen uptake that was completely inhibited by KCN and for KCN resistant oxygen consumption, i.e., cyanide resistant oxygen uptake was observed consistently at a constant rate for 5-10 min.

Carbon Dioxide Determination

Experiments were conducted in which the release of CO_2 from internalized D-fructose was monitored. A. pyridinolis was grown to various

cell densities on D-fructose. Samples were harvested, washing in MS medium, and resuspended to equal cell densities of about 70 Klett units. Radioactive D-fructose (D- U-^{14}C fructose, 10 μM final concentration, 100 μCi/ml) was added to the suspension for 3 min. After 3 min 1 ml of cells was filtered through a 0.45 μm Millipore filter and washed with 10 ml of MS. The rest of the suspension was immediately centrifuged at 14K rpm for 5 min; the supernatant was discarded and the pellet resuspended to its original volume in fresh MS medium. This suspension was then placed in a 25 ml Erlenmeyer flask, with a magnetic stirring bar, and tightly capped with a rubber septum. The amount of CO_2 evolved was determined by the method of Cederbaum et al. (115). After 20 min of incubation at room temperature, the reactions were terminated by the addition of 15% TCA. CO_2 was trapped with 1 ml of hyamine hydroxide. Results were expressed as percent CO_2 released from internalized fructose.

Perchloric Acid Extraction of Whole Cells

For the purpose of measuring intermediary metabolites within the cell, perchloric acid extraction was employed. Several protocols were examined, and the most consistent results were obtained using the method of Hong et al. (116). Cells from a 1 liter culture were harvested, washed in ice cold MS (Note: When L-malate grown cells were to be used for L-malate measurements, 3 washes were necessary to remove residual L-malate) and resuspended to yield a sufficiently thick suspension to give at least $3x10^{10}$ cells. All suspensions were kept at 0°C, usually with a salt-ice water mixture, throughout the entire procedure. This was especially important for AcCoA assays, since AcCoA was rapidly broken down by a cellular deacylase if the cells were not kept cold. For the actual extraction, 1.0 ml of the thick suspension was added to a tube containing 0.25 ml of 10 M $HClO_4$, mixed

thoroughly and allowed to sit, with occasional mixing, for 30 min at 0°C. The extracts were then neutralized to pH 5.5-6.5 with 5N KOH, with care taken not to allow the extract to get higher than pH 7.0. The precipitate was removed by centrifugation at 2°C. The volume of the supernatant was recorded. Metabolite levels in these extracts were usually measured with 1-2 hrs.

Enzyme Assays

Isocitrate Lyase (EC 4.1.3.1) - Isocitrate lyase was measured by the method of McFadden (117), in which the amount of glyoxylate formed from isocitrate is determined colorimetrically. The reaction was run at 30°C, in 2.0 ml final volume with a reaction mixture containing 75 mM Tris pH 7.7, 2.25 mM $MgCl_2$, 0.0125 mM reduced glutathione, 4.0 mM Na_3-DL-isocitrate and 0.5-1.0 mg of extract protein (prepared as previously described). The reaction was stopped with 1.0 ml 10% TCA. To determine the amount of glyoxylate formed, the precipitated protein was centrifuged. A 1.0 ml aliquot of the clarified reaction mixture was added to 6.0 ml of 8.3 mM oxalic acid plus 0.17% phenylhydrazine-HCl and heated until almost boiling. The reaction mixtures were then cooled. To develop the color 4.0 ml of concentrated (16 M) HCl were added, followed by 1.0 ml of 5% (w/v) potassium ferricyanide. The optical density was read at 520 nm, exactly 7.0 min after addition of the ferricyanide.

Malate Synthase (EC 4.1.3.2) - This enzyme, which carries out the synthesis of malic acid from AcCoA + glyoxylate, was assayed by measuring the appearance of free coenzyme A. The conditions for the assay were essentially those of Dixon and Kornberg (118), except that the cleavage of the thioester bond was not measured at 232 nm. Rather, free coenzyme A was measured using the reagent 5,5'-dithiobis-2-nitrobenzoic acid

(DTNB) to detect free sulfhydryl groups, according to the method of Ellman (119). The reaction was followed at 412 nm, at 30°C and contained (final concentration) 80 mM Tris pH 8.0, 3.2 mM $MgCl_2$, 0.15 mM AcCoA (tri-sodium salt), 0.1 mM DTNB (prepared as a 10 mM solution in 100 mM $KHCO_3$, stored frozen and protected from light), 0.01-0.1 mg of extract protein, and 0.5 mM glyoxylic acid, neutralized. For each assay all the components except the glyoxylate were added to the cuvette and a baseline was obtained for 2-4 min. Glyoxylate was then added to start the reaction, which gave linear rates for at least 10 min.

Citrate Synthase (EC 4.1.3.7) - Citrate synthase was assayed according to the method of Weitzman (120), also utilizing the ability of DTNB to detect free sulfhydryl groups formed by the cleavage of coenzyme A from AcCoA. The reaction mixture contained, in a total volume of 1.0 ml, 100 mM Tris pH 8.0, 0.16 mM AcCoA (tri-sodium salt), 0.1 mM DTNB, 0.01-0.1 mg extract protein and 0.5 mM oxaloacetic acid, neutralized. The assay was conducted at 30°C, and monitored at 412 nm; a baseline was obtained with all the assay components except the oxaloacetate, which was then added to start the reaction.

Acetyl Coenzyme A Synthetase (EC 6.2.1.1) - The method of Webster (121) was used to assay this enzyme. The reaction is first allowed to occur. The reaction mixtures are then deproteinized, and aliquots of the clarified reaction fluid are assayed for disappearance of sulfhydryl groups. Because of the many substrates involved in this reaction, several controls were conducted to test the requirement of each substrate in the reaction for every set of assays done. The reaction mixture, at 30°C, contained in 0.25 ml: 100 mM Tris pH 8.0, 2.5 mM $MgCl_2$, 0.016 mM $NiCl_2$, 2.0 mM di-K-ATP, 1.0 mM potassium acetate, 1.3 mM Li-coenzyme A, 0.2-

0.4 mg extract protein, and the reaction was stopped with 0.06 ml of 15% TCA. The tubes were centrifuged to remove precipitated protein. Then, 0.1 ml of the reaction mixture was added to 4.0 ml of H_2O, 0.9 ml Tris pH 8.0, and 0.02 ml DTNB. After thorough mixing, and 10 min incubation, optical density was read at 412 nm.

Acetate Kinase (Acetokinase) (EC 2.7.2.1) - Acetate kinase was assayed by the procedure of Rose (122), wherein the acyl phosphate formed reacts with hydroxylamine to form hydroxamic acids. These acids are measured colorimetrically as ferric-hydroxamate complexes in acid solution. The reaction mixture consisted of, in 1 ml: 0.77 M K-acetate, 50 mM Tris pH 7.4, 10 mM $MgCl_2$ (added as 0.3 ml of a stock solution made from 3.2 M K-acetate, 1.0 M Tris pH 8.0, 1.0 M $MgCl_2$ [25:5:1 v/v/v]), 0.35 ml of neutralized 14% hydroxylamine hydrochloride, 10 mM Na-ATP pH 7.0 and 0.5-1.0 mg of extract protein diluted as necessary with 0.1 M KPO_4 pH 7.4, 5.0 mM cysteine. Reactions were carried out at 30°C, and were stopped with 1.0 ml 10% TCA. The precipitate was removed by centrifugation. To develop the color, the entire supernatant was added to 4.0 ml of 1.25% $FeCl_3$ in 1.0 N HCl and optical density determined at 520 nm.

Phosphotransacetylase (EC 2.3.1.8) - To measure this enzyme, the coupled assay of Brown et al. (123) was employed. Reactions were carried out at room temperature in 2 ml final volume. The reaction mixture contained (final concentration) 100 mM Tris pH 8.0, 5 mM $MgCl_2$, 0.5 mM β-NAD, 0.5 mM CoA (Li-salt), 5.0 mM L-malate, 10.0 mM Li-K-acetyl phosphate, 12.5 µg malate dehydrogenase, 25 µg citrate synthase and about 0.3 mg of extract protein. The optical density was monitored at 340 nm.

Metabolite Determinations

Acetyl Coenzyme A - Using perchloric acid extracts of cells grown under the appropriate conditions, AcCoA was measured by the method of

Tubbs and Garland (124). The assay, in a final volume in 2.0 ml at room temperature, contained 100 mM Tris pH 8.0, about 1 mg of solid oxaloacetic acid, 0.1 mM DTNB and 0.5 ml neutralized $HClO_4$ extract. A baseline was recorded at 412 nm, and then 0.005 ml of citrate synthase was added. The further increase in optical density was recorded.

L-Malate Determination - L-Malate was determined, using the method of Williamson and Corkey (125), by measuring the formation of NADH upon the addition of malate dehydrogenase to an appropriate mixture. The reaction mixture contained, in 2 ml, 1.5 ml of 0.4 M hydrazine hydrate, plus 0.5 M glycine pH 9.5 (adjusted with 5 N KOH and prepared fresh daily), 0.5 ml $HClO_4$ extract and 0.01 ml β-NAD (80 mg/ml). These components were mixed and a baseline recorded at 340 nm. Then 0.01 ml of L-malate dehydrogenase (10 mg/ml, 720 U/mg) was added and the further increase in optical density recorded. The assay was linear with malate concentration from 2.0 to 75.0 μM.

Adenosine Triphosphate (ATP) Assay - Cells grown to the indicated stage were washed in MS and suspended in distilled water, and ATP was extracted with perchloric acid according to the method of Cole et al. (126). ATP was measured by the firefly assay in a Beckman LS-230 spectrometer, with the coincidence off, as described by Stanley and Williams (127). On each day, a new ATP standard was determined with the sodium salt of ATP and a fresh firefly lantern extract was prepared. The assay was linear over the range of 10^{-9} to 10^{-6} M ATP.

Protein Determination - Protein was determined by the method of Lowry et al. (128) using lysozyme as the standard.

