1987

Characterization Of The Specific Pyruvate Transport System Of Escherichia Coli K12

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CHARACTERIZATION OF THE SPECIFIC PYRUVATE TRANSPORT SYSTEM OF *ESCHERICHIA COLI* K12

by

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Submitted in partial fulfilment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

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September, 1986

ISBN 0-315-36080-1

ABSTRACT

A mutant of Escherichia coli K12 lacking pyruvate dehydrogenase and phosphoenolpyruvate synthase was used to study transport of pyruvate by whole cells. Uptake of pyruvate is maximal with mid-log phase cells and the Michaelis constant for transport is 20μM. Pretreatment of the cells with respiratory chain poisons or uncouplers, with the exception of arsenate, inhibits transport of pyruvate by up to 95%. Lactate and alanine, natural analogues of pyruvate, competitively inhibit transport only at very high concentrations. The synthetic analogues 3-bromopyruvate and pyruvic acid methyl ester are good competitive inhibitors.

In order to further minimize metabolism of pyruvate, membrane vesicles of a wild type E. coli K12 were prepared. Transport is dependent on an artificial electron donor system, phenazine methosulfate and sodium ascorbate, added to the vesicles. Pyruvate is concentrated 7-15 times in these energized vesicles and the Michaelis constant is 15μM. Uptake of pyruvate can also be energized by a phenazine methosulfate and NADH system, but not by the metabolic intermediates lactate, glucose or ATP. Energy poisons, with the exception of arsenate, inhibit the transport of pyruvate. Synthetic analogues such as 3-bromopyruvate are good competitive inhibitors of transport. Lactate initially

appeared to be a good competitive inhibitor of pyruvate transport in vesicles, but under conditions in which the oxidation of lactate to pyruvate is minimized, this apparent inhibition disappears.

Transport of pyruvate in whole cells was also found to be sensitive to osmotic shock, indicating that a periplasmic binding protein was involved in the transport system. Column chromatography techniques were employed in an attempt to isolate this binding protein from osmotic shock fluid. Partial purification of pyruvate binding activity was achieved through the use of an affinity column, but purification to homogeneity was not accomplished with subsequent column steps. The binding protein has been tentatively identified as a 36,000 molecular weight monomer by SDS-polyacrylamide gel electrophoresis.

A brief summary of these results indicates that pyruvate is transported in Escherichia coli by a unique and specific active transport system. This transport system utilizes the respiratory chain to provide the driving force for uptake and appears to have a periplasmic component capable of binding pyruvate.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my supervisor, Dr. Robert. A. Cook, for his guidance, patience and encouragement throughout the course of this work. My advisors, Dr. Theodore Lo and Dr. William Magee, also deserve thanks for their ideas and technical advice.

I would also like to thank Ms. Cheryl Leystra-Lantz for her excellent technical assistance.

This investigation was supported by a grant from the Medical Research Council of Canada.

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NOMENCLATURE

ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
BES	N,N-bis(2-hydroxyethyl)-2-aminoethane-sulfonate
CCCP	Carbonyl cyanide-m-chlorophenylhydrazone
CM cellulose	Carboxymethyl cellulose
DEAE cellulose	Diethylaminoethyl cellulose
DNP	2,4-Dinitrophenol
EDTA	Ethylenediaminetetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonate
LDH	Lactate dehydrogenase
MES	2-(N-morpholino)ethanesulfonate
NADH	Nicotinamide adenine dinucleotide (reduced)
PAGE	Polyacrylamide gel electrophoresis
PAME	Pyruvic acid methyl ester
PEP	Phosphoenolpyruvate
PMS	Phenazine methosulfate
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium dodecyl sulfate
Tricine	N-Tris(hydroxymethyl)methyl glycine
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER 1. TRANSLOCATION OF SUBSTRATES BY GRAM-NEGATIVE BACTERIA

1.1. INTRODUCTION

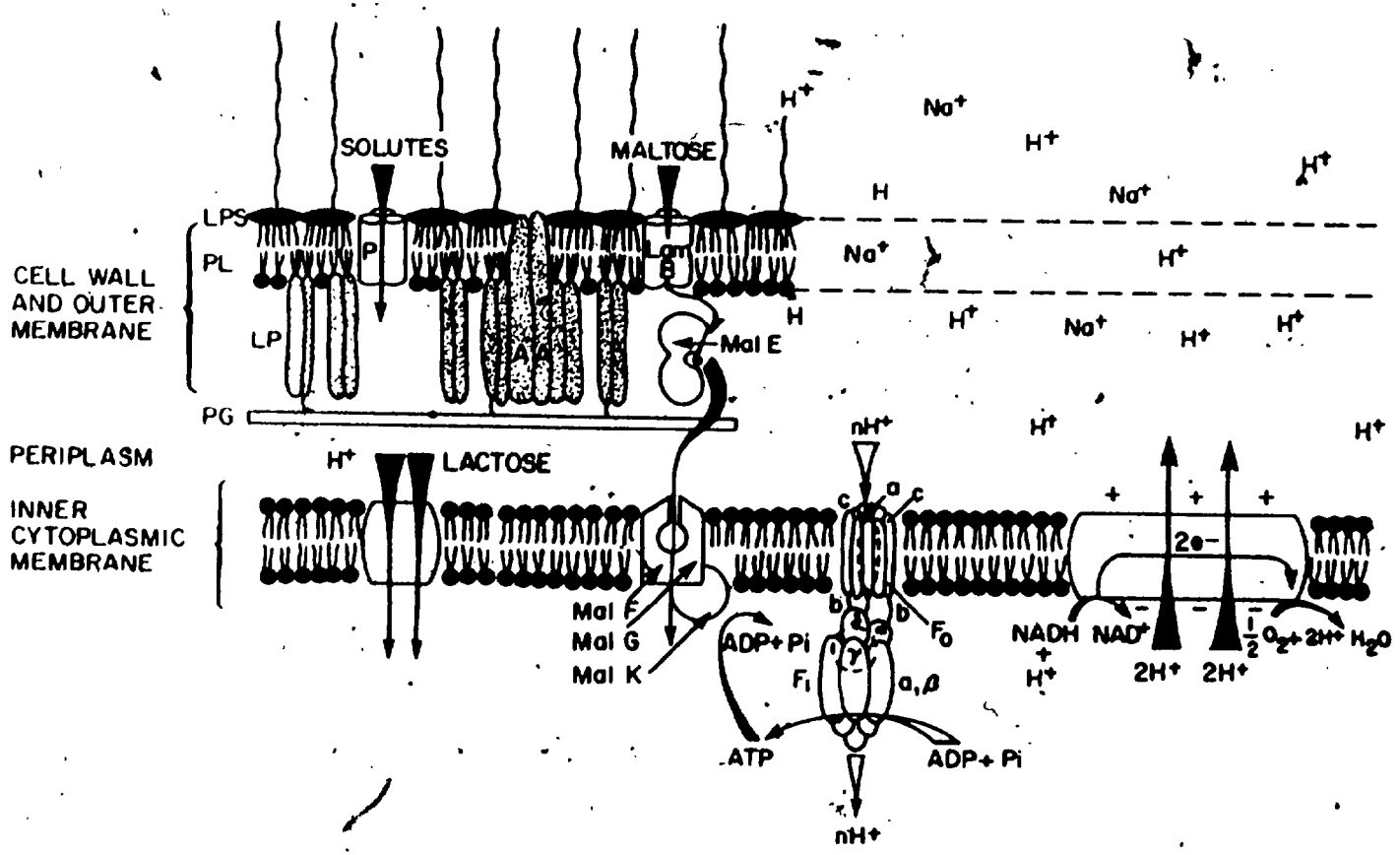
Bacteria have developed a number of mechanisms to bring essential nutrients across their relatively impermeable cytoplasmic membrane. In the past two decades, a number of these translocation mechanisms have been studied intensively and by using a combination of biochemical and genetic techniques, advances have been made in the areas of energy coupling to transport and the molecular mechanisms of transport. Gram-negative bacteria have been studied more intently that any of the bacteria, partially because E. coli are the best understood of the micro-organisms, but also because the complexity of the gram-negative membrane system has resulted in more diverse and intriguing transport mechanisms.

1.2 THE GRAM-NEGATIVE CELL ENVELOPE

A schematic (from Mitchell, 1985) of the gram-negative cell envelope is presented in Figure 1-1 to facilitate discussion of transport concepts. The gram-negative cell envelope is composed of three separate regions: the outer membrane and cell wall, the cytoplasmic membrane, and the periplasm which forms a compartment between the two membranes. The outer membrane, described in detail in a recent review by Lugtenberg and Van Alphen (1983), is a

FIGURE 1-1. SCHEMATIC OF THE GRAM-NEGATIVE CELL ENVELOPE

The basic components of the gram-negative bacterial envelope are represented. LPS = lipopolysaccharide, PL = phospholipid, P = porin, LP = lipoprotein, A = OmpA protein, LamB = maltoporin or lambda receptor protein, PG = peptidoglycan layer, MalE = maltose binding protein, MalF, G, K = membrane-bound or associated components of maltose transport, F_o and F_1 = membrane-bound and catalytic components of ATPase. The respiratory chain is represented in an undefined manner by the large rectangle in the cytoplasmic membrane. Elements of this schematic relevant to solute transport are discussed in more detail in the text.


SOLUTES
MALTOSE
LPS
PL
P
Lam B
CELL WALL AND OUTER MEMBRANE
LP
Mal E
PG
PERIPLASM
LACTOSE
INNER CYTOPLASMIC MEMBRANE
Mal F
Mal G
Mal K
ADP+ Pi
ATP
nH+
NADH
NAD+
2H+
2e−
½ O2+ 2H+ H2O
H+
Na+

bilayer membrane formed by an outer lipopolysaccharide leaflet and an inner leaflet of phospholipid and lipoprotein. EDTA treatment of bacteria, especially in the presence of Tris buffer, removes about half of the lipopolysaccharides from the cell (Leive, 1974), presumably by removing divalent cations which are required for the assembly and maintenance of the lipopolysaccharide interactions (Schindler and Osborn, 1979). This treatment leaves the outer membrane freely permeable to solutes as large as lysozyme. Lipoproteins covalently attach the outer membrane to the rigid peptidoglycan layer (Braun, 1975) of the periplasmic space. The outer membrane also contains several other proteins, represented in the schematic by non-specific pore-forming proteins known as porins, the structural OmpA protein, and the specific pore forming maltoporin complex (also known as the bacteriophage lambda receptor or LamB). The lambda receptor increases the permeability of the outer membrane for maltose and maltodextrans (Boos, 1984).

The peptidoglycan layer is a network of linear chains of alternating N-acetylglucosamine and N-acetylmuramic acid sugar residues which are cross-linked to form a monolayer by tetrapeptide bridges (Schleifer and Kandler, 1972). This peptidoglycan layer forms a rigid structure which enables the cell to withstand osmotic pressure and gives the cell its characteristic rod shape. Lysozyme specifically cleaves

the sugar backbone of the peptidoglycan layer (Ghuysen and Shockman, 1973), creating spheroplasts which are osmotically sensitive (Kaback, 1974).

The periplasmic space was first proposed by Mitchell (1961) to describe a hypothetical compartment existing between the inner and outer membrane in gram-negative cells. Stock et al. (1977) have since confirmed that the periplasm exists as a separate compartment of the whole cell, contains 20-40% of the cell water, and that a Donnan equilibrium exists between the periplasmic space and the external medium. Contained within the periplasmic space are the previously described peptidoglycan layer as well as various enzymes (Beacham, 1979) and binding proteins (Oxender and Quay, 1976).

The cytoplasmic membrane is the most complex of the regions of the cell envelope. The membrane is composed of a phospholipid bilayer in which proteins are either embedded in the lipid bilayer as integral proteins, or loosely associated as peripheral proteins. Some of these proteins are responsible for the transport of solutes across the cytoplasmic membrane, represented in Figure 1 by the lactose permease and the maltose membrane-bound complex. This membrane is also the site of energy transduction in the cell. The respiratory chain is represented in the schematic in an indeterminate manner because bacterial respiratory chains are more variable than those found in mitochondria

(Harold, 1972). The constituents of the respiratory chain are produced in Escherichia coli only when the cells are grown aerobically and the components of the chain vary from organism to organism. Also present in the cytoplasmic membrane is the ATPase, which is capable of both synthesizing and hydrolyzing ATP; functions which are coupled to the flow of protons. The ATPase is composed of a peripheral protein complex (F_1) and an integral membrane protein complex (F_0). Reviews by Downie et al. (1979), and Futai and Kanazawa (1983), discuss the structure, function and genetics of the ATPase in greater detail than can be accommodated within the scope of this thesis.

Transport through the cytoplasmic membrane has been studied by using isolated membrane vesicles as a model system. Membrane vesicles are prepared by forming spheroplasts by the addition of EDTA and lysozyme to cells in 20% sucrose and then lysing the spheroplasts by transfer to a hypotonic medium (Kaback, 1971). This procedure leaves the cell devoid of cytoplasmic contents, the peptidoglycan layer, and with an outer membrane disrupted due to removal of lipopolysaccharide by EDTA chelation. A model system reduced to the elements still retained in the cytoplasmic membrane in this manner has a clear advantage in terms of simplification.

1.3. ENERGY COUPLING TO TRANSPORT IN GRAM-NEGATIVE BACTERIA

Energy coupling to transport has been intensively examined in the past twenty years and the driving force for many of the transported solutes has been elucidated. A brief historical overview of the literature shows steady progress in the understanding of the energetics of transport; from the early comprehensive reviews of Harold (1972); Simoni and Postma (1975); and Hamilton (1975); to the reviews by Kaback et al. (1977) and Konings (1977) that concentrate on energy coupling in membrane vesicles; and finally the more recent reviews by Konings et al. (1984) and Hellingwerf and Konings (1985) that summarize the current status of energization. A brief outline of these methods of energy coupling will aid in understanding the following chapters in this thesis.

Translocation of most solutes is mediated by the energy derived either from an energy-rich phosphate intermediate such as ATP or by the proton motive force. According to the chemiosmotic theory of Mitchell (1968), an electrochemical proton gradient is created across the cytoplasmic membrane by the extrusion of protons to the external medium by electrogenic proton pumps. This electrochemical gradient of protons exerts an inward force known as the proton motive force, and this proton motive force has two components; the pH gradient and the electrical potential. The driving force for translocation of solutes is provided by one or both components of the proton motive force (Hellingwerf and

Konings, 1985).

Extrusion of protons to generate the proton motive force in bacteria occurs primarily via electron transfer in the respiratory chain and via ATP hydrolysis by the cytoplasmic membrane ATPase. However, transport mechanisms have been linked in membrane vesicles of aerobically grown cells to the respiratory chain rather than ATPase activity (Kaback, 1974; Konings et al., 1981). Fermentative bacteria, which lack electron transfer systems, are capable of generating a proton motive force by efflux of an end-product of metabolism (lactate) in symport with a proton (Ten Brink and Konings, 1980; Ten Brink et al., 1985). Solute transport in the phototrophic bacteria Rhodopseudomonas spheroides has also been shown to depend on light-induced cyclic electron flow as well as the proton motive force (Hellingwerf et al., 1975; Elferink et al., 1983).

Energy coupling in the translocation of solutes in systems dependent on ATP or other high-energy phophorylated intermediates is not as well understood as the systems dependent on the electrochemical proton gradient. ATP dependent transport is confined to periplasmic binding protein mediated systems with only a few exceptions (Hellingwerf and Konings, 1985).

1.4. DEFINITIONS OF TRANSPORT

Defining some of the mechanisms by which solute is translocated across the membrane is necessary for clarifying

some of the distinctions made in the following chapters. A brief description of each mechanism, taken from Kaback (1974); and Harold (1972), follows:

Passive diffusion applies when a solute crosses the membrane without the expenditure of metabolic energy or any specific carrier being involved. The solute is not altered or modified during passage through the membrane and accumulation of substrate never exceeds equilibration.

Facilitated diffusion is distinguished from passive diffusion by the involvement of a specific carrier in the membrane. Accumulation of the solute takes place at a faster rate than simple diffusion and saturation kinetics and substrate stereospecificity can be observed. Like passive diffusion, no metabolic energy is expended in the process and accumulation against a concentration gradient does not take place.

Group translocation describes the reaction of an enzyme system oriented across the membrane. The solute is covalently modified during passage across the membrane and translocation is concurrent with the modification. The solute is accumulated against a concentration gradient and the energy expended in this translocation process is chemical. The best example of this mechanism of transport is the well defined phosphotransferase (PTS) system for carbohydrates in enteric bacteria (reviewed by Mitchell, 1985).

Active transport describes translocation systems which are dependent on metabolic energy for accumulation of solute against an electrochemical or osmotic gradient and require a specific membrane carrier. The solute is not altered in the translocation process and saturation kinetics are observed. Wilson (1978) divides the active transport systems in bacteria into two classes; the membrane bound transport systems, and the periplasmic binding protein mediated systems. A brief description of each class of active transport and examples of transport systems typical of the class are outlined below.

1.4.1. Binding protein mediated transport

This class of transport system is distinguished by dependence on ATP for translocation and the presence of a periplasmic protein which carries a specific substrate recognition site for the system. The binding protein mediated transport systems are sensitive to the osmotic shock procedure of Heppel (1967), which selectively removes the periplasmic contents of gram-negative bacteria, and are inactive in vesicles. The membrane carriers of the system have no recognition site for the substrate, which accounts for the lack of transport activity in membrane vesicles.

One of the best defined binding-protein systems is the maltose-transport system in Escherichia coli . A model of this transport system is represented in Figure 1. The system consists of five gene products, with one specific

pore-forming protein in outer membrane (lamB), a periplasmic binding protein (E), two cytoplasmic membrane proteins (F and G) which have no known function, and a peripheral membrane protein (K) which is associated with G (Boos, 1984). The maltose-binding protein interacts with both the outer membrane protein and the inner membrane proteins, and the peripheral K protein is apparently the site of ATP action in the transport system.

1.4.2. Membrane-bound transport systems

Membrane-bound transport systems are retained in membrane vesicles and utilize the proton motive force to drive active transport. The proteins involved in transport are fixed in the membrane and typically only a single carrier protein is involved in transport (Wilson, 1978).

Represented in Figure 1 as an example of a membrane bound transport system is the lactose permease, an inducible membrane protein that acts as a proton symport to translocate lactose and protons with a 1:1 stoichiometry (West and Mitchell, 1973). This system consists of a single integral membrane protein (the lac carrier protein) with a molecular weight of 45,000 and has been purified to homogeneity in a functional state (Newman et al., 1981). Matsushita et al.(1983) and Wilson et al.(1984) have separately reported reconstitution of the lactose carrier protein into proteoliposomes with retention of active

transport. A lactose recognition site and a proton-coupling site have been postulated for the carrier protein, but neither site has been verified.

Also classified by Wilson (1978) as a membrane-bound transport system is the dicarboxylic acid transport system of E. coli . The system transports succinate, fumarate and malate and has been documented in both whole cells and membrane vesicles (Lo et al., 1972; Rayman et al., 1972). However, the molecular mechanism of dicarboxylate transport (Lo, 1977) is more complex than that of the lactose permease. Transport is mediated by three proteins; two integral cytoplamic membrane proteins and one periplasmically associated binding protein. The membrane bound proteins presumably form a channel through the cytoplasmic membrane and each has a substrate recognition site. The proteins are arranged so that one recognition site is exposed on either side of the membrane. Specific binding of substrate to the periplasmic binding protein also takes place, but the exact function of this binding protein is not known.

1.5. PYRUVATE TRANSPORT IN ESCHERICHIA COLI

The history of pyruvate transport in Escherichia coli is remarkably brief. Uptake of pyruvate from the growth medium was examined by Kornberg and Smith (1967) in mutants lacking in phosphoenolpyruvate synthase activity. The pps mutant strains were mutagenized and recombined with another

strain. One of the recombinants (K2-1t-6) obtained was unable to remove pyruvate from the media at a normal rate compared to the parent strain, but was not inhibited in growth by the addition of pyruvate with another carbon source. This mutant (designated usp) was presumed to lack a system for the uptake of pyruvate. This usp strain was recombined again to restore phosphoenolpyruvate synthase activity. A resulting recombinant (K2-6) was able to grow on glucose and lactate, but still unable to grow on pyruvate. This result demonstrated that the usp trait was transferable and that this transport system was not concerned with the uptake of lactate. Uptake of oxaloacetate and α-oxoglutarate was also not affected in the usp mutant.

Uptake of pyruvate in membrane vesicles of Escherichia coli was demonstrated by Matin and Konings (1973). The artificial electron donor system ascorbate-phenazine methosulfate was required to energize transport in these vesicles and pyruvate was accumulated in the vesicles to seven times the external concentration. Pyruvate was also shown to inhibit uptake of D- and L-lactate in Escherichia coli , a result that suggests a common transport system for the monocarboxylic acids. This result contradicts the findings of Kornberg and Smith (1967).

Ogino et al. (1980) used proton correlation nuclear magnetic resonance to follow pyruvate transport in anaerobic

Escherichia coli cells. This technique was used to follow the decrease or increase of metabolites in the cell medium and was checked by lysis of the cells and identification of the accumulated metabolites. The results of these studies indicated that pyruvate is selectively taken up by anaerobic cells and accumulated against a concentration gradient. Uncouplers such as dinitrophenol completely inhibited the transport of pyruvate into the cells, indicating that the energy for uptake originated in the proton motive force. Efflux of pyruvate, mediated by facilitated diffusion, was also observed when the pH of the cell suspension was decreased.

Although these studies indicate the presence of a pyruvate transport system in Escherichia coli , many details such as the kinetics of transport, substrate specificity, and the molecular mechanism of translocation had not been determined. Hopefully, this thesis will answer some of the questions that remain in the area of pyruvate transport and will be used as a basis to elucidate the complete mechanism of transport.

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CHAPTER 2. THE TRANSPORT OF PYRUVATE IN WHOLE CELLS OF AN *ESCHERICHIA COLI* K12 MUTANT.

2.1. INTRODUCTION

The initial stage in the investigation of pyruvate uptake in *Escherichia coli* was to determine if pyruvate accumulation could be demonstrated in whole cells. Once uptake of pyruvate could be demonstrated in the whole cell system, characterization of the transport system could be pursued. A critical step in the characterization process would be establishing the energy dependence of the uptake of pyruvate in order to meet the criteria of active transport processes as outlined in the introductory chapter. The other criteria of active transport, accumulation of the solute unchanged against the concentration gradient, would also ideally be demonstrated, but elimination of all pyruvate metabolism was not possible with *E. coli* strains that were available from the *E. coli* Genetic Stock Center. Elimination of all pyruvate metabolism is theoretically possible by judicious crossing of available mutant strains, but this course of action would entail lengthy testing for the enzyme activities and because of the pivotal position of pyruvate in cell metabolism, success could not be guaranteed. In view of the fact that Matin and Konings (1973) had demonstrated uptake of pyruvate in *E. coli* membrane vesicles, we chose to minimize pyruvate metabolism

in whole cells and use Kaback vesicles, in which the cytoplasmic contents are eliminated, to verify and expand on the whole cell results. Towards this end, we obtained a mutant strain of Escherichia coli K12 which lacks two of the major enzymes in pyruvate metabolism, phosphoenolpyruvate synthetase and pyruvate dehydrogenase.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals

All common chemicals were obtained from commercial sources and were of reagent grade or better purity. [3-^{14}C] pyruvate, sodium salt, 15-20mCi/mmole, was obtained from New England Nuclear. N-ethylmaleimide, 2,4-dinitrophenol, iodoacetimide, carbonyl-m-chlorophenol hydrazone and p-hydroxymercuribenzoate were obtained from Sigma. D(-)lactate, 3-bromopyruvate, and pyruvic acid methyl ester were also received from Sigma. Precoated silica gel thin layer chromatography plates, Si 250 (20cm x 20cm), were obtained from J. T. Baker.

2.2.2. Bacterial strains

The bacterial strain chosen for the whole cell studies was a mutant of Escherichia coli K12, kindly provided by the E. coli Genetic Stock Center (E. C. #5688). This mutant, designated JRG 596 (Langley and Guest, 1977), has the genetic markers pps-1 , relA1 , thyA56 , metB1 , azi-14 , ton-54 , tsx97 and (aroP-aceF)73. A full explanation of

these genetic markers and the appropriate references are given in Bachmann (1983). JRG 596 is defective in two of the major pathways of pyruvate metabolism, pyruvate dehydrogenase and phosphoenolpyruvate sythetase. Pyruvate cannot be converted by the cell to acetyl-coenzyme A due to the deletion ((aroP-aceF)73) of the genes coding for two components of the pyruvate dehydrogenase complex; pyruvate dehydrogenase (E_{1p}) and dihydrolipoamide acetyltransferase (E_{2p}). The other metabolic route, conversion of pyruvate to phosphoenol pyruvate, is blocked by the defect in the phosphoenol pyruvate synthetase gene (pps-1).

2.2.3. Bacterial growth media and methods

The bacteria were routinely grown on M9 minimal salts medium (Miller, 1972), supplemented with magnesium sulfate, calcium chloride, thiamine, methionine, and thymine. Glycerol and acetate were added as the carbon sources. The minimal medium was routinely inoculated with JRG 596 grown on Luria Broth (LB) medium (Miller, 1972) supplemented with thiamine and thymine. The mutant strain was regularly checked for reversions by plating onto a series of nutritional marker plates. These marker plates consisted of M9 salts medium containing magnesium and calcium, solidified by 1% agar and containing all but one of the other required nutrients. Cell growth and concentration were determined with a Klett-Summerson colorimeter using Filter 66. The

Klett readings were correlated to optical density, wet weight and dry weight of the bacterial cells. (See Figure 2-1).

2.2.4. Harvest and preparation of JRG 596

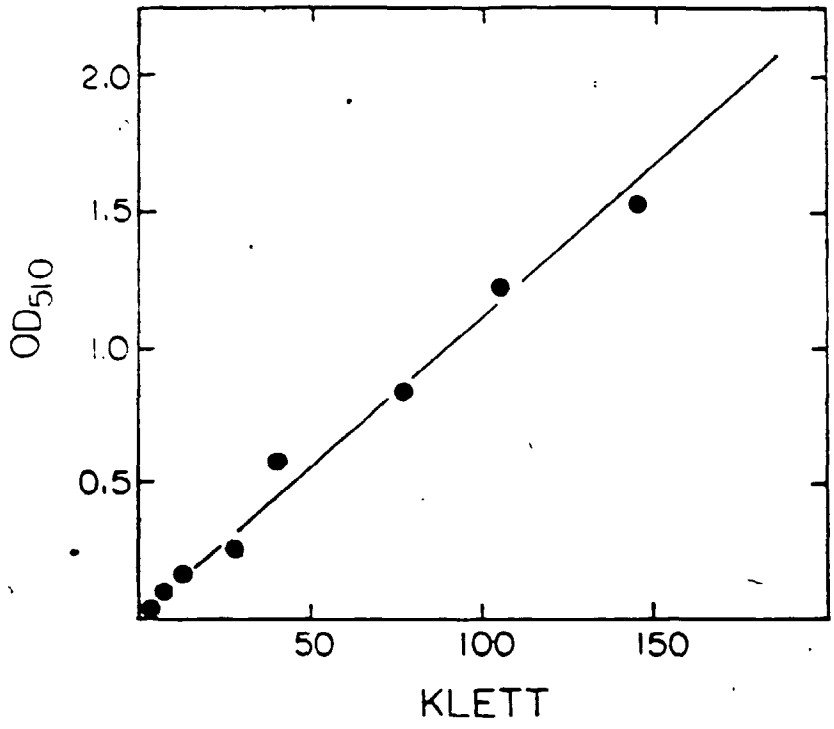
The bacteria were routinely harvested by centrifugation at approximately 10,000 x g for 10 minutes. The pelleted cells were then wasned twice by resuspension in ice-cold 50mM potassium phosphate buffer, pH 7.5, followed by centrifugation. These washed cells were then gently suspended in 50mM potassium phosphate buffer, pH 7.5, containing 0.1mM $MgCl_2$ and 0.1mM $CaCl_2$ and the cell concentration adjusted to 0.36mg dry weight of cells/ml.

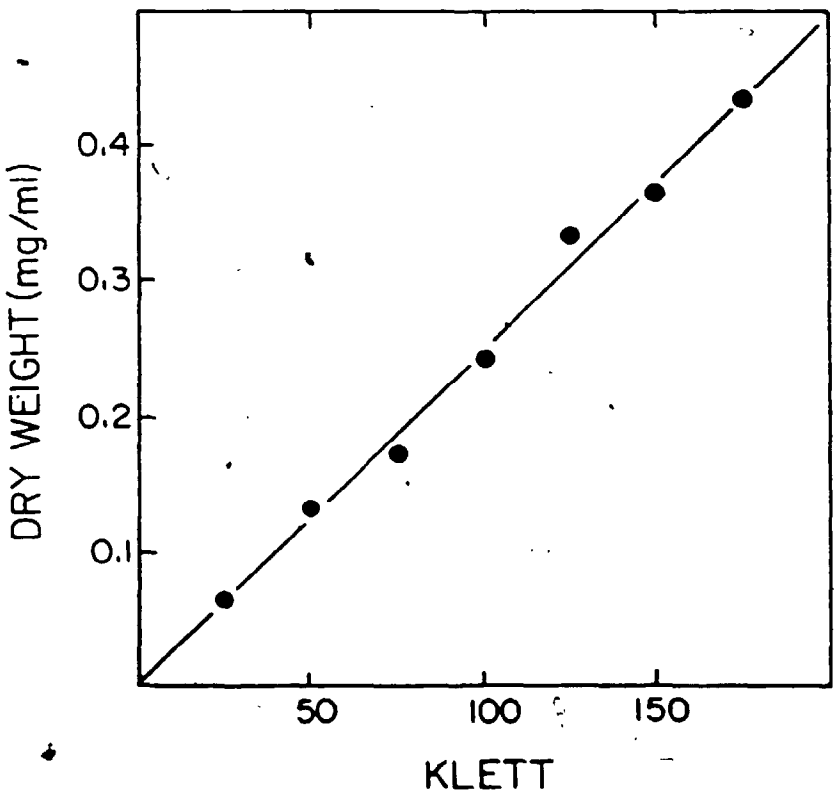
2.2.5. The transport assay procedure

In a typical pyruvate transport assay with strain JRG 596, a small volume of the washed cell suspension was added at 'zero' time, with mixing, to an equal volume of 40µM [3-^{14}C] sodium pyruvate in 50mM potassium phosphate buffer, pH 7.5. Reagents and cells were equilibrated to room temperature (23°C) prior to mixing and held at room temperature during the assays. At various time intervals, aliquots, typically 1.5 ml, of the assay mixture were removed and filtered immediately through 0.45µm Millipore filters held in place on a filtration manifold. The filters were immediately washed twice with 3.5 ml of 50mM phosphate buffer pH 7.5, containing 0.1mM $CaCl_2$ and 0.1mM $MgCl_2$. Each filter was then dissolved in 10 ml of

FIGURE 2-1. CORRELATION OF KLETT READINGS TO OPTICAL DENSITY AND DRY WEIGHT.

These graphs were used to relate Klett readings (from a Klett-Summerson colorimeter) to optical density and to dry weight when needed for calculations or comparative purposes. Klett flasks, which eliminated sampling of the bacteria, were used to generate standard growth curves. Optical density readings were taken at 510 nm on a Gilford 2400 spectrophotometer. Dry weight readings were taken by drying aliquots of bacteria in pre-weighed aluminum weighing boats.


2.0
1.5
1.0
0.5
OD_{510}
50
100
150
KLETT


0.4
0.3
0.2
0.1
DRY WEIGHT (mg/ml)
50
100
150
KLETT

ScintiVerse E (Fisher Scientific) scintillation fluid and the radioactive pyruvate within the bacteria counted by scintillation counting. The amount of pyruvate adhering to either the bacteria and/or the filter was measured by using heat killed cells and extrapolated to zero time. This amount was subtracted from the experimental values at each pyruvate concentration. In experiments in which the concentration was varied, such as the determination of the K_m of transport, the amount of [3-^{14}C] pyruvate was held constant at 300,000 dpm/ml of the assay medium. The initial rate of pyruvate uptake by whole cells was determined by taking samples after 30, 60, 90 and 120 seconds. Early time sampling was required due to the rapid uptake of pyruvate by the bacteria.

2.2.6. Temperature effects on efflux and uptake

The effect of temperature on efflux and uptake of pyruvate was determined by a variation of the basic assay procedure above. Uptake of pyruvate at temperatures ranging from 0 to 50°C was determined by equilibrating the uptake buffer to the temperature under study. The bacteria were then added and transport allowed to occur for ten minutes at that temperature. The cells were then filtered, washed and counted as described previously. For determination of efflux, the bacteria were preloaded with pyruvate by exposing the bacteria to the assay buffer for ten minutes at room temperature. Aliquots of 1.5 ml of the

preloaded bacteria were then centrifuged in an Eppendorf centrifuge and washed twice to remove external pyruvate. Washing and centrifugation were completed within 45 seconds. The bacterial pellet was then resuspended in 1.5 ml of 50mM potassium phosphate buffer, pH 7.5, containing calcium and magnesium, previously equilibrated to the temperature under study, and efflux was allowed to occur at that temperature for 10 minutes. The cells were then centrifuged and a 1.0 ml aliquot of the efflux fluid was counted and corrected to 1.5 ml total volume.

2.2.7. The effect of energy inhibitors and sulfhydryl reagents on pyruvate uptake

Various energy inhibitors and uncouplers were tested for their effect on uptake of pyruvate in JRG 596. The bacteria were preincubated with the inhibitor for 30 minutes at room temperature, then the uptake assay was conducted as described previously. The initial rate of uptake was determined by sampling at 30, 60, 90 and 120 seconds and the percent inhibition was calculated by comparison with an uninhibited control. The pyruvate concentration was held constant at 20μM, the K_m of transport.

Sulfhydryl reagents were preincubated with the bacteria for 30 minutes as in the case of the energy poisons, and the percent inhibitions calculated as described in the previous paragraph.

2.2.8. The effect of analogue inhibitors on pyruvate

uptake

In order to determine the specificity of the pyruvate transport system, various analogues were added simultaneously with the pyruvate to determine which were potential competitive inhibitors of the transport system. The initial rates of transport, in the presence of several different concentrations of the purported inhibitor, were determined, and compared to the initial rate of transport in the absence of the analogue. Percent inhibition values were then calculated from these values. Potential competitive inhibitors were further analyzed by determining the initial rates of uptake at varying pyruvate concentrations and a constant inhibitor concentration. Lineweaver-Burk (1934) plots were graphed using these data.

2.2.9. Thin layer chromatography of the cell contents

The bacteria were loaded with pyruvate according to the basic transport assay and at time intervals the cells were filtered and washed as described in the Methods section. The cytoplasmic contents of the filtered bacteria were then extracted with 0.1 ml of chloroform and 0.2 ml of methanol followed by 2.0 ml of distilled water according to the method outlined by Matin and Konings (1973). This extract was centrifuged at 2,000 x g to remove the chloroform layer and the upper aqueous layer was lyophilized overnight. The lyophilized cytoplasmic extract was redissolved in less than 100 µl of distilled water and spotted on precoated silica

gel thin layer plates. A chromatography tank was prepared previous to spotting the plates according to the procedure of Per Nygaard (1967). Isoamyl alcohol and 5M formic acid (2:1) were thoroughly mixed in a separatory funnel and after separation, the lower acid layer was used to saturate Whatmann 3MM filter paper. This filter paper was then positioned in the chromatography tank in such a way that it was kept clear of the formate-saturated alcohol which was used as the running solvent in the bottom of the tank. The spotted and dried plate was then run in the isoamyl alcohol until the solvent reached appproximately three-quarters of the way up the plate. The plate was then air-dried overnight and the radioactive spots detected by autoradiography on Kodak X-ray film for 5-7 days.