Lactic Acid Separation and Determination - To separate lactic acid from other metabolites in perchloric acid extracts of A. pyridinolis,

the method of Von Korff (129) was used. Dowex-1X8 (Cl^-) resin, 200-400 mesh, was washed and adjusted to pH 6.8. A column packed with 1 cm x 12-13 cm of resin was used. Samples to be added to the column were adjusted to pH 7.0. Elution of the samples was carried out with an HCl gradient, using HCl reservoirs of 0.05 N, 0.1 N and 0.5 N HCl. A flow rate of 0.5 ml/min was used, and approximately 3 ml fractions were collected.

The position at which authentic lactic acid was eluted from the column was determined by the method of Dawes et al. (130). In a 3.0 ml reaction mixture, 2.4 ml of 1.5% glycine plus 2.2% semicarbizide-HCl, pH 10.0, 0.1 ml β-NAD (20 mg/ml) and 0.5 ml of sample were mixed, and the initial optical density determined at 340 nm. Lactic dehydrogenase, 10 µl, was added to each sample at 30°C, for 1 hr and the final optical density then determined.

RESULTS

Patterns of Activity of the Two D-Fructose Transport Systems

If the two transport systems for D-fructose were serving markedly different physiological roles, they might exhibit different patterns of activity *vis a vis* the growth cycle. Therefore, the patterns of activity of the phosphotransferase system and D-fructose uptake were first examined in the wild type strain during growth on D-fructose as sole carbon source. D-Fructose uptake by whole cells sensitive to 10 mM cyanide was used to assay respiration-coupled transport. That such cyanide-sensitive uptake reflects the activity of the respiration-coupled transport system was verified in a control experiment using strain AP243. This enzyme I-deficient strain only shows respiration-dependent uptake of D-fructose (50). In the absence of cyanide, cells of AP243 took up 80 nmoles of D-fructose x min^{-1} x mg $protein^{-1}$; in the presence of 10 mM cyanide, only 2 nmoles of D-fructose x min^{-1} x mg $protein^{-1}$ were taken up. As shown in Fig. 3, cells of the wild type strain growing on 50 mM D-fructose use respiration-coupled transport, almost exclusively, during the first half of logarithmic growth (doubling time, 2 hr). Approximately midway through the log phase, respiration-coupled transport declines dramatically, and PTS activity appears, as shown both by cyanide-insensitive uptake by whole cells and by assays of cell extracts. There is little change in the growth rate during the transition from one type of transport to another. It should be noted that the specific activities measured by the enzymatic assay of the PTS in extracts are much lower than those for whole cell uptake ascribed to the PTS; this is not unreasonable in view of the multicomponent, membrane-involved nature of the PTS.

The finding that PTS activity appears later in the course of growth

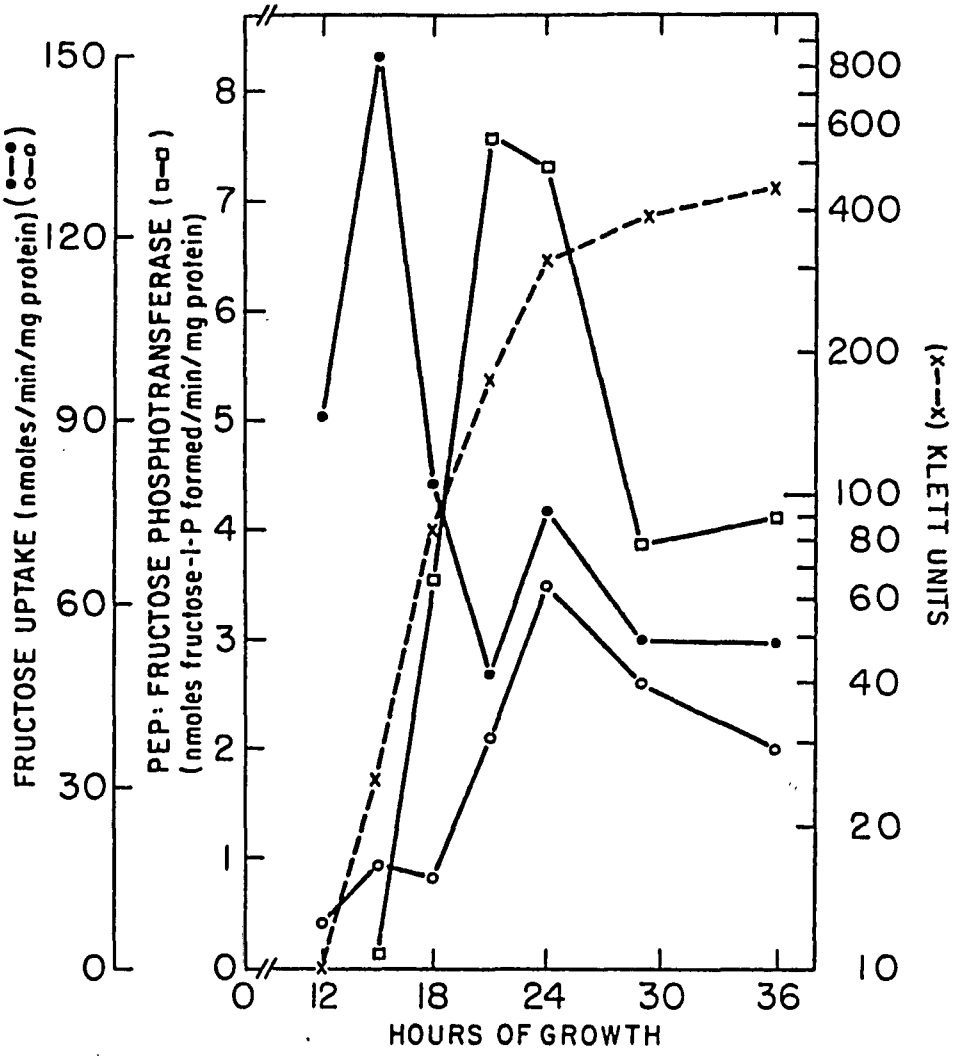

Fig. 3. Patterns of respiration-coupled D-fructose transport and phosphotransferase activity during growth of wild type A.pyridinolis on D-fructose. A.pyridinolis was grown on 50 mM D-fructose; growth was followed turbidometrically. At intervals, samples of the culture were assayed for D-fructose uptake in the presence (0) and absence (●) of 10 mM KCN, and extracts were prepared and assayed for phosphotransferase activity (□).

than respiration-coupled D-fructose transport might relate to a need for sufficient PTS inducer to enter via the respiration-coupled system before induction of the PTS can occur. However, the presence of L-glutamate and especially of L-malate in the medium, in addition to D-fructose, causes a repression of the PTS (Table 1). The level of respiration-coupled transport activity in washed whole cells of AP243 was not similarly affected by L-malate; moreover, addition of 50 mM L-malate to the assay medium increased D-fructose uptake (Table 2). Thus, additions to the growth medium which should increase transport of the PTS inducer, by stimulating respiration-coupled transport, caused repression of the PTS. It therefore seemed likely that the pattern of transport activities observed during growth of the wild type strain on D-fructose was related to controls other than inducer levels and that there controls might correlate with respiratory capacity. To examine this possibility, studies of BJ200, the ALA auxotroph, were undertaken.

Characterization of BJ200, an ALA Auxotroph

Growth experiments with BJ200 showed that it would not grow on various carbon sources with less than 2 μg ALA/ml added; that it grew at an equal rate with the wild type in the presence of 50 μg ALA/ml; and that it showed a growth rate that varied linearly with ALA concentration between 2 and 20 μg ALA/ml, and then increased more slowly and leveled off at 50 μg ALA/ml. As shown in Fig. 4, the cytochrome content of BJ200 increased with increasing concentrations of ALA in the medium up to about 50 μg ALA/ml. At this concentration of ALA, the cytochrome spectrum as well as the growth rate of the ALA auxotroph was similar to that of the wild type strain. By contrast, as shown in Fig. 5 for cells grown for 18 hrs, PTS activity was highest in cells grown on 20-40 μg ALA/ml, concentrations of ALA that were sub-optimal for both growth and total D-fructose uptake. PTS levels were

Table 1. Effect of Krebs cycle intermediates on the level of phosphoenolpyruvate:D-fructose phosphotransferase activity.

Cells of wild type A.pyridinolis were grown for 24 hours on media containing the indicated carbon sources. Extracts were then prepared, dialyzed, and assayed for PTS activity.

Cells grown on:	nmoles D-fructose 1-phosphate formed/min per mg protein
50 mM D-fructose	8.44
50 mM L-glutamate	0.19
50 mM L-glutamate + 50 mM D-fructose	3.81
5 mM L-malate + 50 mM D-fructose	3.02
15 mM L-malate + 50 mM D-fructose	1.88
50 mM L-malate + 50 mM D-fructose	1.51

Table 2. Effect of L-malate on D-fructose uptake in strain AP243.

Cells of AP243 were grown for 24 hours on media containing the indicated carbon sources. The cells were then washed with and resuspended in mineral salts, and were assayed for D-fructose uptake in the presence and absence of 50 mM L-malate.

Cells grown on 50 mM D-fructose plus:	nmoles D-fructose accumulated per min per mg protein in the presence of:	
	No add	50 mM L-malate
50 mM L-glutamate	76.0	95.0
15 mM L-malate	74.0	85.0
50 mM L-malate	78.0	91.0

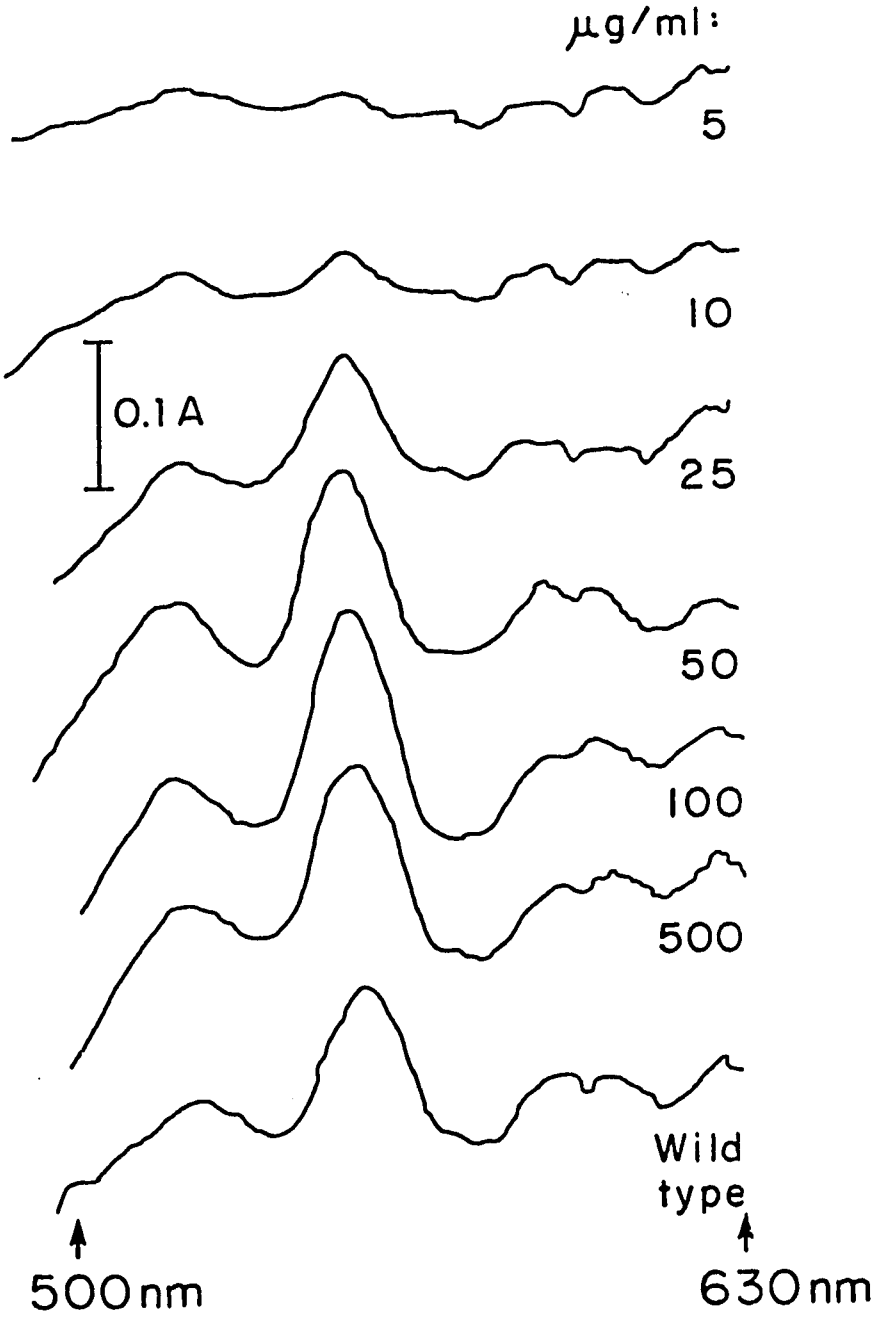

Fig. 4. Spectra of sonic extracts from wild type A. pyridinolis and strain BJ200 grown on various concentrations of δ-aminolevulinic acid for 24 hours. Cells were grown on 50 mM L-malate. ALA was present, at the concentrations indicated, in cultures of BJ200. Cultures were at the same stage of growth when harvested.

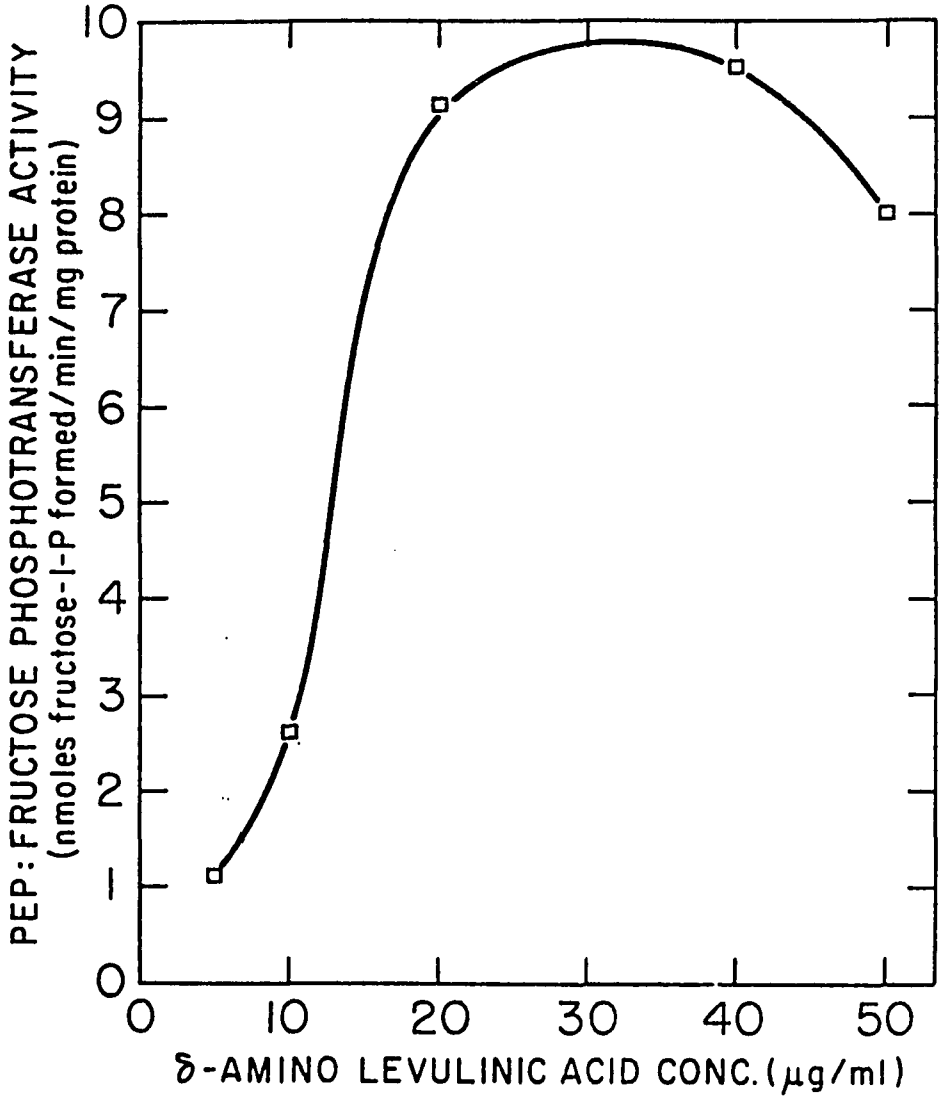

Fig. 5. PEP: D-fructose phosphotransferase activity in BJ200 grown at various concentrations of δ-aminolevulinic acid. Cell of BJ200 were grown for 18 hours on 50 mM D-fructose plus the indicated concentration of ALA.

consistently, but only slightly lower in cells grown on 50 μg ALA/ml. However, although the PTS in BJ200 on 50 μg ALA/ml was not as high as observed at lower ALA concentrations, it was, in fact, much higher than the level of PTS observed in the wild type at 18 hrs (Fig. 3). BJ200 grown on D-fructose plus 50 μg ALA/ml was examined over the course of a growth curve, as had been done with the wild type. As shown in Fig. 6, BJ200 utilizes much less RC-fructose transport than the wild type (note the change in magnitude of fructose uptake axis compared to Fig. 3), as shown by the degree of cyanide-insensitive uptake. This is especially noteworthy in the early stages of growth. Complementing this, and in contrast to the wild type, PTS activity is highest during the early to mid-log phase of growth. The inability of BJ200 to effectively transport ALA, and thus synthesize sufficient levels of cytochromes, during the early stages of growth could possibly explain the data presented in Fig. 6. However, due to high levels of non-specific binding of ALA to whole cells, transport studies provided no useful information. Measurement of cytochrome levels at the early stages of growth was also attempted. The very low density of the cultures during early log phase, and the technical limitations of the spectrophotometer used, however, provided no meaningful spectral data.

Respiration, Cytochromes and Fermentation in A. pyridinolis

The experiments with BJ200 suggested that induction of the PTS was maximal under conditions in which respiration was limited. This in turn raised the possibility that the switch exhibited by the wild type organism, during mid-exponential growth on D-fructose, from utilization of respiration-coupled transport to the PTS represented a switch to a more fermentative mode of metabolism. As a first approach to this possibility, the

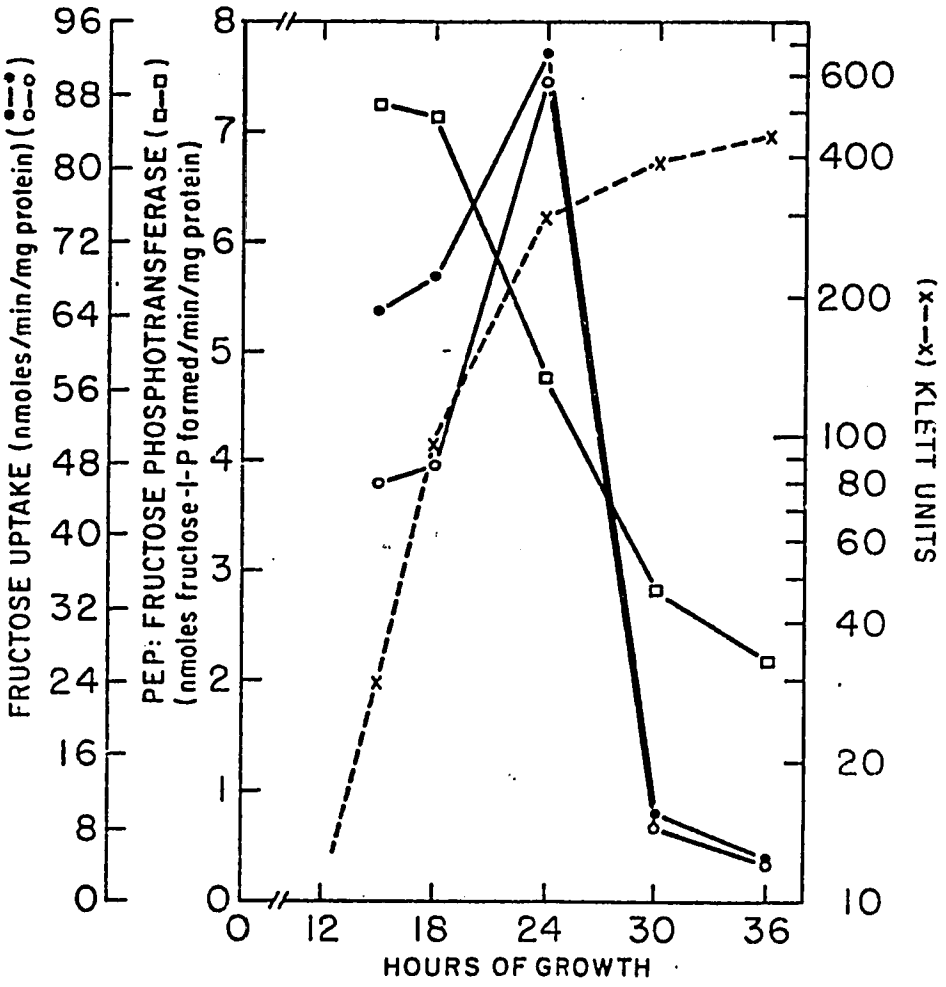

Fig. 6. Patterns of respiration-coupled D-fructose transport and phosphotransferase activity during growth of strain BJ200 on D-fructose with 50 μg δ-aminolevulinic acid/ml. Strain BJ200 was grown on 50 mM D-fructose plus 50 μg ALA/ml; growth was followed turbidometrically. At intervals, samples of the culture were assayed for D-fructose uptake in the presence (0) and absence (●) of 10 mM KCN, and extracts were prepared and assayed for phosphotransferase activity (□).