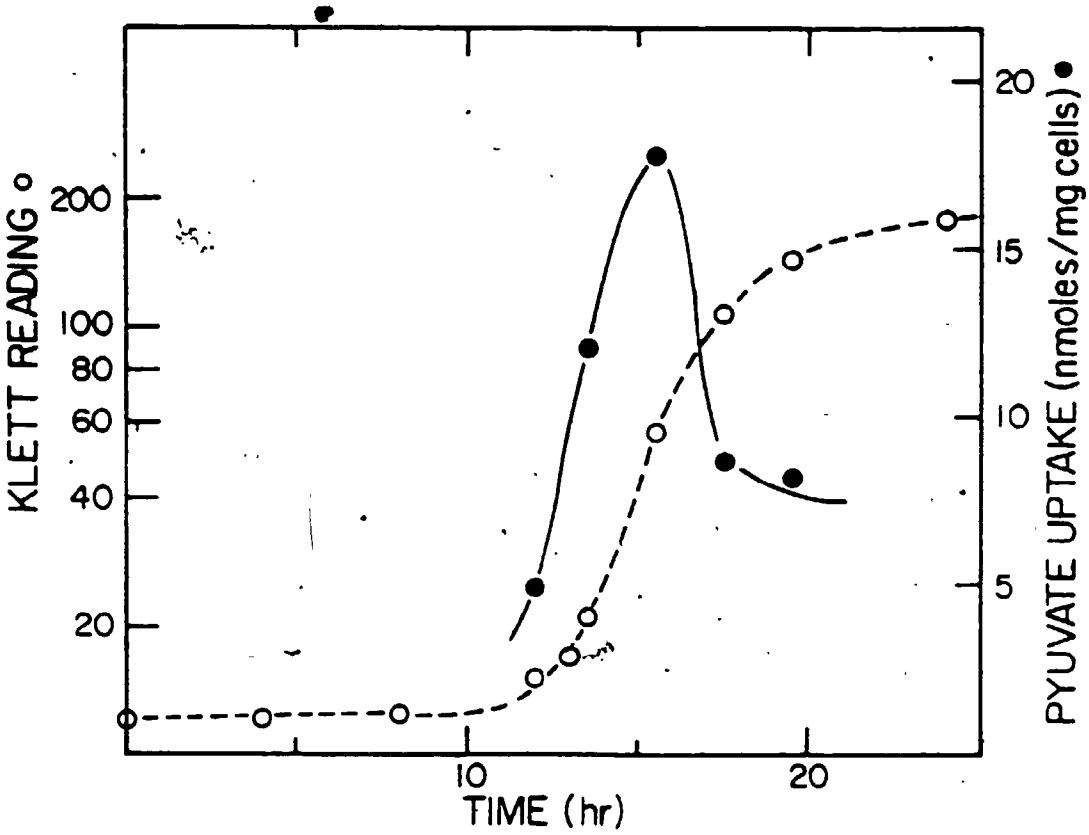
2.3. RESULTS

2.3.1. Characteristics of the uptake of pyruvate in JRG 596

A typical growth curve for JRG 596, the pyruvate metabolism mutant, is shown in Figure 2-2. The cells were grown at 37°C on supplemented M9 as described previously, reaching mid-log phase of growth at approximately 15 hours using a 1% inoculum. The pyruvate uptake activity is also represented on this graph and it is very clear that uptake of pyruvate peaks sharply during the exponential phase of growth. In view of this result, the bacteria were routinely harvested at mid-log phase of growth

FIGURE 2-2. GROWTH CURVE AND PEAK OF TRANSPORT ACTIVITY.

The relationship between cell growth and uptake of pyruvate in JRG 596 was used to optimize transport activity. The open circles indicate the extent of growth at 37°C in minimal M9 medium with acetate and glycerol as carbon sources. The pyruvate uptake was determined from the total uptake of pyruvate in 10 minutes at a concentration of pyruvate of 50μM in the uptake assay. The uptake of pyruvate is expressed in terms of nmoles per milligram dry weight of cells. Complete details of the growth medium and pyruvate transport assay are presented in the Materials and Methods section.


KLETT READING o
200
100
80
60
40
20
10
20
TIME (hr)
20
15
10
5
PYUVATE UPTAKE (nmoles/mg cells) ●

in order to optimize uptake activity.

The time dependence of pyruvate transport is presented in Figure 2-3. Pyruvate is accumulated linearly for about two minutes, then approaches steady state concentrations at approximately 10 minutes. The lower curve represents the results of the pretreatment of the cells with the uncoupler CCCP. There is a significant reduction in uptake activity. A similar reduction in uptake activity can be achieved with heat-killed cells.

2.3.2. The effect of temperature on pyruvate uptake and efflux

The effect of temperature on uptake and efflux of pyruvate is shown in Figure 2-4. Efflux and uptake were determined at steady state (10 minutes) conditions as described in Methods. Uptake of pyruvate increases sharply from 0 to 10°C, then plateaus at a constant high level of uptake until about 25°C, where uptake activity falls off to approach zero at approximately 50°. The level of efflux is stable from 0 to 25°C, at which point efflux increases to peak at 40°C and gradually decreases again.

2.3.3. The Michaelis constant of pyruvate transport

The K_m of pyruvate transport was determined by obtaining the initial rate of transport, as described in Methods, at varying pyruvate concentrations. Two different stages of cell growth were chosen, mid-log and late-log, to determine whether or not other pyruvate transport systems

FIGURE 2-3. THE TIME COURSE OF PYRUVATE UPTAKE BY STRAIN JRG 596 GROWN TO MID-LOG PHASE.

Pyruvate uptake has been determined at a final pyruvate concentration of 50μM in the uptake assay. The solid circles indicate the pyruvate uptake in untreated cells. The open circles indicate the pyruvate uptake by cells preincubated at 23°C for 30 min. in the presence of 1μM CCCP. Pyruvate uptake is expressed in terms of nmoles of pyruvate taken up per mg dry weight of cells.

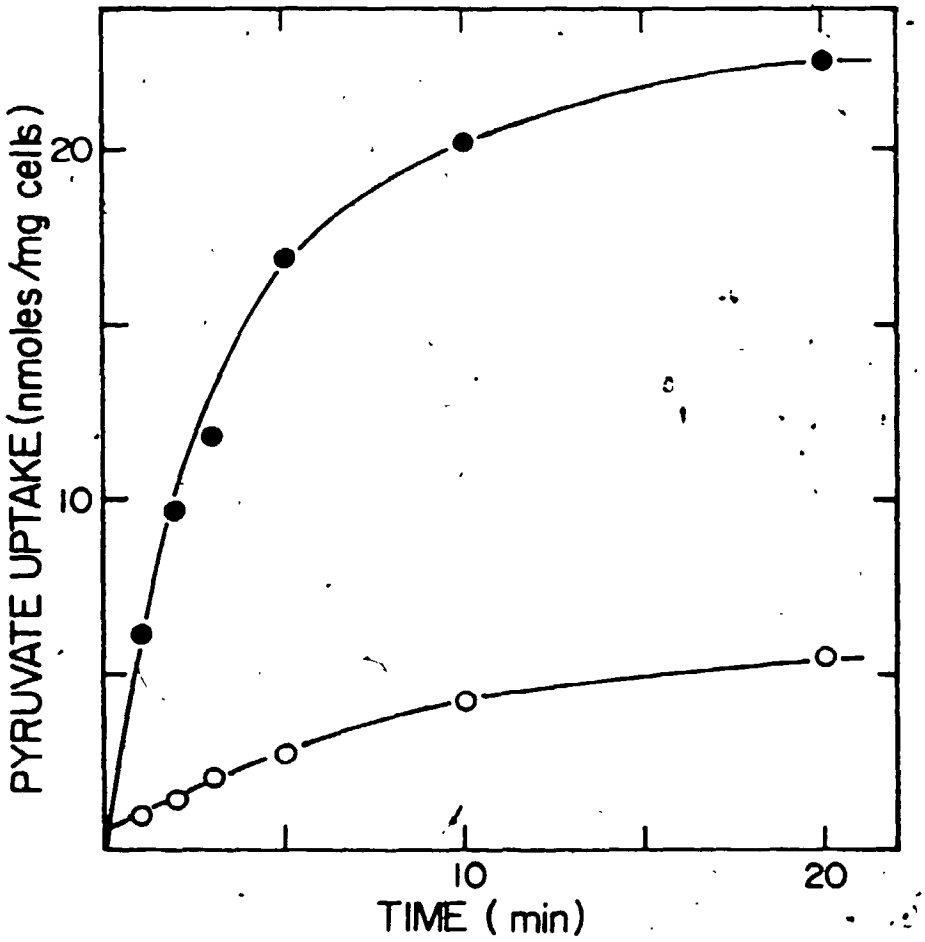
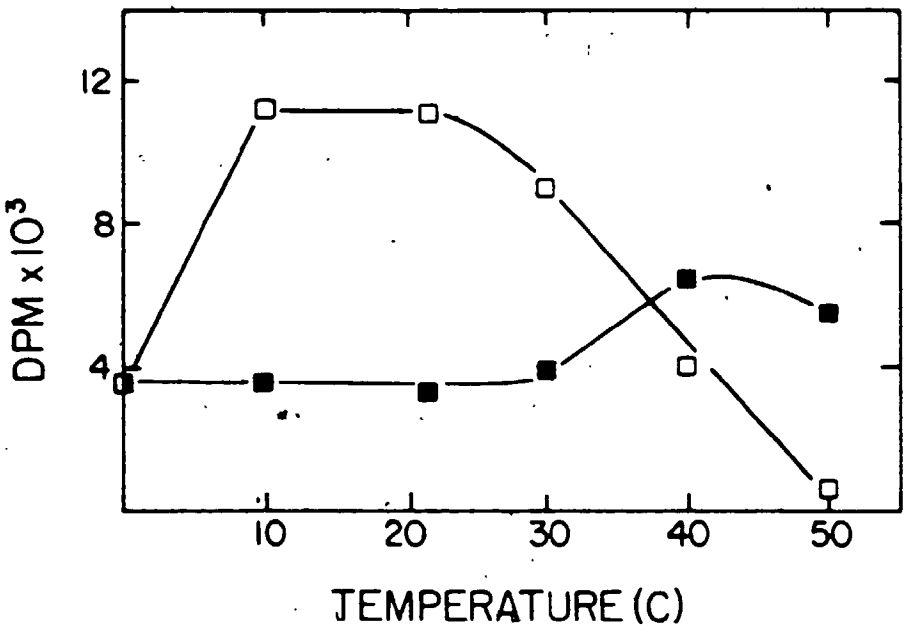

PYRUVATE UPTAKE (nmoles/mg cells)
20
10
10
20
TIME (min)

FIGURE 2-4. THE EFFECT OF TEMPERATURE ON EFFLUX AND UPTAKE OF PYRUVATE.

The open squares denote uptake of pyruvate at various temperatures. The closed squares indicate efflux of pyruvate from preloaded cells over the same temperature range. Complete details of the temperature incubations and the uptake and efflux assays are given in the Materials and Methods section.


12
8
4
DPM x 10³
10
20
30
40
50
TEMPERATURE (C)

exist at various stages of cell growth. It is evident from the results presented in Figure 2-5 that the K_m of transport extrapolates to the same value at both stages of growth; 18-19μM. Both lines were generated by linear regression analysis.

2.3.4. The effect of energy poisons on pyruvate uptake

A variety of energy inhibitors and uncouplers were tested to determine if the transport of pyruvate in E. coli is an energy requiring process. Table 2-1 lists the energy inhibitors which were examined. Potassium cyanide and sodium azide are both fairly effective as inhibitors, but the uncoupling agents 2,4-dinitrophenol and CCCP are both extremely effective at blocking pyruvate uptake. Sodium arsenate shows no inhibitory effect on pyruvate transport in cells starved of ATP by dinitrophenol exposure.

2.3.5. The effect of sulfhydryl reagents on pyruvate transport

The data presented in Table 2-2 outlines the effect of sulfhydryl reagents on pyruvate uptake in E. coli strain JRG 596. All the sulfhydryl reagents had differing levels of inhibition on pyruvate uptake when the cells were preincubated with the reagent for 30 minutes prior to the trial. Of the three reagents studied p-hydroxymercuribenzoate was the most effective inhibitor with appproximately 60% inhibition at 1mM final concentration and N-ethylmaleimide the least effective

FIGURE 2-5. THE MICHAELIS CONSTANT OF PYRUVATE TRANSPORT AT TWO DIFFERENT STAGES OF CELL GROWTH.

Determination of the K_m for the pyruvate uptake in strain JRG 596 grown to mid-log phase (triangles) and late-log phase (circles). The open and closed symbols denote data obtained from two separate experiments. The lines have been determined from linear regression analysis of the data. The velocity represents the initial rate of pyruvate uptake expressed in nmoles per min per mg dry weight of cells. The K_m was determined to be 18-19μM at both stages of cell growth and the V_{max} for the mid-log cells was 10-14nmol per mg dry weight of cells per minute. Specific acitivity of the labelled pyruvate used was 19.8mCi/mmol.

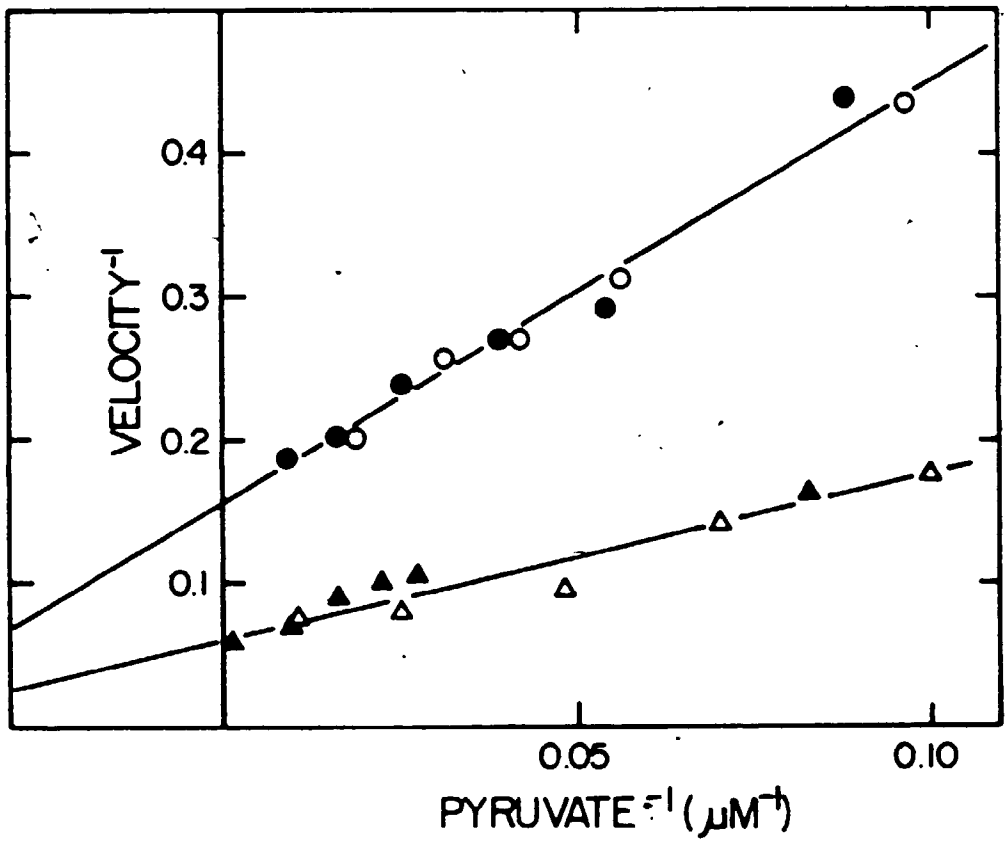

0.4
0.3
0.2
0.1
VELOCITY^{-1}
0.05
0.10
PYRUVATE^{-1} (μM^{-1})

Table 2-1. The Effect of Various Energy Poisons on Pyruvate Uptake in Strain JRG 596.

Inhibitor	Concentration	%Inhibition
Sodium azide	1mM	60
Potassium cyanide	1mM	52
Sodium arsenate[a]	0.2mM	0
2,4-Dinitrophenol	0.8mM	95
CCCP	1µM	75
	10µM	95

Cells were grown to mid-log phase, washed and resuspended in 50mM potassium phosphate buffer, pH 7.5 as described in Methods. The cells were preincubated with the inhibitor for 30 min at room temperature prior to the determination of the initial rate of pyruvate uptake.
The % inhibition was determined by comparing the initial rate of pyruvate uptake in control and inhibited cells at a pyruvate concentration approximating the K_m value of 20µM.

[a]The effect of arsenate was tested in starved cells according to the method outlined by Berger and Heppel (1974). Bacteria were grown normally and then exposed to dinitrophenol for 8 hours. The starved cells were washed extensively, suspended in buffer, and exposed to arsenate for 5 min. An electron donor system, 20mM ascorbate and 200µM phenazine methosulfate, was added and incubated for another 5 min. Transport was initiated by the addition of labelled pyruvate, and samples were taken at 20, 40 and 60s. Transport was dependent on the presence of the electron donor system and percent inhibition was determined by comparison with an uninhibited control.

Table 2-2. The Effect of Various Sulfhydryl Reagents on Pyruvate Uptake in Strain JRG 596.

Inhibitor	Concentration	%Inhibition
N-Ethylmaleimide	1mM	18
Iodoacetamide	1mM	38
p-Hydroxymercuribenzoate	1mM	58

Cells were grown to mid-log phase, washed and resuspended in 50mM potassium phosphate buffer, pH 7.5 as described in Methods. The cells were preincubated with the inhibitor for 30 min. at room temperature prior to the determination of the initial rate of pyruvate uptake.
The % inhibition was determined by comparing the initial rate of pyruvate uptake in control and inhibited cells at a pyruvate concentration approximating the K_m value of 20μM.

inhibitor with 18% inhibition at the same concentration. It is apparent from these results that a sulfhydryl group is involved somewhere in the transport process. The varying response of the transport system to the sulfhydryl reagents may be due to the ability of the reagent to penetrate the cells to the site of action.