respiratory activity of cells growing on L-malate or D-fructose was examined during the course of growth. In these experiments, no electron donors were added to the cell suspensions that were removed from the cultures for assay. As shown in Fig. 7, cells growing on L-malate exhibited increasing rates of oxygen uptake until the end of exponential growth. The rates of oxygen uptake by D-fructose-grown cells were much lower, and declined slightly after the mid-exponential phase. That some respiration occurred throughout growth on D-fructose was consistent with our inability to demonstrate strictly anaerobic growth of A. pyridinolis even when late exponential phase cells and various nutritional supplements were used. In both L-malate and D-fructose grown cells there was an appearance, towards the end of the exponential growth phase, of oxygen uptake activity that was resistant to 3 mM (but not 10 mM) cyanide (Fig. 7). Spectral studies were conducted on cells grown for various times on D-fructose and L-malate in order to determine what changes in cytochrome content were responsible for the appearance of the cyanide-resistant respiration. Reduced vs. oxidized spectra of D-fructose-grown cells are shown in Fig. 8. The most evident cytochromes are b and c type cytochromes with α bands in the peak around 550-565 nm and β bands at about 525 nm (131). The increase in the size of the former peak as a function of the age of the culture may indicate an increase in a cytochrome o (99); this is a cytochrome which functions as a terminal oxidase. The broad overlapping peaks centered at 600 nm are consistent with the presence of cytochrome a_1 (131,132). Finally, the peak at 625 nm, which appears in late exponential phase, could be the α band of cytochrome d, a cyanide-resistant cytochrome (131). The presence of cytochrome d is also suggested by the trough at 445 nm in carbon monoxide spectra (Fig. 9) (132,133).

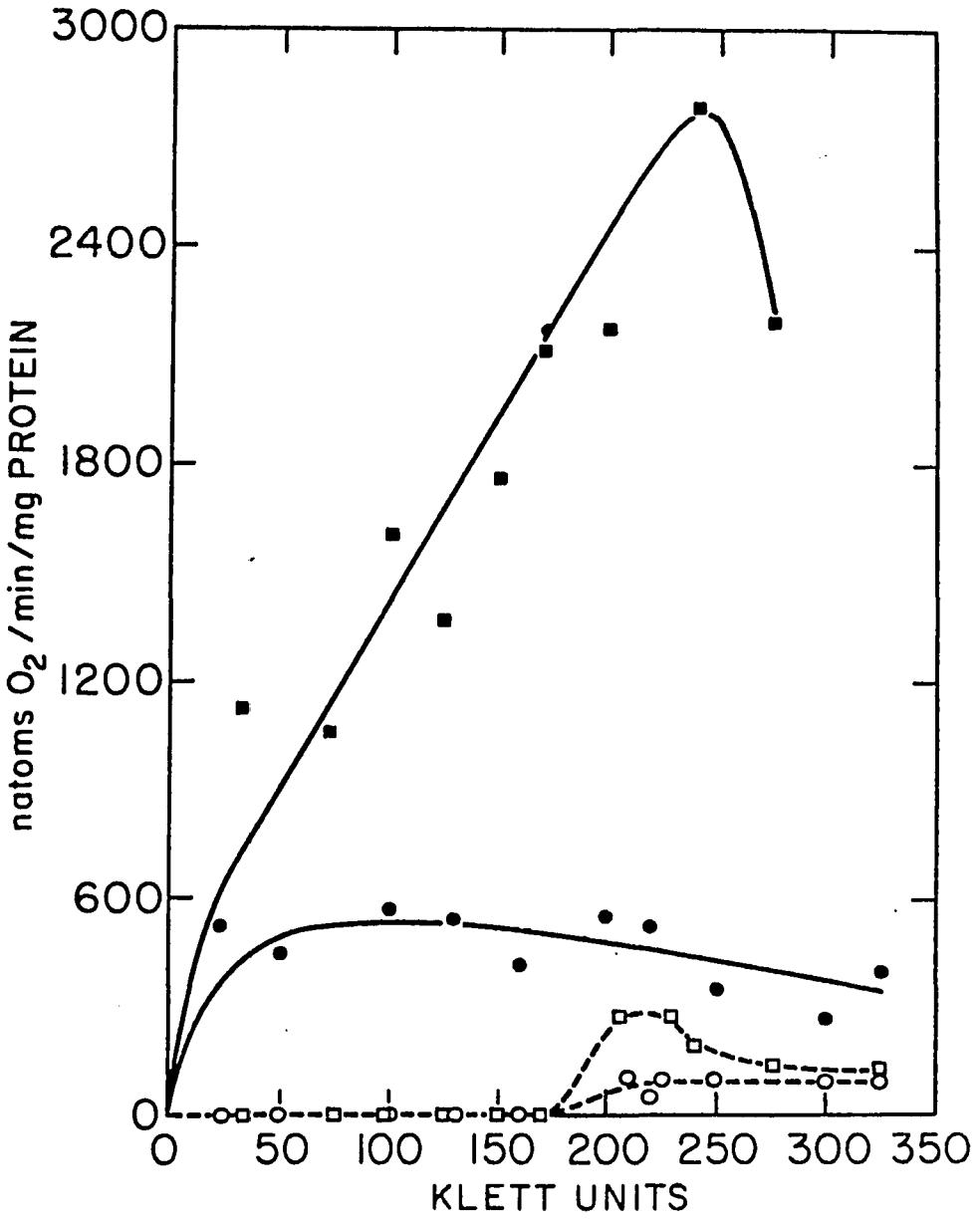

Fig. 7. Oxygen consumption by wild type A.pyridinolis grown to various cell densities on L-malate or D-fructose. Cells were grown on 50 mM L-malate (□ , ■) or 50 mM D-fructose (o, ●) and monitored turbidometrically. At intervals, samples of the cultures were assayed for oxygen consumption in the presence (□ , o) and absence (■ , ●) of 3 mM KCN.

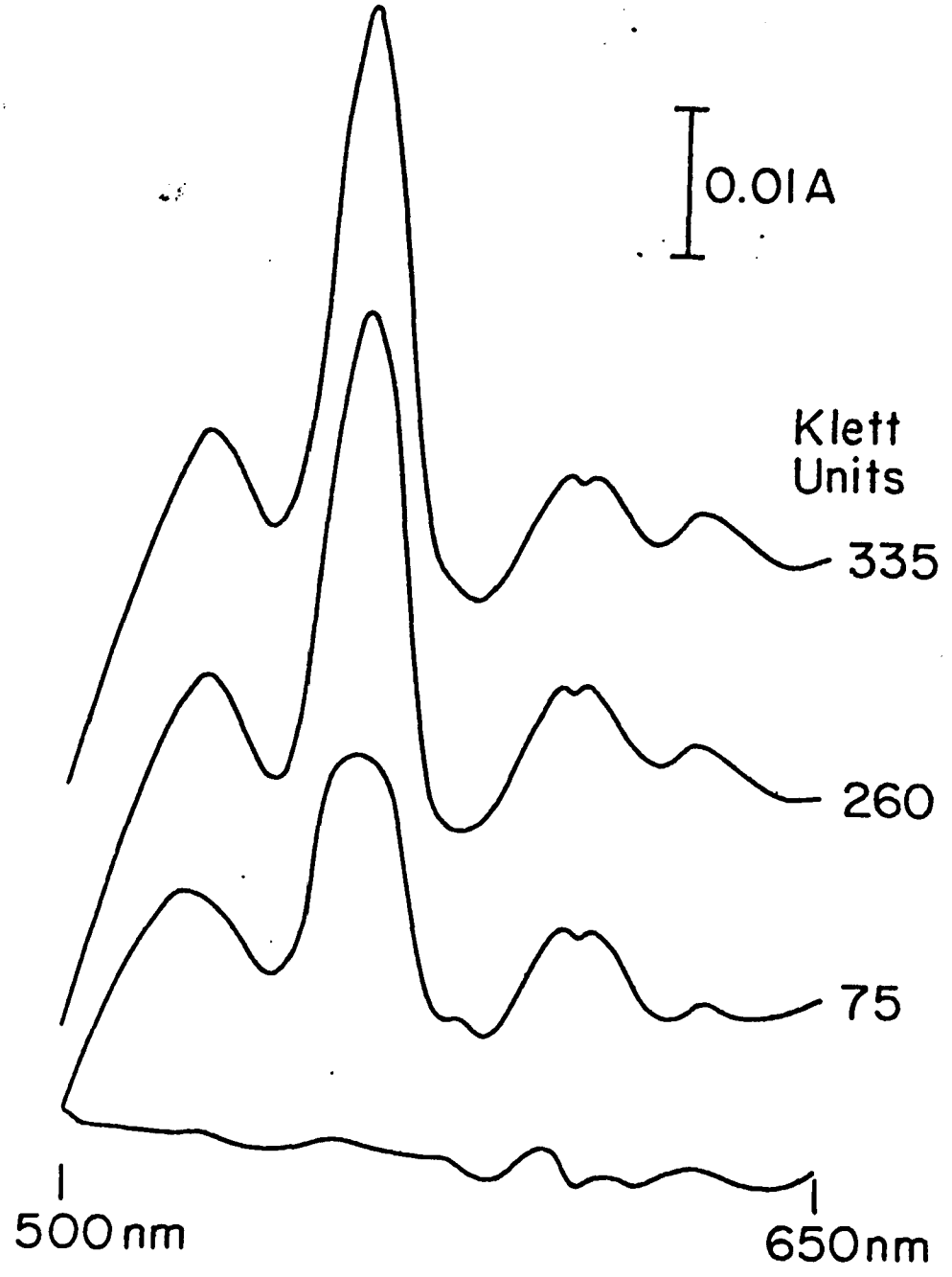

Fig. 8. Reduced vs. oxidized difference spectra of extracts from wild type A. pyridinolis grown on D-fructose. Cells were grown on 50 mM D-fructose and monitored turbidometrically. At intervals, extracts were prepared and spectra were determined as described under Materials and Methods after adjustment of each sample to 5 mg protein/ml.

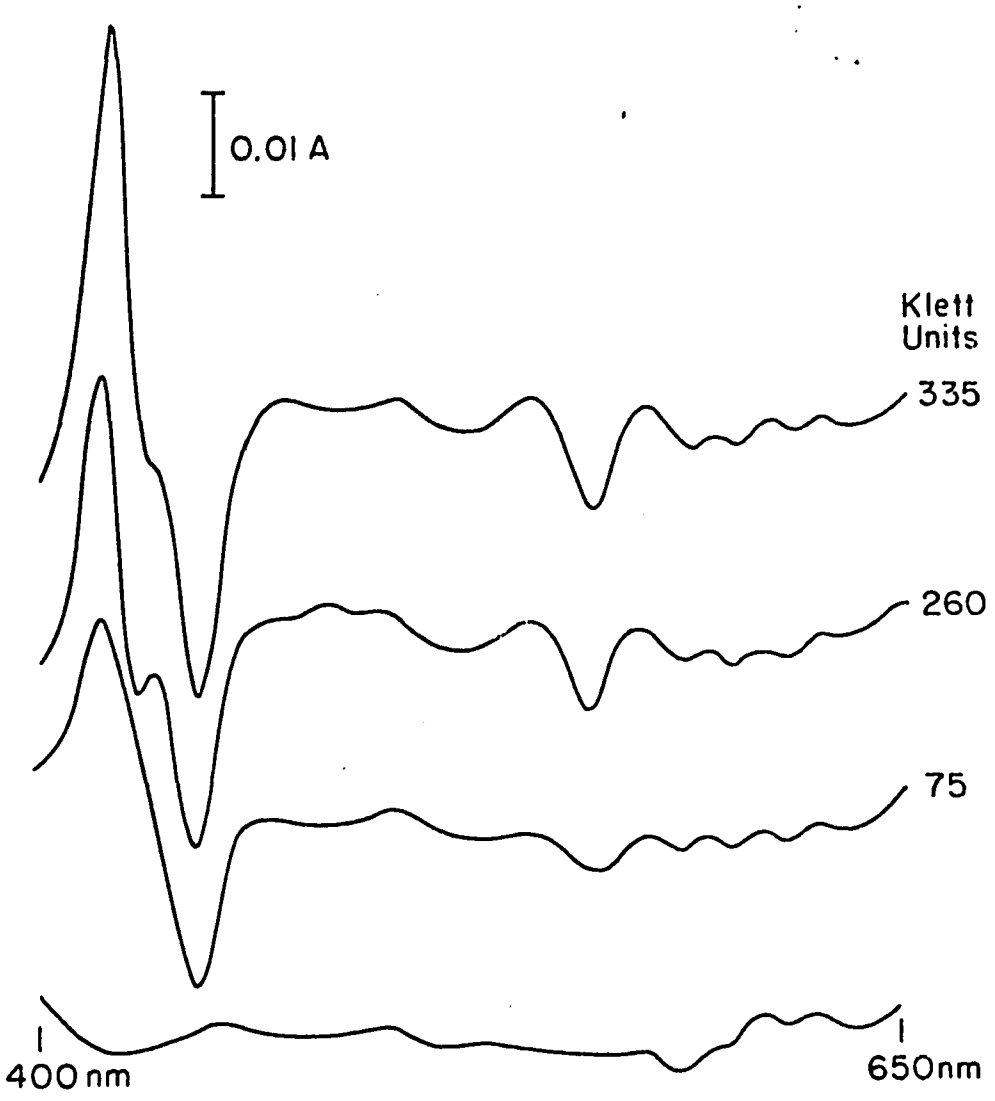

Fig. 9. CO difference spectra of extracts from wild type A. pyridinolis. Cells were grown and extracts were prepared as described in the legend to Fig. 8. CO spectra were determined as described under Materials and Methods.

Moreover, in the carbon monoxide spectra, cytochrome o is identified by the peak in the Soret region at 419 nm and the peaks at about 540 and 570 nm (131,132). Spectra from L-malate-grown cells were qualitatively similar.

The very low respiratory activity of D-fructose-grown cells with a slight decline in older cultures and the appearance of a cyanide-resistant terminal oxidase suggested that, indeed, the latter half of the growth cycle might represent conditions of relative anaerobiosis during which an even more fermentative metabolism occurs than in early exponential cells. However, the respiratory activity was so low throughout the growth cycle on D-fructose that a mid-logarithmic switch in physiology was impossible to substantiate on the basis of that criterion. Experiments in which the release of CO_2 from internalized D-fructose and fructose-1-phosphate was monitored yielded similar results. Cells grown on D-fructose to different densities were washed and resuspended at equal cell concentrations. The cell suspensions were allowed to accumulate radioactive D-fructose (U-[^{14}C]-D-fructose, 10 μM final concentration, 100 μcuries/ml) for 3 min. The cells were then separated from the radioactive medium and were resuspended so that CO_2 evolution could be measured as described in Materials and Methods. The amount of radioactivity internalized by all of the cell suspensions was essentially the same. Evolution of radioactive CO_2 was monitored for 30 min and reached a plateau at 20 min. At that point, suspensions of cells from mid-exponential phase and earlier released 9-10% of the internalized radioactivity as CO_2 while suspensions from late exponential phase cells released 8%. Thus, as with oxygen consumption, the CO_2 released from D-fructose metabolism is low throughout the growth cycle and a marked decline in the latter part of growth cannot be discerned.

In spite of these indications that D-fructose metabolism was not highly aerobic overall, the production of lactic acid from D-fructose was not high relative to aerobic organisms. A protocol similar to that used in the CO_2-evolution experiments was employed for these determinations. At intervals, cell suspensions were extracted with perchloric acid. Neutralized extracts were applied to a Dowex-1 anion exchange column, and eluted with an HCl gradient. Results from this experiment showed that the amount of lactic acid produced from cells in early to mid-log phase was 8-9 mmols lactate per 100 mmols D-fructose, and was slightly less than the amount produced by cells in the late-log stage, which was 10.7 mmols lactate per 100 mmols D-fructose.

The activity of the glyoxylate pathway enzyme isocitrate lyase was examined during the course of growth and wild type A. pyridinolis on D-fructose. This enzyme was chosen because its activity is required for aerobic growth of A. pyridinolis on D-fructose (111); thus a mid-logarithmic decline in its activity would suggest a less aerobic metabolic mode. It was possible that this might represent a more sensitive indicator of such a shift than those examined above. Cells harvested earlier than the mid exponential phase of growth (turbidities of less than 150 Klett units) were found to contain isocitrate lyase at an average specific activity of 6.4 nmoles glyoxylate produced x min^{-1} x mg $protein^{-1}$. By contrast, cells harvested at later times exhibited much less activity, in a range from 0 (non-detectable) to 1.1 nmoles x min^{-1} x mg $protein^{-1}$.

The Glyoxylate Pathway and Metabolite Levels in A. pyridinolis and a Citrate Synthase-Deficient Mutant, JF3

In view of the above findings, it was of interest to investigate metabolic signals which might coordinate changes in the metabolic modes

in A. pyridinolis. From suggestions in the literature (101) as well as its central role in aerobic metabolism, acetyl CoA appeared reasonable to adopt as the focus of these investigations. To facilitate studies on the role of acetyl CoA, a mutant that was deficient in citrate synthase was sought (see Fig. 2). The mutant, JF3, was isolated as a citrate auxotroph after mutagenesis of the wild type as described in Materials and Methods. Strain JF3 required citrate in the medium for growth on various carbon sources, including L-malate and D-fructose. Cells of JF3 grown on 10 mM citrate were found to contain citrate synthase at a specific activity of 0.05 μmoles citrate formed x min^{-1} x mg $protein^{-1}$, as compared to a specific activity of 1.89 μmoles citrate x min^{-1} x mg $protein^{-1}$ in the parent strain grown under the same conditions. Because of the position of the metabolic block in the mutant it was expected that acetyl CoA would accumulate endogenously, and, in fact, this was found (Table 3). Table 3 provides a summary of the data obtained in a comparative study of wild type A. pyridinolis and JF3, grown to the late-log phase. For the wild type organism, only during growth on 50 mM acetate (plus the requisite 2.5 mM citrate) did the wild type exhibit high levels, in fact amy measurable levels, of glyoxylate pathway enzymes. Growth on the required citrate plus either L-malate or D-fructose failed to induce the glyoxylate pathway enzymes. On the other hand, the expression of these enzymes in JF3 was found to be constitutive. Levels of these enzymes in JF3 were consistently found to be at least as high as the levels in acetate-grown wild type cells, regardless of the carbon source.

To examine the plausibility to acetyl CoA as the coordinating metabolite, it was necessary to measure the intracellular levels of this metabolite. At the same time, the endogenous levels of malate and ATP were

Table 3. Levels of glyoxylate pathway enzymes and certain metabolites in the wild type A.pyridinolis and a citrate synthase-deficient mutant, JF3.

A.pyridinolis and JF3 were grown to late log phase on media containing 2.5 mM citrate plus the indicated carbon sources. Sonic extracts were prepared for enzyme assays and perchloric acid extracts for metabolite level assays.