2.3.6. The effect of substrate analogues on pyruvate transport

In order to establish the specificity of the pyruvate transport system, substrate analogues were added simultaneously with the pyruvate in the standard uptake assay. The initial rate of pyruvate uptake in the presence of the analogue was compared to the initial rate of transport in the absence of the inhibitor. The percent inhibition for several different concentrations of analogue was calculated from the initial rate data, and the results are presented in Table 2-3. An increase in the percent inhibition with increasing concentration was interpreted by us to be indicative of a potential competitive inhibitor. Only four of the tested substrates were found to display increasing inhibition with increasing concentration. The synthetic analogues 3-bromopyruvate, pyruvic acid methyl ester and the natural analogues lactate and alanine display this proportionate response. The other analogous substrates listed in Table 2-3 displayed no such increase in inhibition, denoting non-specific inhibition at work.

Table 2-3 The Effect of Various Pyruvate Analogues on Pyruvate Transport in JRG 596.

Inhibitor	Concentration (mM)	%Inhibition
3-Bromopyruvate	0.025	34
	0.050	65
	0.100	85
PAME	0.050	57
	0.100	62
	1.0	94
L-Lactate	1.1	12
	2.1	16
Alanine	0.56	9
	1.1	13
	2.1	27
Acetate	0.56	
	1.1	20.6±5.5
	2.1	
Glycine	1.1	
	2.1	36.5±1.5
Succinate	0.56	
	1.1	30.0±2.6
	2.1	
Malate	0.56	
	1.1	38.0±4.6
	2.1	

Cells grown to mid-log phase in minimal medium were washed and resuspended in 50mM potassium phosphate buffer, pH 7.5. The cells were then added to a mixture of pyruvate and inhibitor at zero time. The % inhibition was determined by comparing the initial rate of pyruvate uptake in control and inhibited cells at a pyruvate concentration approximating the K_m value of 20μM.

2.3.7. Kinetic study of the natural analogues L-lactate and alanine

All potential substrate inhibitors were subjected to kinetic analysis in order to determine the type of inhibitory behavior and an inhibitor constant. The data for the plots was obtained as described in the Methods section, and plotted by the method of Lineweaver and Burk (1934). Although several different concentrations of lactate and alanine were used to determine the inhibitor constant, only one of the lines is represented in Figure 2-6. All the lines plotted were the result of linear regression analysis of the data and intersect the vertical axis at the same point, indicating competitive inhibition. The inhibitor constant (K_i) calculated for alanine was 8.0mM and for lactate was 7.5mM. Considering the K_m of the pyruvate transport system had previously been determined to be 20μM, the millimolar values of the natural analogue inhibitor constants can only be interpreted as being beyond the range of physiologically important substrate inhibition.

2.3.8. Kinetic study of the synthetic analogues 3-bromopyruvate and pyruvic acid methyl ester

Similarly to lactate and alanine, the kinetic studies on the synthetic analogues 3-bromopyruvate and PAME were plotted by the Lineweaver-Burk double-reciprocal method. Figure 2-7 depicts these results. 3-bromopyruvate intersects the vertical axis and the control line at both

FIGURE 2-6. INHIBITION CURVES FOR THE NATURAL PYRUVATE ANALOGUES L-LACTATE AND ALANINE.

Competitive inhibition of pyruvate uptake in strain JRG 596 by the monocarboxylic acids lactate and alanine. The inhibitor was added simultaneously with the pyruvate to the reaction mixture and the initial rates of pyruvate uptake were determined as mentioned in Methods. The velocity is expressed in nmoles of pyruvate taken up per min per milligram dry weight of cells. (●) control, (○) in the presence of 1mM alanine, (▲) in the presence of 1mM L-lactate.

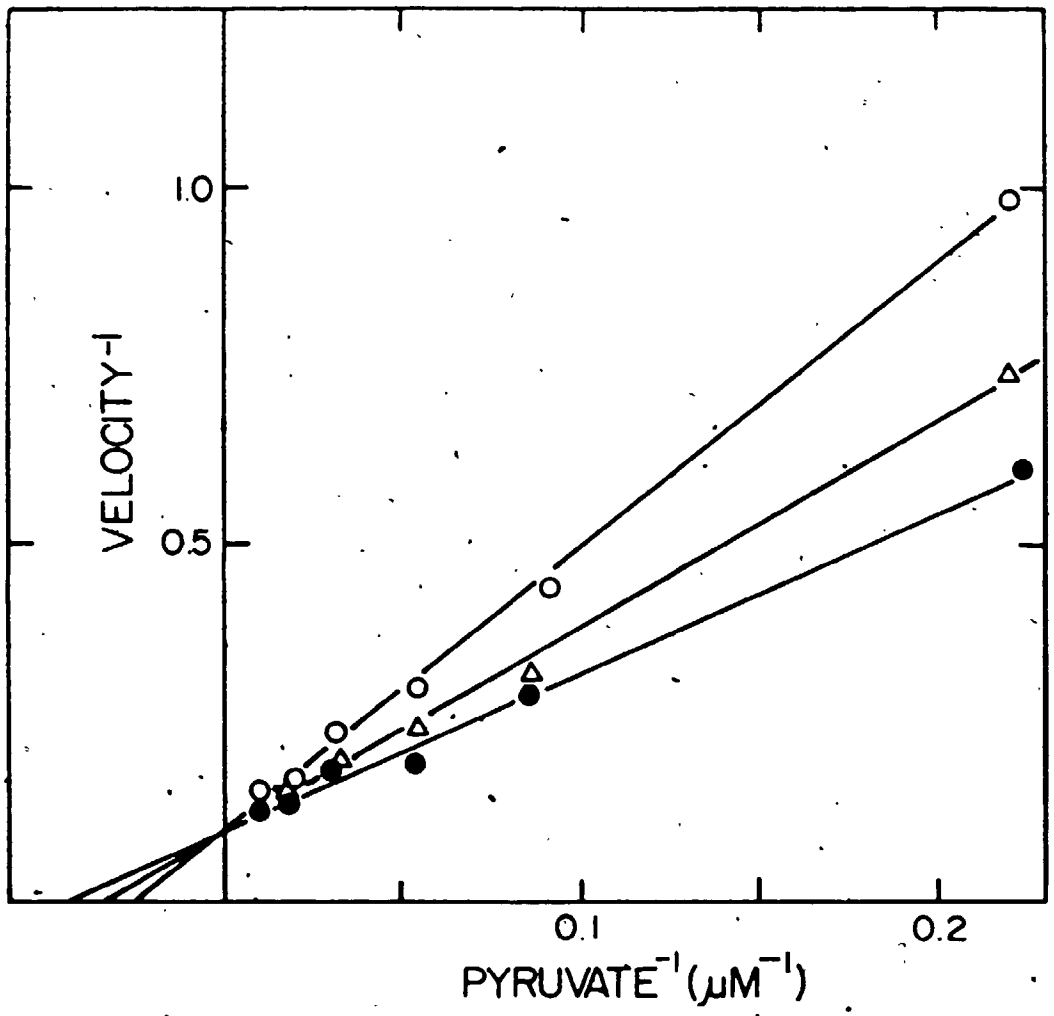
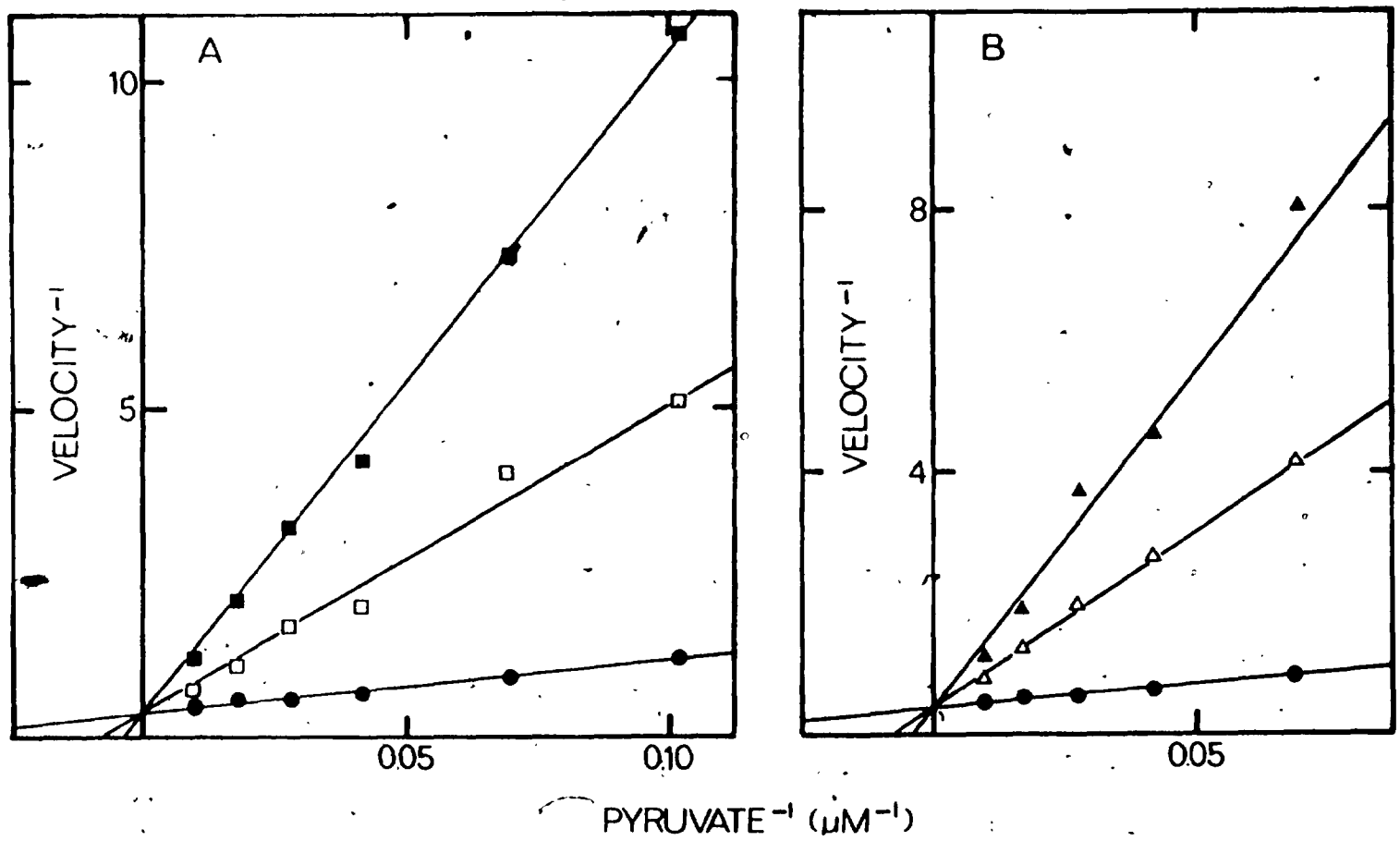

1.0
0.5
VELOCITY-1
0.1
0.2
PYRUVATE^{-1} (μM^{-1})

FIGURE 2-7. INHIBITION CURVES FOR THE SYNTHETIC PYRUVATE ANALOGUES 3-BROMOPYRUVATE AND PYRUVIC ACID METHYL ESTER.

Competitive inhibition of pyruvate uptake in strain JRG 596 by the synthetic analogs 3-bromopyruvate and pyruvic acid methyl ester. The inhibitor was added simultaneously with the pyruvate to the reaction mixture and the initial rates of pyruvate uptake were determined as mentioned in Methods. The velocity is expressed in nmoles of pyruvate taken up per min per mg dry weight of cells.

(A) The effect of 3-bromopyruvate. (●) control, (□) in the presence of 25μM 3-bromopyruvate; (■) in the presence of 50μM 3-bromopyruvate;

(B) The effect of pyruvic acid methyl ester. (●) control; (△) in the presence of 50μM PAME, (▲) in the presence of 200μM PAME.


A
B
10
5
8
4
VELOCITY⁻¹
VELOCITY⁻¹
0.05
0.10
0.05
PYRUVATE⁻¹ (μM⁻¹)

concentrations of inhibitor tested. PAME also displays this behaviour, which is typical of competitive inhibition. The calculated inhibitor constants are 25μM for 3-bromopyruvate and 100μM for PAME. In this case, in direct contrast to the natural analogue results, the synthetic analogues are very good competitive inhibitors of pyruvate transport.

2.3.9. The fate of pyruvate in the cytoplasm of JRG 596

Escherichia coli cells were loaded with pyruvate for various time periods and the cytoplasmic contents extracted as outlined in Methods. The cytoplasmic contents were separated by thin layer chromatography and the position of the resulting radioactive spots compared, by means of autoradiography, to a pyruvate standard. At early time intervals, less than two minutes, pyruvate could be discerned to be the principal radioactive substance extracted from the cytoplasm (results not shown). At ten minutes, several other unidentified bands were easily discernable, but these same bands were at best very faint at two minutes. We interpreted these results to mean that metabolism of the pyruvate did take place, but at the early time intervals during which the kinetic results were obtained, metabolism of pyruvate could not adversely affect the results.

2.4. DISCUSSION

It is clear from the results presented in this chapter that there is ample evidence to support the conclusion that

a unique active transport system serves to take up pyruvate in Escherichia coli K12. Uptake of pyruvate does occur in whole cells as demonstrated in Figure 2-3. This uptake is largely abolished when the cells are heat-killed or treated with an energy inhibitor such as CCCP, indicating that the cells must be viable or capable of producing metabolic energy in order to support transport. Uptake activity peaks during exponential growth of the bacteria, when most metabolic functions are at their highest levels and the requirement for nutrients is also high.

Kinetic studies of the pyruvate transport system (Figure 2-5) revealed that at two different stages of cell growth, only one K_m value of 20μM could be determined. Both of the double reciprocal lines were straight and unbroken, indicating that there was no other system with a different affinity for pyruvate contributing to the transport activity. Although genetic studies have not been carried out to pinpoint the number of pyruvate transport systems operating in E. coli , the kinetic evidence favours a single system.

Studies were carried out with various energy inhibitors in order to determine the energy requirements of the transport system and, if possible, the source of this energy. Table 2-1 lists the inhibitors tried and their effect on uptake. Azide, cyanide, 2,4-dinitrophenol and CCCP are all potent inhibitors of pyruvate transport. The

best of the inhibitors are the uncouplers dinitrophenol and CCCP. The uncouplers act by collapsing the proton gradient and interfering with phosphorylation without impeding electron flow (Boos, 1974). Azide, which inhibits ATPase activity in E. coli (Roisin and Kepes, 1973), possibly by uncoupling the proton motive force from the ATPase, is also an effective inhibitor. Potassium cyanide prevents the reduction of oxygen in the terminal step of electron transport and also inhibits transport of pyruvate. The only inhibitor incapable of impeding the uptake of pyruvate in JRG 596 was found to be sodium arsenate. Arsenate had no effect on the rate of transport in cells starved of endogenous ATP by dinitrophenol exposure. Arsenate inhibits phosphorylation reactions by substituting for inorganic phosphate, usually yielding unstable low energy arsenate compounds. The conclusion which must be drawn from these results is that pyruvate transport is dependent upon the generation of metabolic energy, most probably generation of the proton or pH gradient, since disruption of the gradient by uncouplers has the most deleterious effect on uptake. ATP does not appear to be obligatory for transport since sodium arsenate does not inhibit uptake and has been shown by Berger and Heppel (1974) to eliminate the production of ATP in starved E. coli. Since the whole cells still contain an intact Mg^{2+}-Ca^{2+}-ATPase however, ATP involvement in the transport process cannot be entirely ruled out until

ATPase

deficient mutants (Prezioso et al., 1973) are used to eliminate this possibility.

The action of uncouplers also does not appear to be confined solely to affecting proton conduction. Kaback et al. (1974) has shown that CCCP is capable of acting simply as a sulfhydryl reagent, and inhibition of D-lactate driven transport can be reversed by the addition of sulfhydryl compounds. 2,4-dinitrophenol was shown by Smith and Montie (1975) to affect the transition temperature of bacterial membranes and inhibition of transport does not take place below that temperature. This information can only confuse the obvious conclusion that pyruvate transport is dependent on the generation of the proton motive force hypothesized by Mitchell (1963) as outlined in the first chapter. More rigorous examination of the transport system with Mg^{2+}-Ca^{2+}-ATPase mutants and other energy inhibitors will be necessary to definitely pinpoint the source of the energy upon which pyruvate uptake is dependent.

Sulfhydryl reagents, which inhibit transport of pyruvate to various extents (Table 2-2), would indicate that a sulfhydryl group is involved somehow in the uptake process. This information may be useful in characterizing the components of the transport system at some later date. Kaback and Barnes (1971) had proposed a transport model which utilized the oxidation and reduction of a sulfhydryl group containing carrier protein as the method of

translocation, but this model has since been displaced by the author by one favouring chemiosmosis (Kaback, 1974).

The pyruvate uptake system also appears to be specific for pyruvate. The kinetic results of the analogue studies demonstrated quite clearly that neither of the natural analogues, lactate or alanine, was a candidate for a substrate competitor of uptake. Only the synthetic analogues, PAME and bromopyruvate, had inhibitor constants in the range of a potent competitive inhibitor, clearly demonstrating that the transport system has a narrow specificity range which recognizes small modifications in the pyruvate molecule. Neither of these artificial analogues should have been metabolized to any extent within the cell, and this fact, coupled with the short time interval during which the kinetics were studied, leads to the conclusion that the competitive inhibition takes place at the level of a transport element rather than at the cytoplasmic enzyme level. According to the results from the thin layer chromatography of the cytoplasmic contents, pyruvate was not subject to significant metabolism during the first two minutes of uptake, so competitive inhibition of the cytoplasmic enzymes by the synthetic analogues does not explain competitive inhibition results obtained within that time period.