STRAIN	CARBON SOURCE (50 mM)	GLYOXYLATE PATHWAY ENZYMES		METABOLITES		
		ISOCITRATE LYASE	MALATE SYNTHASE	ACETYL CoA	MALATE	ATP
		nmols x min^{-1} x mg $prot^{-1}$		nmols x mg $protein^{-1}$		
Wild type	Acetate	351.0	113.5	--	--	--
Wild type	L-Malate	1.0	1.0	0.74	3.28±.81	5.05±.95
Wild type	D-Fructose	1.0	1.0	0.64±.04	--	4.24±.67
JF3	Acetate	382.0	--	--	--	--
JF3	L-Malate	688.0	85.9	13.4±1.5	0.89±.22	5.89±1.9
JF3	D-Fructose	402.1	171.9	11.5±1.4	0.65±.23	3.22±.87

measured to see if there was a correlation between these other metabolites and the induction or constitutive expression of the glyoxylate pathway. Cells were grown exactly in parallel for measurement of enzyme activities, except that perchloric acid extracts instead of sonic extracts were prepared, for measurement of the metabolites. As shown in Table 3, the level of acetyl CoA in JF3 was almost 20 times higher than the level found in the wild type. The high levels of endogenous acetyl CoA in JF3 correlate well with the constitutive expression of the glyoxylate cycle enzymes. At the same time, the levels of L-malate and ATP measured in these cells did not show any correlation with the induction of isocitrate lyase and malate synthase. These data strongly suggest that acetyl CoA is, in fact, responsible for induction of the glyoxylate cycle in A. pyridinolis.

The Effect of an Acetyl CoA Synthetase Deficiency on Glyoxylate Pathway Induction

To obtain further evidence that acetyl CoA might induce the glyoxylate pathway, an acetyl CoA synthetase-deficient mutant was isolated. This enzyme catalyzes the activation of free acetate to acetyl CoA for entry into the central metabolic pathways. Mutant strain AC17 was isolated through mutagenesis of the wild type as described in Materials and Methods, and subsequent selection for its inability to grow on acetate while retaining the ability to grow on other carbon sources. When both AC17 and the wild type were grown on 15 mM malate (Table 4), the wild type showed a significant level of acetyl CoA synthetase activity, while AC17 showed none; neither strain exhibited isocitrate lyase under these conditions. Upon induction with a high concentration of acetate, acetate transport in whole cells was shown to be normal in both strains. However, while the

wild type showed pronounced induction of both acetyl CoA synthetase and isocitrate lyase, AC17 showed no induction of these enzymes at all.

The work of Hong et al. (116) indicates another possible route for the synthesis of acetyl CoA in the presence of acetate. In E. coli acetate can be converted to acetyl phosphate by the enzyme acetate kinase (in the presence of ATP). Acetyl phosphate can in turn be converted to acetyl CoA by the enzyme phosphotransacetylase, which exchanges the phosphate for coenzyme A. These enzymes, however, were not detectable in cell free, dialyzed extracts of A. pyridinolis when grown on 50 mM acetate, as well as other carbon sources.

PTS Induction and Metabolite Levels in A. pyridinolis and JF3

Because acetyl CoA appeared to play a role in the induction of the glyoxylate pathway, and hence aerobic metabolism of A. pyridinolis, it was possible that a converse role existed for acetyl CoA in regulation of the PTS. To explore this possibility, the following experiment was conducted: Both the wild type and JF3 were grown to mid-log phase on 2.5 mM citrate plus 50 mM glutamate (a relatively non-repressing carbon source). The cells were then harvested, and induced for PTS activity by incubation in the presence of D-fructose. As shown in Table 5, while the wild type PTS was induced to the same level of activity as found in earlier studies, JF3 had no more than 50% of the wild type activity. When the levels of endogenous metabolites were measured, malate and ATP were found to have no correlation with PTS activity in either strain. However, the acetyl CoA levels in JF3 were again more than 15 times greater than those found in the wild type.

Table 4. Failure of an acetyl CoA synthetase-deficient mutant to produce isocitrate lyase upon induction.

Wild type A.pyridinolis and an acetyl CoA synthetase-deficient mutant, AC17, were grown to late log phase on media containing 15 mM L-malate, and to the indicated flask 50 mM acetate was added for 2 hrs. Acetate transport was measured in whole cells, while enzyme assays were performed on dialyzed, sonic extracts.

STRAIN	INDUCED W/ACETATE	ACETATE TRANSPORT	ACETYL CoA SYNTHETASE	ISOCITRATE LYASE
		nmols/min mg prot.	μmols AcCoA min/mg prot.	nmols/min mg protein
Wild type	NO	--	33.2	1.0
Wild type	YES	70.0	102.3	235.0
AC17	NO	--	1.0	1.0
AC17	YES	66.2	1.0	1.0

Table 5. Levels of phosphoenolpyruvate:D-fructose phosphotransferase activity and certain metabolites in wild type A.pyridinolis and JF3 during PTS induction.

A.pyridinolis and JF3 were grown on 2.5 mM citrate plus 50 mM L-glutamate overnight, harvested aseptically and re-inoculated into media containing 50 mM D-fructose for 4 hrs. Sonic extracts were then prepared for enzyme assays and perchloric acid extracts for metabolite levels.

STRAIN	PTS	METABOLITES		
		ACETYL CoA	MALATE	ATP
	nmols x min^{-1} x mg $prot.^{-1}$	nmols x mg $protein^{-1}$		
Wild type	10.14	0.48±.11	1.10±.66	3.57±1.3
JF3	4.78	7.85±.84	1.05±.59	3.15±1.8

DISCUSSION

The alternate transport systems for D-fructose in A. pyridinolis appear to function under different physiological conditions. In wild type cells, respiration-coupled transport occurs during the early log phase with PTS activity appearing only after respiration-coupled transport has declined. Compounds which stimulate respiration-coupled transport, e.g., L-malate, repress the PTS. In the ALA-auxotroph, activities of the two transport systems show a similarly inverse relationship to one another. PTS induction is optimal under conditions in which the concentration of ALA for growth and respiration-coupled transport is limiting. Morever, even in the presence of 50 μg ALA/ml, at which growth of BJ200 is comparable to the wild type, respiration-coupled transport is greatly reduced during early log phase with a concomitant increase in PTS activity at that time. Presumably exogenous ALA is not incorporated as efficiently as endogenously produced ALA; this may well reflect poor ALA transport by D-fructose-grown cells that are limited in respiration. However, as stated previously, attempts to measure ALA transport and cytochrome content in BJ200 during the early log phase were unproductive, largely due to technical limitations.

Apparently the two transport systems for D-fructose are controlled such that factors favoring respiration-coupled transport cause repression, and perhaps also inhibition, of the PTS. It is notable that the mid-logarithmic change from use of respiration-coupled transport to use of the PTS in wild type cells occurs with no apparent change in growth rate, and only the slightest indication of a decrease in oxygen consumption and in CO_2 release from internalized D-fructose and fructose-1-phosphate, accompanied by a small increase in lactic acid production. However, there are

several indications that the later stages of growth on D-fructose, when the phosphotransferase system is the principal form of transport, represent a more fermentative physiological mode, perhaps an adaptation to conditions of greater anaerobiosis. These include a decline in oxygen consumption, albeit a small decline; the appearance of a cyanide-resistant cytochrome, probably cytochrome d; the increase in cytochromes, especially b-type cytochromes, as cultures get more dense; the marked decrease in isocitrate lyase activity which is required for replenishment of Krebs cycle intermediates during aerobic growth of A. pyridinolis; and the small increase in lactic acid production. The increase in cytochrome levels observed as the oxygen tension of a culture decreases has been well documented in other bacteria. Thomas et al. (92), working with glucose grown E. coli, found that both cytochromes b_1 and d increase substantially as the oxygen tension of the culture decreases; they then declined rapidly, but not to zero, when the cultures are completely anaerobic. Sinclair and White (95) observed that the concentration of cytochromes in H. parainfluenzae was much greater at an oxygen concentration of 20 μM as compared to an oxygen concentration of 150-200 μM. As measured in P. putida from log- to stationary-phase cells (when almost no dissolved oxygen was measured), cytochrome o increased three-fold, and while no cytochrome d was evident in log phase cells, a significant amount was present in stationary phase cells (99). Increases in the amounts of cytochrome o and the appearance of cytochrome d in late-log phase cultures of A. pyridinolis is in agreement with the findings of these other workers. Although cytochrome o is probably the primary oxidase, under conditions of limited oxygen cytochrome d may function to insure more efficient utilization of available oxygen. While lactic acid formation did increase slightly in the later stages of growth, the levels produced were not very high. When

allowed to utlize D-glucose fermentatively, E. coli produced 80 mmol lactate per 100 mmol D-glucose (134), Leuconostoc mesenteroides produced 89 mmol lactate per 100 mmol D-glucose (130), and when utilized by the aerobic bacterium Bacillus subtilis, 10 mmol lactate per 100 mmol D-glucose were produced.

Are there positive effectors of aerobic metabolism that may coordinately repress or inhibit the PTS? The results with the citrate synthase-deficient strain, in which the constitutive synthesis and high levels of activity of the glyoxylate pathway enzymes are shown to exist concomitantly with intracellular levels of acetyl CoA twenty times higher than those found in the wild type, strongly implicate acetyl CoA as the inducer of the glyoxylate pathway. Furthermore, the inability of an acetyl CoA synthetase-deficient strain to induce this pathway provides additional support to this conclusion.

While acetyl CoA may appear to be the inducer of the glyoxylate pathway in A. pyridinolis, this has been shown not to be the case in E. coli. Kornberg (107) noted that isocitrate lyase, the key enzyme of the glyoxylate pathway, was fully induced only in the presence of acetate. He then proposed that the induction-like effect of acetate might be due to one of three causes: (1) Acetate (or acetyl CoA) might be the inducer; (2) acetate (or acetyl CoA) might combine with some other metabolite and form an inducer; or (3), acetate (or acetyl CoA) might combine with another metabolite to relieve repression of isocitrate lyase synthesis. Using a citrate synthase mutant of E. coli, Kornberg demonstrated that isocitrate lyase was not induced by acetate in the mutant, under conditions in which this enzyme was induced in the wild type. When the mutant's ability to activate acetate to acetyl CoA was demonstrated,

Kornberg ruled out acetyl CoA as the direct inducer. Postulate (3) was demonstrated to be correct, and malate or some metabolite closely related to it was proposed as the repressor. Thus the combination of acetate (acetyl CoA) with OAA to form citrate would relieve the repression of isocitrate lyase. As discussed earlier, the repressor and allosteric regulatory of isocitrate lyase in E. coli was identified as PEP (115). Clearly though, as noted by Mukkada et al. (84), the regulatory signals need not be the same in all organisms.

The data presented suggest that in addition to its role in induction of the glyoxylate pathway, acetyl CoA may mediate a repressive effect on the PTS. It is not known whether this effect is direct or indirect. Although acetyl CoA levels appear to have a correlation with PTS activity, the data are not as clear-cut as they are for the glyoxylate pathway. While JF3 does exhibit acetyl CoA levels 15 times higher than the wild type under PTS induction conditions, the effect is to repress the PTS only 50%. The effect of such high levels of acetyl CoA on the glyoxylate pathway were far more dramatic. Morgan and Kornberg (135) have implicated acetyl CoA as an inhibitor of sugar transport in E. coli. They found that PEP synthase-deficient mutants (pyr + ATP → PEP + AMP + P_i) grown on certain sugars were inhibited by added pyruvate, while pyruvate had no effect when they were grown on other sugars. They then showed that 10 mM pyruvate in the wild type and 1 mM pyruvate in a citrate synthase-deficient mutant could severely inhibit 2-deoxyglucose uptake, while a pyruvate dehydrogenase mutant was not affected by 1 mM pyruvate. It was concluded that acetyl CoA was the inhibitory agent.

The isolation of a pyruvate dehydrogenase mutant of A. pyridinolis has been attempted; however, the isolation of a stable mutant has not been

possible up to this point. It was believed, as indicated by Morgan and Kornberg's data, that such a mutant would provide a complement to JF3, the citrate synthase-deficient mutant. In our organism, a pyruvate dehydrogenase mutant would require acetate for growth, and obtain its acetyl CoA via acetyl CoA synthetase; the inability to produce acetyl CoA without added acetate (due to the metabolic block) should drastically reduce or abolish the intracellular levels of acetyl CoA. If grown under PTS induction conditions, as in Table 5, substituting acetate for citrate, I would expect a pyruvate dehydrogenase mutant to have higher, perhaps constitutive, levels of PTS activity compared to the wild type, if in fact acetyl CoA serves as a repressor of the PTS. It would also be interesting to utilize such a mutant in an attempt to grow A. pyridinolis anaerobically on D-fructose, especially if the PTS could be constitutively expressed.

It appears relevant, then, that high levels of L-malate as well as acetyl CoA have negative effects on the PTS, and could relate to the recent findings that the protonmotive force regulates the PTS activity. This topic has been discussed in the Literature Review section at length, but several observations are noteworthy in this regard: (1) addition of L-malate, a substrate of the respiratory chain, represses the PTS; (2) a α-aminolevulinic acid auxotroph exhibits high levels of PTS activity when its respiration is limited; and (3) when high levels of acetyl CoA, providing a constitutive, aerobic, glyoxylate pathway are present, the PTS is also repressed. These observations could all be linked to the magnitude of the protonmotive force; whether a true correlation exists in these cells awaits further investigation.

Even though A. pyridinolis is an obligate aerobe and cannot be grown

strictly anaerobically, under some growth conditions it manifests a set of biochemical changes, with regulatory signals, which result in a relatively fermentative physiology. These changes may represent an adaptation which provides the organism with enhanced ecological versatility and resistance to environmental variations. It will be of interest to determine whether other estensibly obligate aerobes exhibit these properties, and whether they correlate with the observed range of growth habitats and/or survival capacities.

ABBREVIATIONS

ALA	δ-aminolevulinic acid
ATP	adenosine 5'-triphosphate
CoA	coenzyme A
cyclic AMP	adenosine 3',5'-monophosphate
diam.	diameter
DNP	2,4-dinitrophenol
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
EMS	ethyl methane sulfonate
HPr	histidine-containing protein of PTS
hr	hour(s)
K	x 1000
α-MG	α-methyl glucoside
min	minutes(s)
MS	mineral salts
mol. wt.	molecular weight
β-NAD	β-nicotinamide adenine dinucleotide (oxidized)
OAA	oxaloacetic acid
PEP	phosphoenolpyruvate
P-HPr	phospho-HPr
PTS	phosphotransferase system
PYE	peptone-yeast extract
RC	respiration-coupled
rpm	revolutions per minute
sec	second(s)

REFERENCES

1. Mitchell, P. 1957. Nature 180:134.

2. Mitchell, P. 1963. In The Structures and Function of the Membranes and Surfaces of Cells, D. Bell and J. Grant, eds., University Press, Cambridge, England.

3. Kundig, W., S. Ghosh and S. Roseman. 1964. Proc. Natl. Acad. Sci. 52:1067.

4. Kundig, W. and S. Roseman. 1971. J. Biol. Chem. 246:1393.

5. Kundig, W. and S. Roseman. 1971. J. Biol. Chem. 246:1407.

6. Saier, M.H., Jr., R.D. Simoni and S. Roseman. 1970. J. Biol. Chem. 245:5870.

7. Simoni, R.D., J.B. Hays, T. Nakazawa, and S. Roseman. 1973. J. Biol. Chem. 248:957.

8. Saier, M.H., Jr. and S. Roseman. 1976. J. Biol. Chem. 251:6598.

9. Saier, M.H., Jr. and S. Roseman. 1976. J. Biol. Chem. 251:6606.

10. Postma, P. and S. Roseman. 1976. Biochem. Biophys. Acta 457:213.

11. Kundig, W. 1974. J. Supramol. Struc. 2:695.

12. Saier, M.H., Jr. 1977. Bacteriol. Rev. 41:856.

13. Hays, J.B. 1977. In Bacterial Transport, B.P. Rosen, ed., Marcel Dekker, Inc., New York.

14. Saier, M.H., Jr. and E.G. Moczydlowski. 1977. In Bacterial Transport, B.P. Rosen, ed., Marcel Dekker, Inc., New York.

15. Weigel, N., E. Waygood, A. Nakazawa, M. Kukuruzinska, and S. Roseman. 1977. Proc. Can. Fed. Biol. Soc. 20:54.

16. Simoni, R., T. Nakazawa, J. Hays, and S. Roseman. 1973. J. Biol. Chem. 248:932.

17. Anderson, B., N. Weigel, W. Kundig, and S. Roseman. 1971. J. Biol. Chem. 246:7023.

18. Simoni, R., J. Hays, T. Nakazawa, and S. Roseman. 1973. J. Biol. Chem. 248:957.

19. Ullah, A. and V. Cirillo. 1976. J. Bacteriol. 127:1298.

20. Simoni, R., M. Smith and S. Roseman. 1968. Biochem. Biophys. Res. Commun. 31:804.

21. Nakazawa, T., R. Simoni, J. Hays, and S. Roseman. 1971. Biochem. Biophys. Res. Commun. 42:836.

22. Wolfson, E.B. and T.A. Krulwich. 1974. Proc. Natl. Acad. Sci. 71: 1739.

23. Levinson, S. and T.A. Krulwich. 1973. Arch. Biochem. Biophys. 160: 445.

24. Hanson, T. and R. Anderson. 1968. Proc. Natl. Acad. Sci. 61:269.

25. Walter, R. and R. Anderson. 1973. Biochem. Biophys. Res. Commun. 52:93.

26. Saier, M., B. Feucht and S. Roseman. 1971. J. Biol. Chem. 246:7819.

27. Lengeler, J. 1976. J. Bacteriol 124:26.

28. Lengeler, J. 1976. J. Bacteriol. 124:39.

29. Solomon, E. and Lin, E.C.C. 1972. J. Bacteriol. 111:566.

30. Tanaka, S. and Lin, E.C.C. 1967. Proc. Natl. Acad. Sci. 57:913.

31. Kelker, N. and R. Anderson. 1971. J. Bacteriol. 105:160.

32. Maryanski, J. and C. Wittenberger. 1975. J. Bacteriol. 124:1475.

33. Tanaka, S., D.G. Fraenkel and E.C.C. Lin. 1967. Biochem. Biophys. Res. Commun. 27:63.

34. Tanaka, S., S. Lerner and E.C.C. Lin. 1967. J. Bacteriol. 93:642.

35. Simoni, R.D., M. Levinthal, F.D. Kundig, B. Anderson, P.E. Hartman, and S. Roseman. 1967. Proc. Natl. Acad. Sci. 58:1963.