Ideally, transport of a substrate should take place in the absence of metabolism in order to demonstrate true

active transport. According to the detailed analysis of the effect of metabolism on the kinetics of transport by Christensen (1975), metabolism of substrate should not affect the kinetic results, providing the initial rates of transport are taken at early time intervals. However, without the absence of metabolism, concentration of the substrate against the gradient is subject to question. Calculating the concentration of pyruvate within the whole cells at steady state (10 minutes), using an internal volume of 2.7 µl per milligram dry weight (Winkler and Wilson, 1966), results in a value of 8.1mM. Compared to the external concentration of 50µM, it would initially appear that pyruvate is concentrated over 150 times against the gradient. However, according to the thin layer chromatography of the cell contents at ten minutes, pyruvate had been partially metabolized to several unknown compounds. The amount of unmetabolized pyruvate could have been estimated by scraping the labelled bands and counting, but the variable efficiency of both the extraction and separation procedures would still have led to inaccuracy in the calculation of the amount of unmetabolized pyruvate in the cells. Instead of relying on the whole cell system, with enzyme systems still capable of metabolizing pyruvate to demonstrate the accumulation of pyruvate against the concentration gradient, we decided to repeat and confirm the whole cell transport studies using membrane vesicles. The

membrane vesicle results are presented in the following chapter.

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CHAPTER 3. THE TRANSPORT OF PYRUVATE IN MEMBRANE VESICLES OF ESCHERICHIA COLI K12.

3.1. INTRODUCTION

The previous chapter presented the results from pyruvate uptake studies using a mutant strain of E. coli K12 defective in pyruvate metabolism. With this mutant we were able to demonstrate that pyruvate is taken up by an energy dependent process. No kinetic evidence of more than one uptake system could be presented and all the analogue inhibitor studies indicated that the transport system was specific for pyruvate alone. Since catabolism of pyruvate was not totally eliminated in the mutant strain of E. coli , one of the criteria of active transport, accumulation of substrate against the concentration gradient, could not be substantiated to our satisfaction. Matin and Konings (1973) had demonstrated that uptake of pyruvate in membrane vesicles of E. coli K12 did occur, but did not go on to characterize this pyruvate uptake system. Membrane vesicles are a system devoid of the cytoplasmic contents and consequently the soluble enzymes. Catabolism of pyruvate would therefore be greatly reduced, if not eliminated, by using such a system for the demonstration of active transport of pyruvate. We decided to use membrane vesicles to repeat and enlarge on the information already obtained from the whole cell studies. This chapter will describe

these studies.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals

All common chemicals, such as the cation sources, were obtained from commercial sources and were of reagent grade or better purity. [3-^{14}C] pyruvate, sodium salt, 15-20mCi/mmole, was obtained from New England Nuclear. The isotope was routinely checked by scintillation counting for concentration corrections after dilution. The quantity of pyruvate delivered varied from the stated weight from vial to vial and a correction using the specific activity was necessary to preserve accuracy. The purity of the pyruvate was periodically checked by thin layer chromatography. Phenazine methosulfate was obtained from Sigma and was diluted just prior to use. The diluted PMS was kept in the dark whenever possible. Ascorbic acid was also diluted and the pH adjusted to 6.6 immediately before the assays were started. Other energy sources, such as NADH and D(-)lactate were also obtained from Sigma. The electron transport inhibitors, such as CCCP and 2,4-dinitrophenol, the sulfhydryl reagents such as N-ethylmaleimide and the pH buffers were also received from Sigma. Precoated silica gel thin layer chromatography plates, Si 250 (20cm x 20cm), were obtained from J. T. Baker.

3.2.2. Bacterial strain and growth media

The bacterial strain chosen for the preparation of the

membrane vesicles was a wild type E. coli K12, designated Her H. The bacteria were routinely grown in 12 liter batches in New Brunswick fermentors on Luria Broth (Miller, 1972). This enriched broth was inoculated to 1% with an E. coli K12 culture also grown on LB medium to mid-log phase of growth. The fermentor cultures were grown to mid-log stage of growth (approximately three hours) and harvested by means of a Pellicon filtration unit (Millipore).

3.2.3. Preparation of the membrane vesicles

The membrane vesicles were prepared by the method of Kaback and Stadtman (1966). These Kaback vesicles were prepared by forming spheroplasts by resuspending washed bacterial cells in a lysozyme-EDTA buffer, followed by lysis of the spheroplasts in hypotonic buffer containing DNase and RNase. The resultant vesicles were extensively washed by differential centrifugation and resuspended in 0.1M potassium phosphate buffer, pH 6.6. The protein concentration was determined by the method of Lowry et al. (1951) and the protein concentration of the vesicles adjusted to 5-6 mg per ml. The vesicles were then divided into small aliquots and stored at -70°C. Aliquots of the vesicles were thawed at room temperature immediately prior to use.

3.2.4. Standard assay for pyruvate uptake in membrane vesicles

The standard assay for pyruvate uptake in membrane

vesicles was adapted from the methods of Rayman et al. (1972) and Matin and Konings (1973). Aliquots of the membrane preparation, containing 0.1 to 0.2 mg of protein, were diluted in 50mM potassium phosphate buffer, pH 6.6, and supplemented with magnesium sulfate to 50mM. The buffered membrane vesicles were then preincubated for fifteen minutes at room temperature (23°C). After fifteen minutes, an artificial electron donor system, 20mM sodium ascorbate, pH 6.6 and 200uM phenazine methosulfate was added. [3-^{14}C]-sodium pyruvate was added to initiate the reaction at a concentration of 20uM. Total volume of the reaction mixture was 0.1 ml. At time intervals, the reaction was stopped by dilution by the addition of 3.5 ml of 0.1M LiCl and filtered through 0.45um Nucleopore filters on a ten place filtration manifold. The filtered vesicles were washed twice by 3.5 ml of 0.1M LiCl. Filtration and washing were completed within 30 seconds. The filters were then dissolved in 10 ml of Scintiverse E scintillation fluid (Fisher) and the radioactive pyruvate contained within the vesicles was measured by scintillation counting. Each sample was corrected by a 'zero' time control obtained by diluting the reaction mixture with LiCl prior to the addition of the labelled pyruvate and filtering and counting as above.

3.2.5. The pH profile of pyruvate uptake in vesicles

The pH profile of the uptake of pyruvate in membrane

vesicles was determined by using buffers with effective pH ranges which fell within the range of interest. The buffers of choice and their pH ranges are as follows: MES, 2-[N-morpholino]ethanesulfonate; pH 5.8-6.5, BES, (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonate); pH 6.6-7.6, Tricine, (N-tris[hydroxymethyl]methyl glycine); pH 7.6-8.8. The standard transport assay as outlined above was followed using these buffers at the appropriately adjusted pH instead of the standard phosphate buffer. Uptake was allowed to proceed for ten minutes and then duplicate samples were filtered, washed and counted.

3.2.6. The effect of various energy sources and cations on pyruvate transport in membrane vesicles

Various energy sources were used in the standard uptake assay in an effort to determine the energy dependence of the transport system. The energy sources were simply substituted for the PMS-ascorbate electron donor system in the assay mix.

The cation dependence of the transport system was also examined. Various cation sources were substituted for $MgSO_4$ and preincubated with the membranes for fifteen minutes prior to the addition of the electron donor source. The rest of the standard assay procedure followed as outlined above.

3.2.7. The effect of energy inhibitors and sulfhydryl reagents on pyruvate uptake in vesicles

Energy inhibitors and sulfhydryl reagents were preincubated with the membrane vesicles for fifteen minutes prior to the addition of the pyruvate and electron donors. The standard assay was then followed for ten minutes (steady state), filtered and counted. The inhibitor assays were then compared to an uninhibited control, assayed under identical conditions, and the percent inhibition calculated from these two values.

3.2.8: The effect of analogue inhibitors on pyruvate uptake in vesicles

Preliminary studies were carried out to determine which pyruvate analogues were potential inhibitors of pyruvate uptake in the membrane vesicles. In these studies, the analogue was added simultaneously with the pyruvate and the amount of pyruvate inside the vesicles was counted at ten minutes. Percent inhibition of transport was calculated by comparison of the analogue values to an uninhibited control. The concentration of the analogue was varied to determine the effect of increasing concentration on the extent of inhibition.

Those analogue inhibitors which displayed potential competitive inhibition were examined in more detail by kinetic analysis. These studies were carried out either by varying the inhibitor concentration at a constant pyruvate concentration or by varying the pyruvate concentration at a constant inhibitor concentration. The resultant graphs were

used to determine the nature of the inhibition, either competitive or non-competitive, and an inhibitor constant.

3.2.9. The fate of pyruvate in membrane vesicles

Membrane vesicles were also assayed to determine whether or not pyruvate was metabolized during transport or while accumulating within the vesicles. The vesicles were exposed to labelled pyruvate as described above in the transport assay, but the concentration of pyruvate was increased to either 100µM or 500µM. In the case of the higher pyruvate concentration, [^{14}C]-pyruvate was maintained at 100µM and unlabelled pyruvate was added to make up the final concentration of 500µM. At time intervals, 20 µl aliquots of the assay mixture were spotted directly on thin layer chromatography plates and air dried according to the procedure of Kaback and Milner (1970). The plates were then immediately developed by the chromatography method of Nygaard (1967) as described in the previous chapter. The radioactive spots were detected by autoradiography by exposure to Kodak X-ray film for 7 days.

3.3. RESULTS

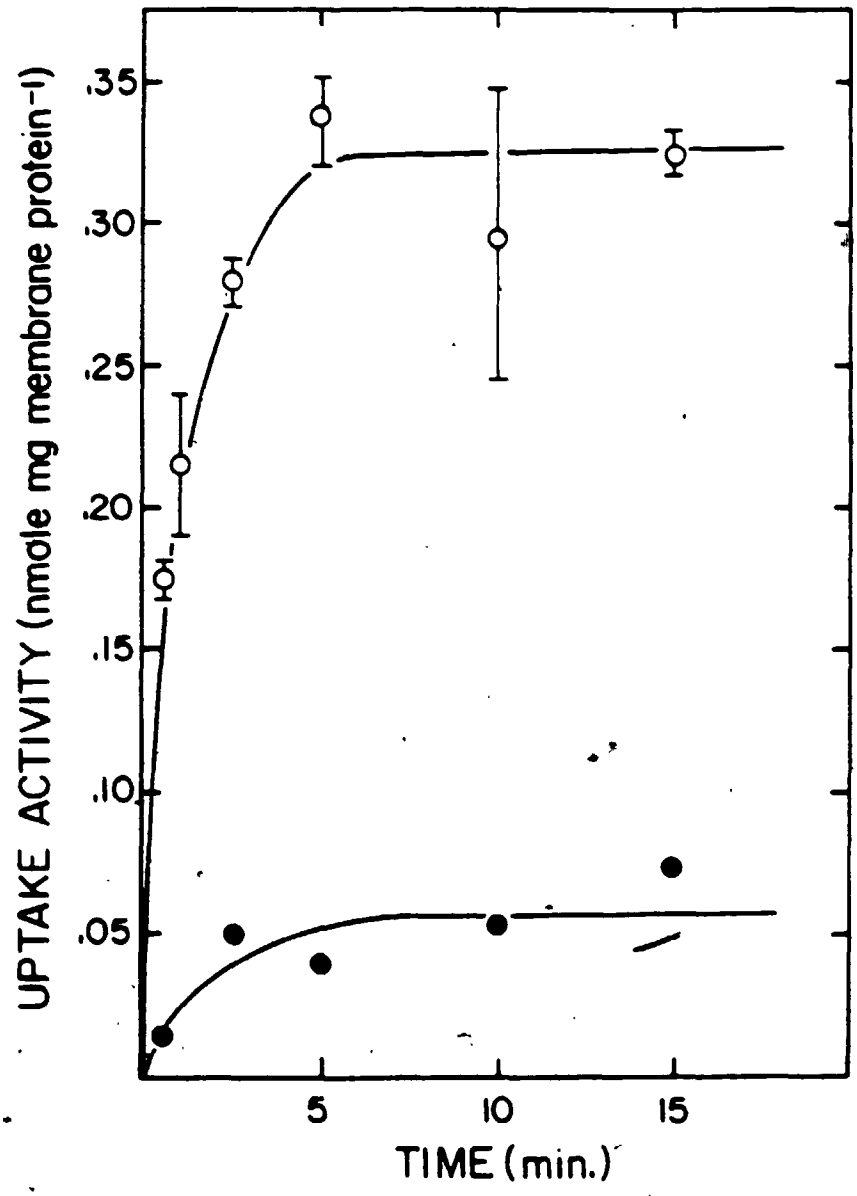
3.3.1. Characterization of the pyruvate transport system in membrane vesicles

The initial stage in this study was to establish that transport of pyruvate in membrane vesicles did indeed occur. Matin and Konings (1973) showed that uptake of pyruvate took place in vesicles of E. coli K12, but duplication of this

was required before proceeding with the characterization of the transport system. Optimal pyruvate uptake occurred in vesicles prepared by means of a Teflon and glass motor-driven homogenizer. The results of the time course of pyruvate uptake in membrane vesicles prepared in this manner are presented in Figure 3-1. It is evident that uptake of pyruvate in E. coli membrane vesicles is stimulated by the artificial electron donor system ascorbate-PMS. Pyruvate is accumulated rapidly and linearly for roughly one minute and then the rate of uptake tapers off gradually and reaches steady state at about five minutes. It can be calculated that the internal concentration of pyruvate in these vesicles at steady state is 110uM, assuming that the internal volume of the vesicles is 3 µl per milligram membrane protein (Konings and Freese, 1972). Comparing this internal concentration value to the external pyruvate concentration of 15µM yields a figure of seven times the external concentration. In the absence of electron donor, the concentration of pyruvate inside the vesicles is roughly 20uM, a value very close to that of the external concentration, and implying a simple equilibration process was involved. Other membrane vesicle preparations were observed to take up different quantities of pyruvate. A maximum value of fifteen times the external pyruvate concentration was achieved with one of these other batches.

FIGURE 3-1. THE TIME COURSE OF PYRUVATE UPTAKE IN MEMBRANE VESICLES.

Pyruvate uptake in E. coli K12 membrane vesicles was determined at a final pyruvate concentration of 15μM. The open circles indicate pyruvate uptake in the presence of the electron donor system 20mM sodium ascorbate, pH 6.6 and 200μM phenazine methosulfate. The closed symbols denote the uptake of pyruvate in vesicles in the absence of the electron donor system.


UPTAKE ACTIVITY (nmole mg membrane protein-1)
.35
.30
.25
.20
.15
.10
.05
5
10
15
TIME (min.)

Due to the variability in uptake activity found in the vesicle preparations, experiments in which comparison of results was a critical factor were completed with the same batch of vesicles.

The pH optimum of pyruvate uptake was then determined in order to verify that the vesicle assay conditions were appropriate for the substrate. Figure 3-2 shows the results of this study. Uptake displays a broad peak of activity between pH 5.5 to 7.5, with maximum activity at pH 6.0. The assay pH of 6.6 was retained for future assays however, due to the presence of pyruvate oxidase activity found in membrane vesicles of E. coli B wild type bacteria (Shaw-Goldstein et al., 1978). The K_m of this pyruvate oxidase, which converts pyruvate to acetate and CO_2, was 80mM at pH 7.5, but decreased to 3.2mM at pH 6.2. In order to minimize the activity of the pyruvate oxidase and lessen the chances of pyruvate being metabolized, the standard pH value of 6.6 was used, although a slightly lower uptake activity would be the result.

Although uptake activity can be energized with the artificial electron donor system ascorbate-PMS (Figure 3-1), other energy sources were tested for their ability to stimulate uptake of pyruvate in the vesicles. The results of these studies are shown in Table 3-1. It is clear from these results that PMS-ascorbate is the best of the energy sources for stimulation of pyruvate transport. The only

FIGURE 3-2. THE pH OPTIMUM OF PYRUVATE TRANSPORT IN VESICLES.

To optimize the transport assay in membrane vesicles, the pH optimum of uptake was determined. The transport assay was carried out essentially as described in the Materials and Methods section, except for the substitution of the phosphate buffer by the buffers indicated at the bottom of the graph. The transport assay was allowed to proceed for 10 minutes to steady state conditions and the transport activity (nmoles pyruvate per milligram membrane protein) of duplicate samples was determined. All the samples were corrected for 'zero' time controls, in which the amount of pyruvate adhering to the exterior of the vesicles or the filters was determined by stopping the assay before the addition of the labelled pyruvate. The buffering range of the substitute buffers is indicated by the arrows at the bottom of the graph. The electron donor system used was PMS-ascorbate.

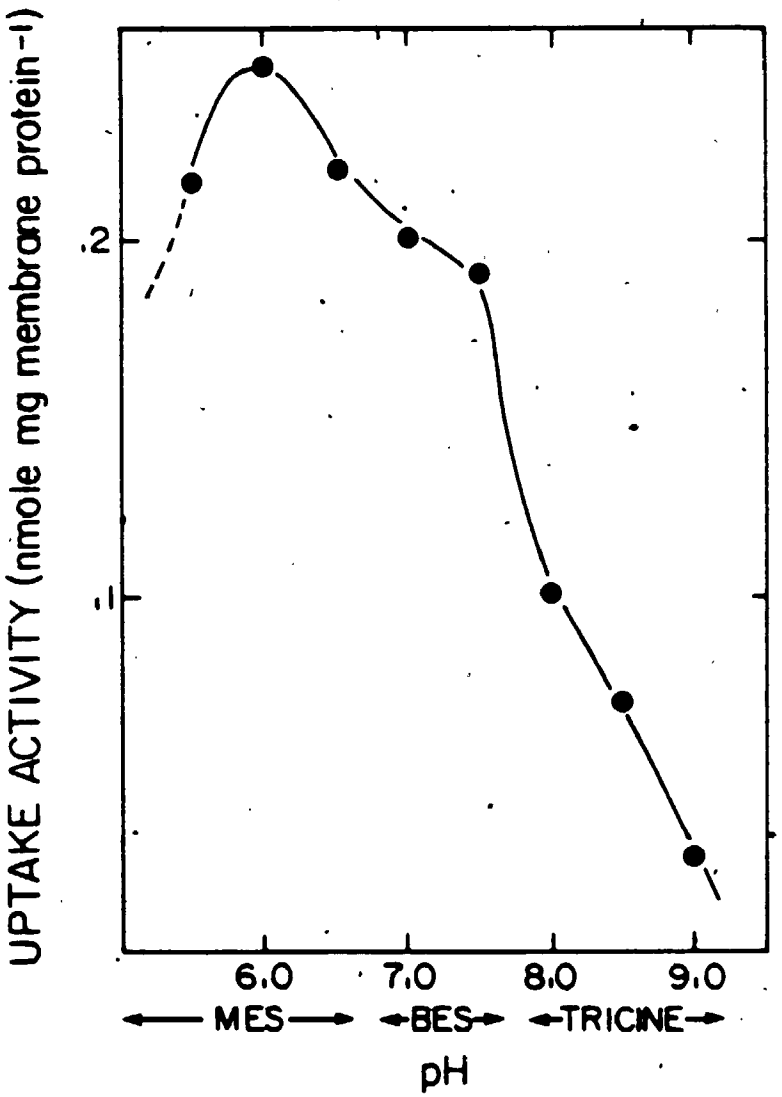

UPTAKE ACTIVITY (nmole mg membrane protein-1)
2
1
6.0
7.0
8.0
9.0
MES
BES
TRICINE
pH

Table 3-1. The Effect of Various Energy Sources on the Uptake of Pyruvate by Membrane Vesicles of *E. coli* K12.

Energy source	Concentration (mM)	Uptake Activity (nmoles pyruvate/ mg protein/10 min.)
None	20	0.073
Sodium ascorbate	20	0.110
PMS	0.2	0.044
Ascorbate + PMS	20	0.712
NADH	20	0.064
NADH + PMS	20	0.560
D(-)Lactate	20	0.014
Glucose	20	0.045
PEP	20	0.075
Formate	20	0.093
ATP	3	0.059
ADP	3	0.014
cAMP	1	0.030

The assay was performed as described in the Methods section. The various energy sources were added immediately prior to the addition of pyruvate and uptake was allowed to proceed for 10 min. The pyruvate content of the vesicles was then determined. All the assays contained equivalent amounts of vesicle protein and a final pyruvate concentration of 20uM. When phenazine methosulfate was used in conjunction with another energy donor, PMS was added to a final concentration of 0.2mM.

other energy donor capable of substituting for PMS and ascorbate is NADH and PMS. NADH, like ascorbate, reduces phenazine methosulfate spontaneously and the reduced PMS can feed electrons into the electron transport chain of E. coli below the level of cytochrome b_1 (Konings et al., 1971). Metabolic intermediates, such as glucose, PEP, and formate do not stimulate uptake and in the case of D(-)lactate, even inhibit transport. None of the adenine nucleotides tested was capable of supporting uptake of pyruvate.

The role of cations in the transport assay was also examined. Table 3-2 lists the various cations added to the assay mixture in place of magnesium in order to determine the cation dependence of the transport system. The baseline uptake activity was established by adding no cation to the membrane vesicles during the fifteen minute preincubation period (see Methods). Magnesium sulfate generated the highest uptake activity, which agrees with the findings of Rayman et al. (1972) for the succinate system, and is consistent with the choice of cation by Matin and Konings (1973). Other cations, such as magnesium chloride, potassium chloride and manganous chloride can substitute for magnesium sulfate to some extent, but none of these salts could stimulate uptake to the level of magnesium sulfate. Still others of the cations tried, such as nickel and zinc, were inhibitors of the uptake process. Another observation that arose from these data is the effect of other

Table 3-2. The Effect of Various Cations on Pyruvate Transport in E. coli K12 Membrane Vesicles.

Cation Added	Uptake Activity (nmoles pyruvate/ mg protein/ 10 min).
None	0.058
$MgSO_4$	0.125
$MgCl_2$	0.085
NaCl	0.073
KCl	0.087
LiCl	0.069
$MnCl_2$	0.084
$Ca(NO_3)_2$	0.034
$FeSO_4$	0.073
$ZnCl_2$	0.028
$BaCl_2$	0.050
$NiCl_2$	0.027
$CuSO_4$	0.015

The values represent the steady state concentrations of pyruvate in the vesicles in the presence of the various metals. The experimental conditions were as described in Methods except that the membrane vesicles were suspended in 50mM HEPES buffer, pH 6.5. The final concentration of all the cations used was 10mM,, except $ZnCl_2$, which was used at 1mM. The concentration of pyruvate in all cases was 20μM.

buffers, in this case HEPES, on transport activity. The maximum uptake, with magnesium, in the HEPES buffer was 0.125 nmole pyruvate/mg protein/10 minutes. The comparable value in the phosphate buffer, under identical conditions, was 0.712 (see Table 3-1, PMS and ascorbate). This deleterious effect of other buffer systems can also be seen in uptake levels of the pH curve (Figure 3-2). In view of these observations, transport studies were carried out in the original 50mM potassium phosphate buffer whenever possible.

3.3.2. The Michaelis constant of pyruvate uptake in membrane vesicles

The Michaelis constant of the pyruvate transport system was determined for the membrane vesicles. Figure 3-3 is the result of the compilation of the data from four different K_m determinations. The K_m obtained by linear regression analysis was 15μM. This value compares favourably with the K_m of 20μM found in the whole cell studies. The best line generated from these data showed no obvious breaks (biphasic kinetics) that would indicate another transport system operating at a different affinity.

3.3.3. The effect of various energy inhibitors and sulfhydryl reagents on pyruvate uptake by vesicles

Various energy inhibitors and uncouplers were preincubated with the vesicles to determine the energy dependence of the transport system. The results of these

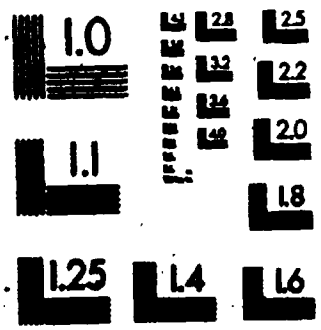
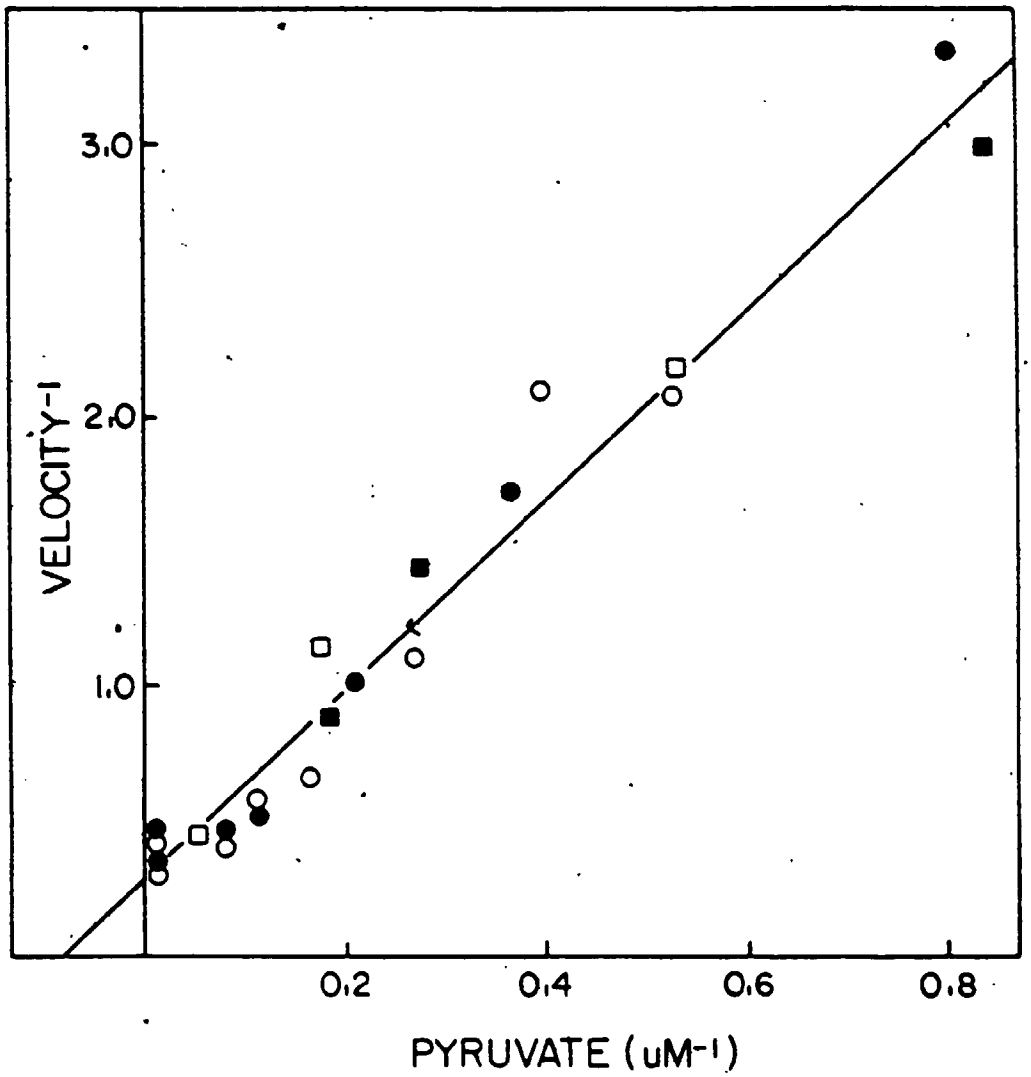


FIGURE 3-3. THE MICHAELIS CONSTANT OF PYRUVATE UPTAKE IN KABACK VESICLES.

The open and closed symbols represent four different determinations of the K_m of transport. Experimental details are essentially as outlined in the Materials and Methods section. Each point was calculated from the initial velocity of transport assayed at 30, 60, and 90 seconds in duplicate. Each initial velocity value was corrected by a 'zero' time control in which the amount of pyruvate adhering to the filter and vesicle exterior was determined by the addition of pyruvate to diluted vesicles immediately prior to filtration. The slope of the line was determined by linear regression (coefficient of correlation = 0.984) and the K_m obtained was 15uM. Velocity was expressed as nmoles pyruvate taken up per milligram vesicle protein per minute. The V_{max} of pyruvate uptake in vesicles was found to be 3-5 nmoles per mg membrane protein per minute. The specific activity of the pyruvate used was 15.6mCi/mmole. The electron donor system used was 20mM sodium ascorbate and 200uM PMS.


3.0
2.0
1.0
VELOCITY-1
0.2
0.4
0.6
0.8
PYRUVATE (uM-1)

assays, presented in Table 3-3, clearly show that uptake of pyruvate is an energy dependent process. Only sodium arsenate shows a non-specific type of inhibition, unrelated to increasing concentration. This result is not unexpected, since it is unlikely that the vesicles retain the cytoplasmic precursors which are inhibited in phosphorylation reactions by arsenate. The uncouplers CCCP, DNP are particularly effective in preventing uptake of pyruvate. Azide, which has been described as an uncoupler and an ATPase inhibitor in E. coli (Roisin and Kepes, 1973) was also an effective inhibitor of translocation. The electron transport chain inhibitors antimycin A and potassium cyanide also display inhibitory properties. It is evident from these results that transport of pyruvate in E. coli membrane vesicles is an energy dependent process.

The inhibition of transport resulting from the exposure of vesicles to sulfhydryl reagents is shown in Table 3-4. All of the reagents tested were effective inhibitors of transport, but silver nitrate and mercuric chloride were found to be the best inhibitors of transport. These results would indicate that a sulfhydryl group is involved somewhere in the transport system, especially in view of the information provided by Kaback and Barnes (1971) that respiration induced by PMS-ascorbate is not affected by N-ethylmaleimide treatment.

3.3.4. The specificity of the pyruvate transport system in

Table 3-3. The Effect of Various Energy Poisons on the Transport of Pyruvate in Vesicles.

Inhibitor	Concentration (mM)	% Inhibition
Sodium arsenate[a]	0.5	18.3
	5.0	18.3
Sodium azide	0.5	29.6
	5.0	69.4
Potassium cyanide	0.5	26.8
	5.0	50.3
2,4-Dinitrophenol	0.05	30.1
	0.5	88.6
Oligomycin[b]	0.005	4.1
	0.05	33.6
CCCP[b]	0.0005	33.7
	0.005	88.8
Antimycin A[b]	0.05	5.0
	0.2	61.0

The standard vesicle uptake assay was followed as described in Methods. The uptake assays were allowed to proceed to steady state at 10 min. The inhibitors were preincubated with the membranes for 15 min. at room temperature prior to the addition of the electron donors and labelled pyruvate. The % inhibitions were calculated by comparison of the inhibited uptake assays to an uninhibited control. The pyruvate concentration in these experiments was held at 20uM.

[a]The sodium arsenate experiment was carried out in HEPES buffer, pH 6.5, instead of the normal phosphate buffer and compared to a HEPES buffered control.

[b]Dissolved in dimethyl sulfoxide and compared to a control assay containing an equivalent amount of the solvent.

Table 3-4. The Effect of Various Sulfhydryl Reagents on the Transport of Pyruvate in Vesicles.

Inhibitor	Concentration (mM)	% Inhibition
N-ethylmaleimide	0.05	21.5
	0.5	33.4
Iodoacetate	0.5	0.0
	5.0	35.3
Silver nitrate	0.008	17.6
	0.08	98.5
Mercuric chloride	0.01	20.7
	0.1	99.2

The standard vesicle uptake assay was followed as described in Methods. The uptake assays were allowed to proceed to steady state at 10 min. The inhibitors were preincubated with the membranes for 15 min. at room temperature prior to the addition of the electron donors and labelled pyruvate. The % inhibitions were calculated by comparison of the inhibited uptake assays to an uninhibited control. The pyruvate concentration in these experiments was held at 20μM.

vesicles

The initial stage in establishing the specificity of the pyruvate translocation system was to expose the vesicles to several concentrations of substrate analogues, and to calculate the inhibition resulting from addition of the analogue. The results of these studies are shown in Table 3-5. As expected from the whole cell results, bromopyruvate, fluoropyruvate and pyruvic acid methyl ester show increasing inhibition with increasing concentration, a good indication of competitive inhibition. Most of the natural analogues, represented in Table 3-5 by oxaloacetate and alanine, display either non-specific or no inhibition. D-Lactate and L-lactate both show indications of being good competitive inhibitors. Oxalic acid and glyoxylate are included in the table for convenience but their significance will be discussed in a later section of these results.

All of the analogues that produced a pattern of increasing inhibition with increasing concentration were examined by kinetic means in order to establish the type of inhibition involved and an inhibitor constant. By this means the specificity of the pyruvate transport system in E. coli membrane vesicles could be determined. The graphic methods of kinetic analysis chosen were the double-reciprocal plot of Lineweaver-Burk (Lineweaver and Burk, 1934) and the method of Dixon (Dixon and Webb, 1964).

Table 3-5. The Effect of Various Pyruvate Analogues on the Uptake of Pyruvate in Membrane Vesicles.

Inhibitor	Concentration (mM)	% Inhibition
3-Bromopyruvate	0.05	93.2
	0.1	95.1
3-Fluoropyruvate	0.05	10.8
	0.1	59.3
	0.2	88.5
Pyruvic acid methyl ester	0.05	23.4
	0.1	40.6
Oxaloacetate	0.05	28.1
	0.1	25.7
D(-)Lactate	0.05	38.9
	0.1	80.6
	0.2	97.7
L(+)Lactate	0.5	78.7
	1.0	90.6
Oxalic acid	10.0	19.8
Glyoxylate	1.0	15.9

The experimental conditions of the assays were as outlined in the Methods section. The inhibitor was added simultaneously with the labelled pyruvate and uptake allowed to proceed to steady state at 10 min. The % inhibiton was calculated by comparison of the inhibited uptake values to an uninhibited control. The final pyruvate concentration in these assays was 20µM.

Pyruvic acid methyl ester was found to be a competitive inhibitor of pyruvate transport by the double-reciprocal method and Figure 3-4 indicates that the K_i of PAME was 52μM. Figure 3-4 also shows a plot of the initial velocity of transport versus substrate concentration at a constant bromopyruvate concentration. The K_i of bromopyruvate was determined to be 6μM. These results agree favourably with the whole cell values presented in Chapter 2 (K_i's of 100μM and 25μM respectively).

From the results obtained from the energy donors (Table 3-1) and the analogue studies (Table 3-5), inhibition of transport by D-lactate was a forgone conclusion. The nature of the inhibition and its extent was determined by exposing the transport assay system to various concentrations of D-lactate, the results of which are presented in Figure 3-5. Two concentrations of pyruvate were chosen for the assays and both resulting lines extrapolate to intersect at the x-axis, which denotes non-competitive inhibition in a Dixon plot. The inhibitor constant (K_i) determined for D-lactate from linear regression analysis was 360μM.

L-lactate was determined to be a competitive inhibitor from the Lineweaver-Burk plot shown in Figure 3-6. This was not unexpected in view of the whole cell results presented in Chapter 2. However, the K_i of L-lactate in membrane

FIGURE 3-4. THE INHIBITION CURVES OF THE SYNTHETIC PYRUVATE ANALOGUES 3-BROMOPYRUVATE AND PYRUVIC ACID METHYL ESTER.

The inhibitor was added simultaneously with the labelled pyruvate and the initial velocity of transport was determined in duplicate as described in the Methods section and corrected for a 'zero' time control. The lines were determined by linear regression analysis of the data. The velocity is expressed in nmoles pyruvate transported per milligram membrane protein per minute. The dashed line is the uninhibited control. (○) In the presence of 50μM PAME; (●) In the presence of 10μM 3-bromopyruvate. The K_i values determined were 52μM for PAME and 6μM for 3-bromopyruvate. Specific activity of the pyruvate used was 18mCi/mmole. Sodium ascorbate and PMS were added to each assay as the electron donor source.