36. Kaback, H.R. 1968. J. Biol. Chem. 243:3711.

37. Romano, A.H., S.J. Eberhard, S.L. Dingle, and T.C. McDowell. 1970. J. Bacteriol. 104:808.

38. Romano, A.H. and J.D. Trifone. 1976. Abstr. Ann. Mtg. Amer. Soc. microbiol. p. 153.

39. Andrews, K.J. and E.C.C. Lin. 1976. Fed. Proc. 35:2185.

40. Delobbe, A., H. Chalumeau and P. Gay. 1975. Eur. J. Biochem. 51:503.

41. Delobbe, A., H. Chalumeau, J.-M. Claverie, and P. Gay. 1976. Eur. J. Biochem. 66:485.

42. Gay, P. and A. Delobbe. 1977. Eur. J. Biochem. 79:363.

43. Marquet, M., M.-C. Creignou and R. Dedonder. 1976. Biochimie 58:435.

44. Sawyer, M.H., P. Baumann, L. Baumann, S.M. Berman, J.L. Canovas, and R.H. Berman. 1977. Arch. Microbiol. 112:49.

45. Sawyer, M.H., P. Baumann and L. Baumann. 1977. Arch. Microbiol. 112:169.

46. Sobel, M.E., E.B. Wolfson and T.A. Krulwich. 1973. J. Bacteriol. 116:271.

47. Sobel, M.E. and T.A. Krulwich. 1973. J. Bacteriol. 113:907.

48. Krulwich, T.A., M.E. Sobel and E.B. Wolfson. 1973. Biochem. Biophys. Res. Commun. 53:258.

49. Wolfson, E.B., M.E. Sobel and T.A. Krulwich. 1973. Biochim. Biophys. Acta 321:181.

50. Wolfson, E.B., M.E. Sobel, R. Blanco, and T.A. Krulwich. 1974. Arch. Biochem. Biophys. 160:440.

51. Levinson, S.L. and T.A. Krulwich. 1976. J. Gen. Microbiol. 95:277.

52. Adler, J. and W. Epstein. 1974. Proc. Natl. Acad. Sci. 71:2895.

53. Fox, C.F. and G. Wilson. 1968. Proc. Natl. Acad. Sci. 59:988.

54. Pastan, I. and R. Perlman. 1969. J. Biol. Chem. 244:5836.

55. Saier, M.H., Jr., R.D. Simoni and S. Roseman. 1976. J. Biol. Chem. 251:6584.

56. Saier, M.H., Jr. and B.U. Feucht. 1975. J. Biol. Chem. 250:7078.

57. Saier, M.H., Jr., B.U. Feucht and L.J. Hofstadter. 1976. J. Biol. Chem. 251:883.

58. Castro, L., B.U. Feucht, M.L. Morse, and M.H. Saier, Jr. 1976. J. Biol. Chem. 251:5522.

59. Harwood, J.P., C. Gazdar, C. Prasad, A. Peterkofsky, S.J. Curtis, and W. Epstein. 1976. J. Biol. Chem. 251:2462.

60. Gonzalez, J.E. and A. Peterkofsky. 1977. J. Supramol. Struc. 6:495.

61. Peterkofsky, A. and C. Gazdar. 1978. J. Supramol. Struct. 9:219.

62. Peterkofsky, A., J.E. Gonzalez and C. Gazdar. 1978. Arch. Biochem. Biophys. 188:47.

63. Peterkofsky, A. and C. Gazdar. 1979. Proc. Natl. Acad. Sci. 76:1099.

64. Hoffee, P. and E. Englesberg. 1962. Proc. Natl. Acad. Sci. 48:1759.

65. Hoffee, P., E. Englesberg and F. Lamy. 1964. Biochim. Biophys. Acta 79:337.

66. Halpern, Y.S. and M. Lupo. 1966. Biochim. Biophys. Acta 126:163.

67. Hagihira, H., T.H. Wilson and E.C.C. Lin. 1963. Biochim. Biophys. Acta 78:505.

68. Gachelin, G. 1972. Ann. Inst. Pasteur 122:1099.

69. Haguenauer, R. and A. Kepes. 1971. Biochimie 53:99.

70. Hernandez-Asensio, M., J.M. Ramirez and F.F. del Campo. 1975. Arch. Microbiol. 103:155.

71. del Campo, F.F., M. Hernandez-Asensio and J.M. Ramirez. 1975. Biochem. Biophys. Res. Commun. 63:1099.

72. Fields, K.L. and S.E. Luria. 1969. J. Bacteriol. 97:57.

73. Plate, C.A., J.L. Suit, A.M. Jetten, and S.E. Luria. 1974. J. Biol. Chem. 249:6138.

74. Jetten, A.M. 1976. Biochim. Biophys. Acta 440:403.

75. Gilchrist, M.J.R. and J. Konisky. 1975. J. Biol. Chem. 250:2457.

76. Konisky, J. 1975. J. Bacteriol. 124:1439.

77. Reider, R., E.F. Wagner and M. Schweiger. 1979. Proc. Natl. Acad. Sci. 76:5529.

78. Hamilton, W.A. and E.A. Dawes. 1960. Biochem. J. 76:70P.

79. Hamilton, W.A. and E.A. Dawes. 1961. Biochem. J. 79:25P.

80. Ng, F.M.-W. and E.A. Dawes. 1967. Biochem. J. 104:48P.

81. Ng, F.M.-W. and E.A. Dawes. 1973. Biochem. J. 132:129.

82. Tiwari, N.T. and J.J.R. Campbell. 1969. Biochim. Biophys. Acta 192:395.

83. Midgely, M. and E.A. Dawes. 1973. Biochem. J. 132:141.

84. Mukkada, A.J., G.L. Long and A.H. Romano, 1973. Biochem. J. 132:155.

85. Guymon, L.F. and R.G. Eagon. 1974. J. Bacteriol. 117:1261.

86. Hylemon, P.B. and P.V. Phibbs, Jr. 1972. Biochem. Biophys. Res. Commun. 48:1041.

87. Krulwich, T.A. and J.C. Ensign. 1969. J. Bacteriol. 97:526.

88. Krulwich, T.A. and N.J. Pelliccione. 1979. Ann. Rev. Microbiol. 33:95.

89. Morris, J.G. 1975. Adv. Microbial Physiol. 12:169.

90. Gray, C.T., J.W.T. Wimpenny, D.E. Hughes, and M.R. Roseman. 1966. Biochim. Biophys. Acta 117:22.

91. Gray, C.T., J.W.T. Wimpenny and M.R. Roseman. 1966. Biochim. Biophys. Acta 117:33.

92. Thomas, A.D., H.W. Doelle, A.W. Westwood, and G.L. Gordon. 1972. J. Bacteriol. 112:1099.

93. Ruch, F.E., J. Lengeler and E.C.C. Lin. 1974. J. Bacteriol. 119:50.

94. Ruch, F.E. and E.C.C. Lin. 1975. J. Bacteriol. 124:348.

95. Sinclair, P.R. and D.C. White. 1970. J. Bacteriol. 101:365.

96. de Vries, W., W.M.C. van Wijck-Kapteijn and A.H. Stouthamer. 1972. J. Gen. Microbiol. 71:515.

97. Haddock, B.A. and C.W. Jones. 1977. Bacteriol. Rev. 41:47.

98. Doelle, H.W. and N.W. Hollywood. 1978. Microbios 21:47.

99. Sweet, W.J. and J.A. Peterson. 1978. J. Bacteriol. 133:217.

100. Kornberg, H.L. 1966. Biochem. J. 99:1.

101. Kornberg, H.L. 1965. Symp. Soc. Gen. Microbiol. 15:8.

102. Utter, M.F. and H.M. Kolenbrunder. 1972. In The Enzymes, vol. 6, P.D. Boyer, ed., Academic Press, New York.

103. Kornberg, H.L. and S.R. Elsden. 1961. Adv. in Enzymol. 23:401.

104. McFadden, B.A. and W.V. Howes. 1962. J. Bacteriol. 84:72.

105. McFadden, B.A. and W.V. Howes. 1962. Biochim. Biophys. Acta 65:366.

106. Ashworth, J.M., H.L. Kornberg and R.L. Ward. 1965. Biochem. J. 94: 28P.

107. Kornberg, H.L. 1963. Biochim. Biophys. Acta 73:517.

108. Ashworth, J.M. and H.L. Kornberg. 1963. Biochim. Biophys. Acta 73: 519.

109. Cooper, R.A. and H.L. Kornberg. 1965. Biochim. Biophys. Acta 104:618.

110. Ramanandra Rao, G. and B.A. McFadden. 1965. Arch. Biochem. Biophys. 112:294.

111. Wolfson, P.J. and T.A. Krulwich. 1972. J. Bacteriol. 112:356.

112. Krulwich, T.A., B.I. Sharon and L.S. Perrin. 1976. J. Bacteriol. 127:179.

113. Krulwich, T.A., R. Blanco and P.A. McBride. 1977. Arch. Biochem. Biophys. 178:108.

114. Hegeman, G.D. 1966. J. Bacteriol. 91:1140.

115. Cederbaum, A.I., C.S. Lieber and E. Rubin. 1975. Arch. Biochem. Biophys. 169:29.

116. Hong, J.-S., A.G. Hunt, D.S. Masters, and M.A. Lieberman. 1979. Proc. Natl. Acad. Sci. 76:1213.

117. McFadden, B.A. 1969. In Methods in Enzymology, J.M. Lowenstein, ed., vol. 13, Academic Press, New York.

118. Dixon, G.H. and H.L. Kornberg. 1962. In Methods in Enzymology, S.P. Colowick and N.O. Kaplan, eds., vol. 5, Academic Press, New York.

119. Ellman, G.L. 1959. Arch. Biochem. Biophys. 82:70.

120. Weitzman, P.D.J. 1969. In Methods in Enzymology, J.M. Lowenstein, ed., vol. 13, Academic Press, New York.

121. Webster, L.T., Jr. 1969. In Methods in Enzymology, J.M. Lowenstein, ed., vol. 13, Academic Press, New York.

122. Rose, I.A. 1955. In Methods in Enzymology, S.P. Colowick and N.O. Kaplan, eds., vol. 1, Academic Press, New York.

123. Brown, T.D.K., M.C. Jones-Mortimer and H.L. Kornberg. 1977. J. Gen. Microbiol. 102:327.

124. Tubbs, P.K. and P.B. Garland. 1969. In Methods in Enzymology, J.M. Lowenstein, ed., vol. 13, Academic Press, New York.

125. Williamson, J.R. and B.E. Corkey. 1969. In Methods in Enzymology, J.M. Lowenstein, ed., vol. 13, Academic Press, New York.

126. Cole, H., J.W.T. Wimpenny and D.E. Hughes. 1967. Biochim. Biophys. Acta 143:445.

127. Stanley, P.E. and S.G. Williams. 1969. Anal. Biochem. 29:381.

128. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. J. Biol. Chem. 193:265.

129. Von Korff, R.W. 1969. In Methods in Enzymology, J.M. Lowenstein, ed., vol. 13, Academic Press, New York.

130. Dawes, E.A., D.J. McGill and M. Midgely. 1971. In Methods in Microbiology, J.R. Norris and D.W. Ribbons, eds., vol. 6a, Academic Press, New York and London.

131. Jones, C.W. and E.R. Redfearn. 1967. Biochim. Biophys. Acta 143: 340.

132. Poole, R.K. and B.A. Haddock. 1975. Biochem. J. 152:537.

133. Haddock, B.A. and H.U. Schairer. 1973. Eur. J. Biochem. 35:34.

134. Woods, W.S. 1961. In The Bacteria, I.C. Gunsalus and R.Y. Stanier, eds., vol. 2, Academic Press, New York and London.

135. Morgan, M.J. and H.L. Kornberg. 1979. FEBS Lett. 3:53.