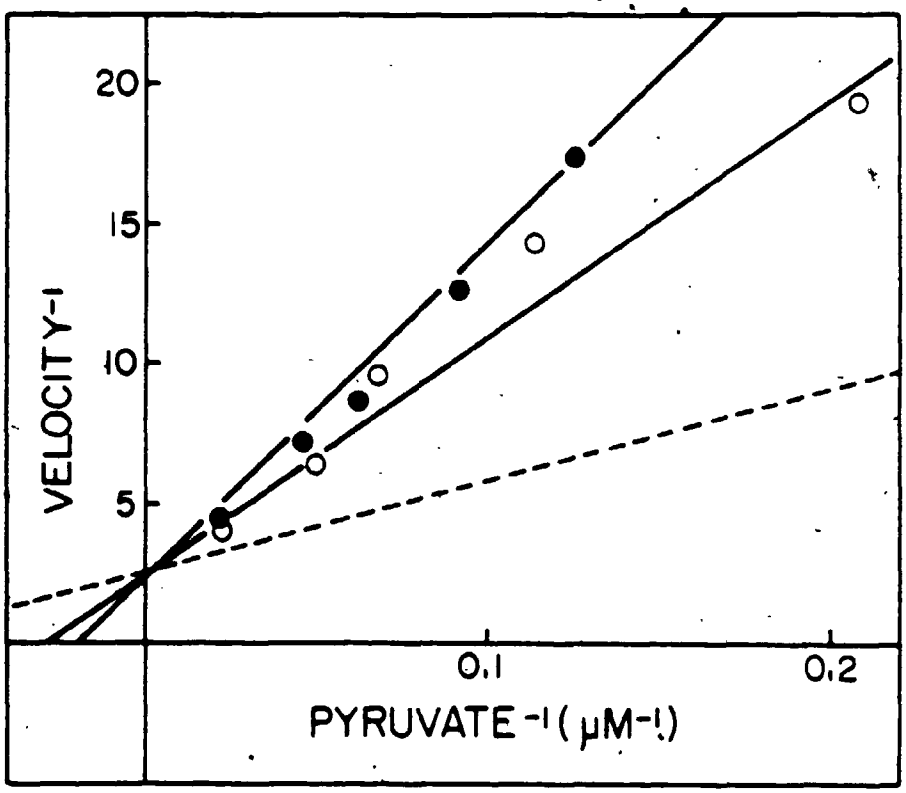

20
15
10
5
VELOCITY-1
0.1
0.2
PYRUVATE -1 (μM-1)

FIGURE 3-5. THE INHIBITION OF PYRUVATE TRANSPORT BY D-LACTATE.

The inhibition of pyruvate transport in membrane vesicles was determined from the initial rates of transport as described in the Materials and Methods section. Transport was initiated by the addition of labelled pyruvate simultaneously with the lactate and energized by the electron donor system ascorbate-PMS. Velocity (V) is expressed in nmoles pyruvate transported per milligram membrane protein per minute. (△) D-lactate at a constant 7.5uM pyruvate concentration; (▲) D-lactate at a constant 20uM pyruvate concentration. Specific activity of the pyruvate used was 16.7mCi/mmole.

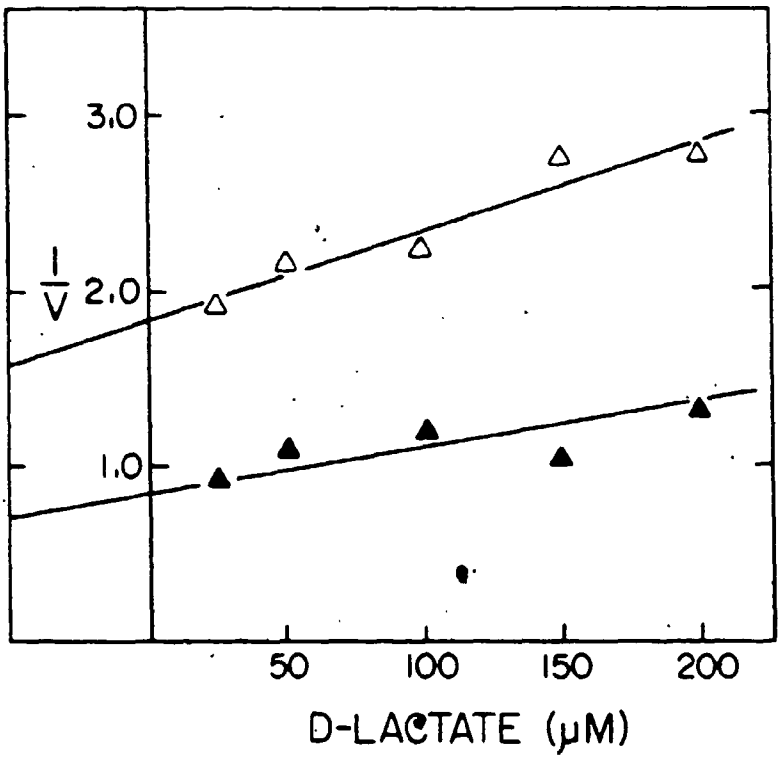
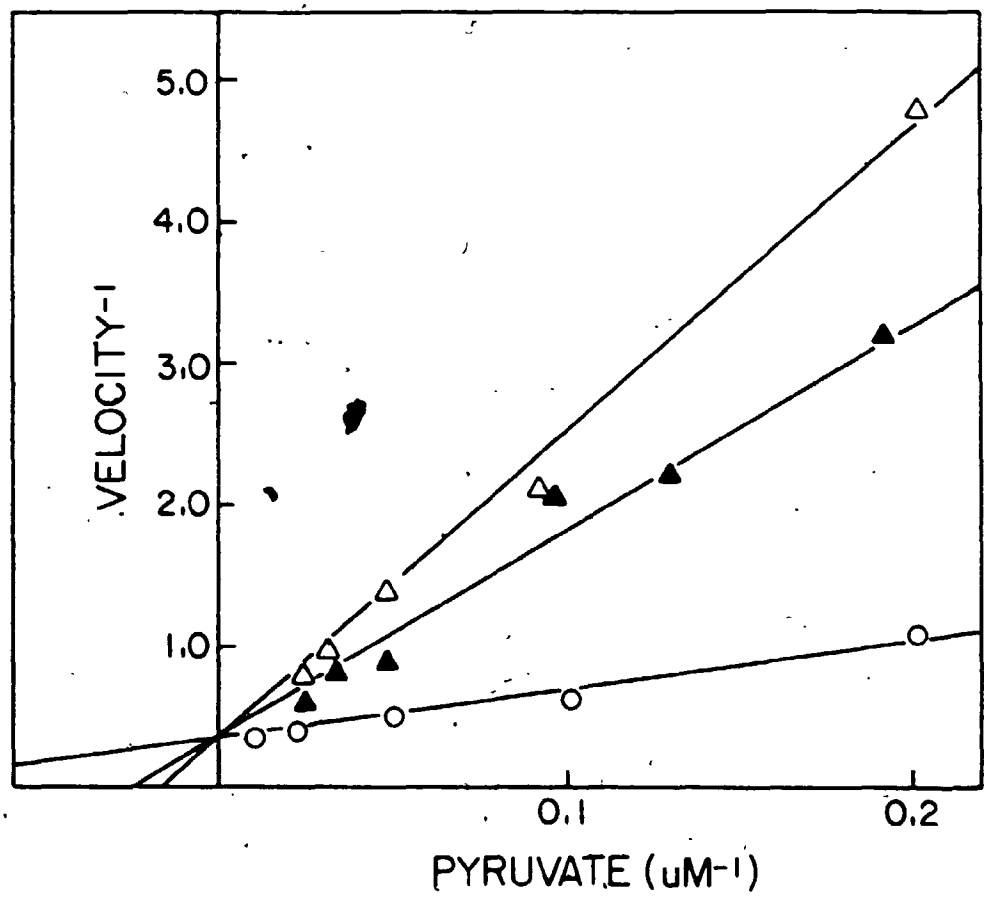

3.0
1/V 2.0
1.0
50
100
150
200
D-LACTATE (μM)

FIGURE 3-6. THE INHIBITION OF PYRUVATE TRANSPORT BY L-LACTATE.

The inhibitor was added simultaneously with labelled pyruvate to initiate transport. The initial velocity of transport was determined as described in the Methods section, and the lines determined by linear regression analysis. The velocity is expressed in terms of nmoles pyruvate transported per milligram membrane protein per minute. (△) 200uM lactate; (▲) 100uM lactate; (○) control. The specific activity of the pyruvate was 18mCi/mmole. Transport was assayed in the presence of 20mM sodium ascorbate and 200uM PMS as the electron donor source.

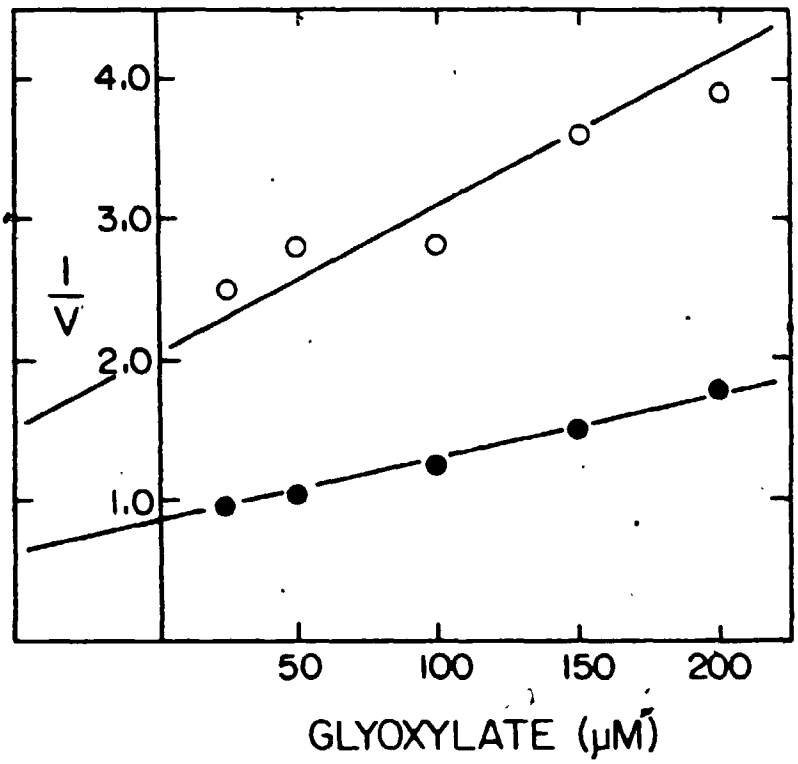

5.0
4.0
3.0
2.0
1.0
VELOCITY-1
0.1
0.2
PYRUVATE (uM-1)

vesicles was 37-40μM, which is significantly different from the value of 7.5mM determined for lactate inhibition in whole cells. The K_i of lactate in the vesicles was also close enough to the K_m of transport of pyruvate to make lactate a likely substrate for the pyruvate transport system. This conclusion was proposed by Matin and Konings (1973) in their study of lactate transport in vesicles, but the high inhibitor constant of lactate in whole cells prevented us from concurring with that supposition. Since there was such a discrepancy between the effect of lactate in whole cells and membrane vesicles, more information was needed before the specificity of the pyruvate transport system could be established.

Matin and Konings (1973) had found that 1mM glyoxylate was capable of inhibiting transport of both D- and L-lactate by 55% and 74% respectively. Similar results were obtained with 1mM pyruvate, leading to their supposition that these monocarboxylic acids share the same transport system. Glyoxylate was obtained and added to the pyruvate transport assay system in order to find out if 1mM glyoxylate inhibited pyruvate transport to a similar extent. Table 3-5 shows that 1mM glyoxylate inhibits pyruvate by only 16% however. Various concentrations of glyoxylate were then added to the pyruvate uptake assay and the initial velocity results, at two different pyruvate concentrations, are presented in Figure 3-7. Glyoxylate is a non-competitive

FIGURE 3-7. THE INHIBITION OF PYRUVATE TRANSPORT BY GLYOXYLATE.

The inhibitor was added simultaneously to the assay mixture with labelled pyruvate. Initial velocity of transport was determined by sampling at early time points as described in the Materials and Methods section. Both lines were the result of linear regression analysis of the data. Velocity (V) is expressed as nmoles pyruvate taken up per milligram membrane protein per minute. (○) Glyoxylate at a constant 7.5μM pyruvate concentration; (●) Glyoxylate at a constant 20μM pyruvate concentration. PMS and ascorbate were added to each assay as the electron donor source.


4.0
3.0
2.0
1.0
1/V
50
100
150
200
GLYOXYLATE (μM)

inhibitor of pyruvate translocation with a K_i of 215μM. From these two pieces of information, it is unlikely that the lactate transport system described by Matin and Konings and our pyruvate transport system are identical. Other possible explanations for the very effective competitive inhibition by L-lactate of pyruvate transport in membrane vesicles were then explored.

E. coli has been shown to have both D- and L-lactate dehydrogenase activities in the membrane (Kline and Mahler, 1965). These dehydrogenases oxidize their respective lactate stereoisomers to pyruvate in normal E. coli membrane vesicles (Konings, 1977). Matin and Konings (1973) were able to demonstrate that uptake of D-lactate was not dependent on oxidation of the lactate by inhibiting the activity of the membrane-bound D-lactate dehydrogenase (D-LDH) up to 98% by the addition of the analogue inhibitor oxalic acid. Konings (1977) also stated that uptake of L-lactate is not dependent on oxidation. In this case, membrane vesicles were prepared that were not induced for L-LDH (Kline and Mahler, 1965). Snoswell (1963) described NAD-independent D- and L-lactate dehydrogenases in Lactobacillus arabinosus, enzymes similar to the E. coli lactate dehydrogenases, which are also inhibited by oxalic acid. Therefore we decided to add oxalate to the pyruvate transport assay to see what effect, if any, inhibition of D-LDH and L-LDH had on pyruvate transport. Table 3-5 shows

that 10mM oxalate alone inhibits pyruvate translocation no more than 20%. This result is consistent with a shared transport system since transport of the lactates and pyruvate appears to be independent of oxidation. 10mM oxalic acid was then added to an inhibition experiment to determine what effect oxalate had on inhibition of pyruvate transport by L-lactate. The results of this experiment are presented in Figure 3-8. Surprisingly, the addition of oxalate abolished the inhibition of transport by L-lactate. The velocity of uptake remained the same, within experimental error, for up to 200μM lactate. This result implies that the inhibition of pyruvate transport is dependent on L-lactate oxidation, rather than transport of L-lactate.

3.3.5. Fate of pyruvate in the membrane vesicles

The vesicle contents were separated by thin layer chromatography as described previously in Chapter 2 and the Methods section. The resulting autoradiogram is shown in Figure 3-9. Each sample applied to the silica gel plate essentially contained a pyruvate standard, since the pyruvate in the assay mixture was not separated from the internalized pyruvate before application. Even at the higher pyruvate concentration (500μM), no new products are produced in the vesicles, since no bands other than those present in the standard are visible. There is also no increase in intensity of any of the minor bands over time,

FIGURE 3-8. THE INHIBITION OF PYRUVATE TRANSPORT BY L-LACTATE IN THE PRESENCE OF OXALATE.

L-lactate was added simultaneously with 20μM pyruvate and 10mM oxalic acid to the transport assay mixture. Each initial velocity curve was done in duplicate as outlined in the Materials and Methods section. Velocity (V) is expressed as nmoles pyruvate taken up per milligram membrane protein per minute. Increasing the lactate concentration had essentially no effect on the initial velocity of transport. The energy source was PMS-ascorbate and the specific activity of pyruvate used was 18mCi/mmole.

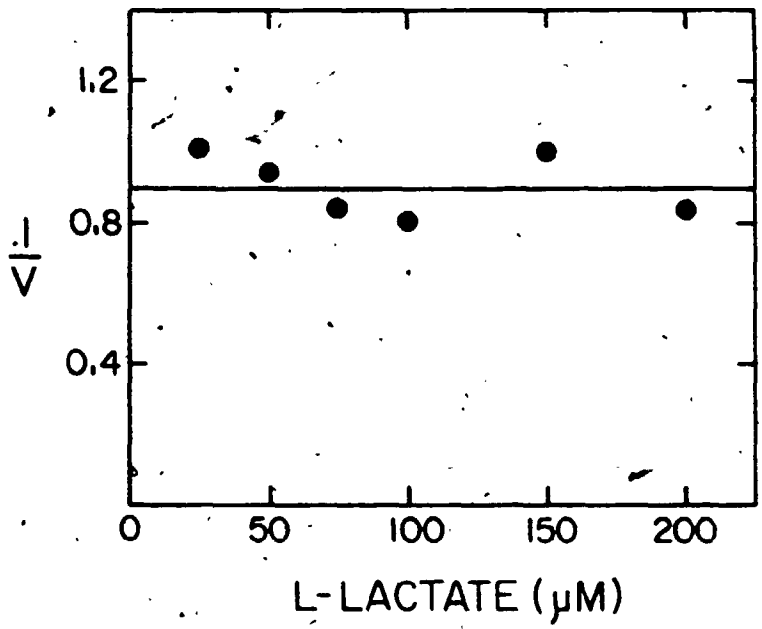

1.2
0.8
0.4
1/V
0
50
100
150
200
L-LACTATE (μM)

FIGURE 3-9. THE FATE OF PYRUVATE IN MEMBRANE VESICLES.

Aliquots of the vesicle assay mix were spotted directly on thin layer chromatography plates, developed, and autoradiographed as described in the Methods section. The time course study was conducted at two pyruvate concentrations, 0.1mM and 0.5mM, as denoted at the bottom of the autoradiograph. The quantity of labelled pyruvate was equivalent at both pyruvate concentrations. Each sample was spotted at 30 seconds, 1.5 and 5.0 minutes after initiation of transport. A pyruvate standard (STD) was also spotted for comparison.

30s 1.5min 5.0min 30s 1.5min 5.0min STD

0.1mM 0.5mM

which effectively rules out metabolism of pyruvate in the vesicles to a degradation product already present in the pyruvate standard. This method of analysis was chosen to avoid extraction and concentration of the vesicle contents, procedures which invariably resulted in some degree of degradation of the pyruvate molecule.

3.4. DISCUSSION

Membrane vesicles are a well defined model system (Kaback, 1974; Kaback et al., 1977) devoid of cytoplasmic contents and therefore provide a great advantage over whole cells as a method of study in as far as determination of energy source and limitation of substrate metabolism. Energy sources must be provided exogenously, and the only enzymes in vesicles are restricted to the membrane itself.

It is clear from the cumulative evidence presented in this chapter that pyruvate is transported by an active transport process in membrane vesicles. A non-physiological energy donor source, ascorbate-PMS, was necessary to stimulate uptake of pyruvate beyond simple equilibration and steady state conditions were observed in the time course study. Pyruvate was accumulated up to 15 times the external concentration inside the vesicles, fulfilling the criteria of active transport of accumulation against the concentration gradient. The lack of metabolism of pyruvate, shown by thin layer chromatography (Figure 3-9), agreed with

the finding of Kaback and Milner (1970), that pyruvate, produced from D-lactate by D-lactate dehydrogenase, was not significantly metabolized inside membrane vesicles. Shaw-Goldstein et al.(1978) had found that E. coli B membrane vesicles, when exposed to millimolar concentrations of pyruvate, were capable of converting pyruvate to acetate and CO_2 by the activity of a pyruvate oxidase. However, due to the low quantities of pyruvate oxidase found in vesicles, and the high K_m (see section 3.3.1) of the oxidase, it was highly unlikely that the oxidase would metabolize pyruvate at the concentrations used in the transport assays.

The pyruvate transport system also appears to be unique in E. coli . The best evidence for this assumption is the monophasic kinetics displayed in the double-reciprocal plot (Figure 3-3) used to determine the K_m of transport. As mentioned in the preceding chapter, different transport systems usually have different K_m's of transport, resulting in a biphasic plot. The Michaelis constants of transport found in the whole cells and membrane vesicles were also very similar (20 and 15μM respectively), implying that the same pyruvate transport system was being examined in each of the bacterial preparations. However, without additional evidence, preferably a thorough examination of the genetic background of the transport system in regard to the number, products, and location of the genes involved in

pyruvate translocation, we cannot state conclusively that only one pyruvate transport system exists in E. coli .

It is apparent from the results presented in this chapter that pyruvate transport in membrane vesicles is dependent on metabolic energy. Translocation of pyruvate does not proceed without the addition of an exogenous electron donor system to activate the respiratory chain. From the results obtained by exposure of the vesicles to the various energy poisons, especially the high degree of inhibition of transport in response to addition of the uncoupling agents, the driving force for translocation appears to be the proton motive force predicted by the chemiosmotic hypothesis of Mitchell (1963). This hypothesis was outlined in the introductory chapter. There is not enough evidence, however, to completely dismiss the possiblility that ATP is somehow involved in the transport process. Neither ATP or ADP was capable of stimulating transport when added exogenously to the vesicles, but there are conflicting reports on the ability of the adenine nucleotides to penetrate to the cytoplasmic side of the plasma membrane to the site of the ATPase (Weissbach et al., 1971, Brockman and Heppel, 1968). In view of the fact that normal vesicles cannot catalyze oxidative phosphorylation (Klein et al., 1970), and arsenate does not inhibit transport, it is highly improbable that ATP is obligatory to the translocation process. However, at this stage of our

investigation, it is not possible to state unequivocally that the energy coupled to the translocation process originates solely in the proton motive force generated by the respiratory chain. Experiments will have to be done using membrane vesicles prepared from uncA mutants lacking the Ca^{2+}, Mg^{2+}-ATPase (Butlin et al. , 1971) and vesicles preloaded with ATP in order to eliminate the possibility that the ATPase can contribute to the generation of the proton gradient and drive transport as well.

The specificity of the transport system was the most intriguing problem presented during this study. As expected from the whole cell results, both the non-metabolizable synthetic analogues, 3-bromopyruvate and PAME, were good competitive inhibitors of uptake. D-lactate was found to be a non-competitive inhibitor of transport, a not unexpected result since D-lactate is transported into the vesicles by the lactate transport system and converted to pyruvate by the membrane bound D-LDH (Konings, 1977). D-lactate would therefore be responsible for raising the level of pyruvate within the vesicles. The resulting inhibition is apparently not due to competition for a common carrier, but is probably due to the dilution of the labelled pyruvate that had been translocated. The case of L-lactate is more complex, especially in regard to the whole cell results.

L-lactate was a competitive inhibitor of pyruvate transport in whole cells, but the high K_i led us to

conclude that only very high concentrations of lactate could overcome the low affinity of a common carrier for lactate. Such was not the case in the membrane vesicles. The K_i of L-lactate competitive inhibition in vesicles was comparable to the K_m of transport of pyruvate, indicating a shared uptake system. Since Kaback vesicles are essentially an artificial system and the whole cell response to inhibition of pyruvate transport by L-lactate was known to deviate from the vesicle results, other possible explanations for the vesicle response to lactate inhibition were sought.

As stated in the Results section (3.3.4), Matin and Konings (1973) had found glyoxylate to be a potent inhibitor of transport of both D- and L-lactate. Glyoxylate, at the same concentration, inhibited pyruvate transport only marginally (see Table 3-5). It is unlikely that the pyruvate and lactate transport systems are identical considering this lack of cross-inhibition. Based on this information, two possible explanations could account for the lack of inhibition by glyoxylate:

(i) Another pyruvate-lactate transport system, insensitive to glyoxylate, exists separately from the D-and L-lactate transport system of Matin and Konings (1973) and has a very similar K_m for L-lactate transport.

(ii) The kinetic results, although apparently showing competitive inhibition by lactate, may be misleading and

lactate may not be a true substrate inhibitor. Lactate may be affecting transport of pyruvate by some mechanism other than competition for the same substrate binding site, but the method used for kinetic analysis was not able to distinguish between the two behaviors. The most likely source for lactate inhibition is the conversion of lactate to pyruvate by L-lactate dehydrogenase, leading to dilution of the labelled pyruvate pool within the vesicles.

The first of these explanations is plausible, but is inconsistent with our whole cell results and with the finding of Matin and Konings (1973) that D-lactate and L-lactate shared a single transport system. Resolution of the transport systems would require a full analysis of the number of genes involved in lactate and pyruvate transport. Since this analysis would require a fair length of time, the second of the explanations was examined first by the simple expedient of adding oxalate to the transport assay as described in the Results section.

The results achieved with the oxalate exposure indicate that the second explanation is the most probable one. Oxalate, a potent inhibitor of lactate dehydrogenases, was shown to eliminate the inhibition of pyruvate transport by up to 200μM lactate. It would appear that an active L-LDH is essential to the inhibition of pyruvate translocation by L-lactate and the transport system is apparently specific for pyruvate in E. coli .

Since the K_m of L-lactate dehydrogenase is 33μM (Matin and Konings, 1973) it is possible that transport of lactate through the lactate transport system, followed by oxidation to pyruvate, is sufficiently fast to act as another pyruvate transport system and exerts some form of 'feedback' control on pyruvate transport. Although it is interesting to speculate, further work needs to be done before any controlling link between pyruvate transport and L-LDH can be substantiated, however. The effect of oxalate on L-LDH should be established as a specific inhibitor of the lactate dehydrogenase only. Ogino et al. (1980) concluded that efflux of pyruvate in anaerobic E. coli cells takes place by means of carrier-mediated facilitated diffusion. This carrier, which is activated by a decrease in the pH gradient, may also be responsive to oxalate treatment. Repetition of the effect of oxalate on lactate inhibition by using the covalently bound lactate dehydrogenase inhibitor 2-hydroxy-3-butynoate (Walsh and Kaback, 1974) would also be a major step in confirming this link. Preparation of vesicles from E. coli lacking L-LDH activity, either using mutants or growing cells under conditions in which L-LDH is not induced (Kline and Mahler, 1965), would also be an easy test of the effect of LDH on pyruvate transport. If inhibition of pyruvate transport by lactate does not occur in these vesicles, then control of pyruvate transport linked to the dehydrogenase could be

confirmed. In fact, the difference between the whole cell response to L-lactate as an inhibitor of pyruvate transport and the vesicle response may be the simple reason that the whole cells are grown on a defined medium containing glycerol which should not induce L-lactate dehydrogenase (Kline and Mahler, 1965) and the vesicle cells were grown on a complex medium which contained sufficient lactate to cause induction of the LDH.

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CHAPTER 4. PARTIAL PURIFICATION OF A PERIPLASMIC PYRUVATE BINDING PROTEIN.

4.1. INTRODUCTION

In the early stages of the characterization of pyruvate translocation in Escherichia coli K12, the transport system was tested for osmotic shock sensitivity. The bacteria were subjected to the osmotic shock procedure of Nossel and Heppel (1966), which selectively removes the periplasmic proteins from gram-negative cells, and the pyruvate transport activity of these 'shocked' cells was assayed. Pyruvate transport activity after osmotic shock treatment was sharply reduced, indicating that a periplasmic component of the transport system had been lost. Shock-insensitive proline transport was also reduced by the osmotic shock procedure, but with a much lower percentage loss of activity, indicating that damage to the bacteria cells was not solely responsible for the reduction of pyruvate transport activity. This chapter outlines the methods used in the attempts to purify the periplasmic protein involved in pyruvate transport in whole cells.

4.2. MATERIALS AND METHODS

4.2.1. Chemicals

All common chemicals were purchased from commercial sources and were of reagent grade or better. [3-^{14}C] pyruvate, sodium salt, 15-20mCi/mmole, and [2,3-^{3}H] proline, 36Ci/mmole, were obtained

from New England Nuclear. Acrylamide and other chemicals used for polyacrylamide gel electrophoresis were obtained from Biorad and were of electrophoresis purity grade. 1,4-butanediol diglycidyl ether was obtained from ICN and all column chromatography resins were obtained from Pharmacia. 3-Hydroxypyruvate, CCCP and phenylmethylsulfonyl fluoride were purchased from Sigma.

4.2.2. Bacterial strains, culture media and growth conditions

The bacterial strains used for the isolation of the periplasmic binding protein were the E. coli mutant JRG 596 described in Chapter 2, and the wild type E. coli K12 designated HfrH. The mutant strain was grown on the supplemented M9 minimal medium described in section 2.2.3., and the wild type E. coli K12 was grown on minimal A medium (Miller, 1972) with glucose or pyruvate added as the carbon source. The bacteria were routinely grown in 12 liter batches in a New Brunswick fermentor at 37°C, with vigorous agitation and aeration. At mid-log stage of growth, the cells were harvested by ultrafiltration using a Pellicon (Millipore) filtration unit.

In experiments in which induction of the pyruvate binding protein in JRG 596 was the objective, the M9 minimal media was supplemented with pyruvate and one of the Krebs cycle intermediates as well as the standard carbon sources glycerol and acetate.

4.2.3. Preparation of shock fluid by the osmotic shock procedure

The initial phase of the purification of the periplasmic binding protein was the isolation of the periplasmic proteins of the gram-negative cells by the osmostic shock procedure of Nossel and Heppel (1966). This basic procedure was modified for exponential phase cells and large quantities of cells (Rosen and Heppel, 1973) as required. In a typical preparation procedure, harvested cells were washed two times with 10mM Tris-HCl buffer, pH 7.5 and the cells pelleted by centrifugation at 10,000 g for 10 minutes. These washed cells were rapidly dispersed into 30 volumes of 30mM Tris-HCl, pH 7.2, 20% sucrose and 0.1mM potassium EDTA and stirred at room temperature for 10 minutes. These cells were then pelleted by centrifugation, and shocked by gentle dispersal into ice-cold 0.5mM $MgCl_2$ and stirred on ice for 10 minutes. The shocked cells were centrifuged down and the supernatant fluid, hereafter referred to as the shock fluid, was collected. The shock fluid was buffered by the addition of potassium phosphate and sodium arsenate, pH 6.6, to 25mM concentration of each. To prevent proteolysis, potassium EDTA and PMSF, pH 6.6, were added to 5mM and 0.1mM respectively. At this stage of the purification protocol, cell fragments and remaining whole cells in the shock fluid were removed by filtration through a 0.22 µm Duropore membrane (Millipore).

Protein concentration of the shock fluid and subsequent purification steps was estimated by the method of Bradford (1976), using bovine serum albumin as a protein standard.

4.2.4. Transport assays in whole and shocked cells

The assays for the transport of pyruvate and proline were carried out in the wild type E. coli HfrH. Use of the wild type E. coli rather than the pyruvate metabolism mutant JRG 596 aided in comparison of the results by maintaining normal metabolism of both of the substrates. Suspension of the shocked and whole cells and transport of pyruvate in both whole cells and shocked cells was carried out by the procedure outlined in section 2.2.4. Uptake of L-proline was measured by the method of Kaback and Milner (1970). The shocked cells used were the pelleted fraction remaining after the removal of the shock fluid as described above.

4.2.5. Preparation of the hydroxypyruvate affinity column

Preparation of epoxy-activated Sepharose 6B was carried out by the method of Sundberg and Porath (1974). Sepharose 6B was extensively washed and suction dried on a coarse scintered glass filter. Twenty grams of the dried gel wete mixed with 20 ml of 1,4-butanediol diglycidyl ether and 20 ml of 0.6M NaOH containing 40 mg sodium borohydride. The suspended chromatography resin was then swirled for 8-10 hours at room temperature. This epoxy-activated Sepharose was then extensively washed with distilled water and suction dried. Linking the 3-hydroxypyruvate to the activated resin

was carried out by the method outlined by Pharmacia. The epoxy-activated Sepharose was washed with 1 liter of 0.1M sodium carbonate buffer, pH 9.0 and the suction-dried gel was mixed with 100 ml of 0.1M hydroxypyruvate in the 0.1M carbonate buffer. This suspension was incubated at 37°C in a shaking water bath for approximately 18 hours. The hydroxypyruvate-coupled gel was then washed extensively with 0.1M sodium carbonate buffer, pH 9.0, followed by 0.1M sodium bicarbonate buffer, pH 8.0 and finally by 0.1M sodium acetate buffer, pH 4.0. Excess active groups which had not reacted with the hydroxypyruvate were blocked by exposure of the affinity resin to 1M ethanolamine overnight. This hydroxypyruvate affinity gel was washed extensively with distilled water and stored at 4°C until required.

4.2.6. Affinity column chromatography of the shock fluid

The affinity resin was equilibrated to room temperature and poured in a 1.5 by 30 cm column. The poured column was equilibrated before each use by extensive washing with column buffer, which contained 25mM potassium phosphate, 25mM sodium arsenate, 20mM NaCl, 5mM EDTA and 0.1mM PMSF. The shock fluid was concentrated down to approximately 50 ml by means of a 10,000 molecular weight cut-off filter in an Amicon ultrafiltration apparatus and the protein concentration of the concentrated shock fluid was determined as described above. A quantity of protein, typically less than 10 ml and 40 mg, was loaded on the affinity column.

When large quantities of protein were present, multiple affinity column separations were run, either sequentially or concurrently. Protein not capable of binding to the hydroxypyruvate was removed from the affinity column by elution with column buffer. The bound protein was then removed either by elution with a 0 to 0.2M sodium pyruvate gradient added to the column buffer or by the addition of NaCl, either as a 0 to 0.5M gradient or straight 0.5M NaCl, to the column buffer. The column profile was determined by reading the optical density of the fractions at OD_{280}. When pyruvate was used as the eluent, protein in the collected fractions was estimated by the dye binding method of Bradford (1976), since pyruvate absorbs strongly in the OD_{280} range. Any peaks eluted by NaCl or pyruvate were pooled and either used immediately or frozen for later study.

4.2.7. Purification of pyruvate binding protein by other column chromatography methods

Other column chromatograhy methods were also tried to improve on the partial purification achieved with the hydroxypyruvate affinity column. The resins used included chromatofocusing, G-100 Sephadex, G-75 Sephadex, CM cellulose, DEAE cellulose, and Sepharose 4B. All these different chromatograhy resins and columns were prepared according to information provided by Pharmacia or other suppliers. Details of separation procedures will be

discussed later in the text where necessary.

4.2.8. Binding assays

Activity of the pyruvate binding protein was assayed by measuring the ability of the isolated protein to bind [^{14}C] pyruvate. This binding activity was measured by two different methods. When qualitative rather than quantitative data was of interest, binding activity was measured by the simple expedient of dialyzing a small quantity of the sample protein, in dialysis tubing, against a volume of buffer containing labelled pyruvate. The dialysis buffer contained 25mM potassium phosphate-25mM sodium arsenate, pH 6.6, 100μM PMSF, 10μM CCCP, 100μM pyruvic acid and approximately 100,000 dpm of [^{14}C] pyruvate. The protein was exposed to the dialysis buffer for 24 hours to allow complete equilibration. CCCP was included in the buffer to eliminate uptake of pyruvate by any whole cells that may have contaminated the protein preparation. (See Chapter 2 for the effect of CCCP on transport in whole cells).

The second method of determining binding activity was equilibrium dialysis. In equilibrium dialysis, equal volumes of protein and the labelled pyruvate buffer described in the last paragraph were added to wells separated by dialysis membrane. The dialysis cells were then rotated at 4°C until the label had equilibrated across the membrane. Equilibration time was accurately

determined by sampling, at various time intervals, a series of wells containing buffer instead of protein. After equilibration, aliquots of each of the wells were measured by scintillation counting.

4.2.9. Polyacrylamide gel electrophoresis

Progress of the purification was followed by separation of the purified protein by SDS polyacrylamide electroporesis. The PAGE system used was taken from Laemmli (1970) and a Protean (Biorad) electrophoresis unit was used for the separations. The gel bands were stained either with Coomassie brilliant blue or by the silver staining procedure of Merril et al. (1982).

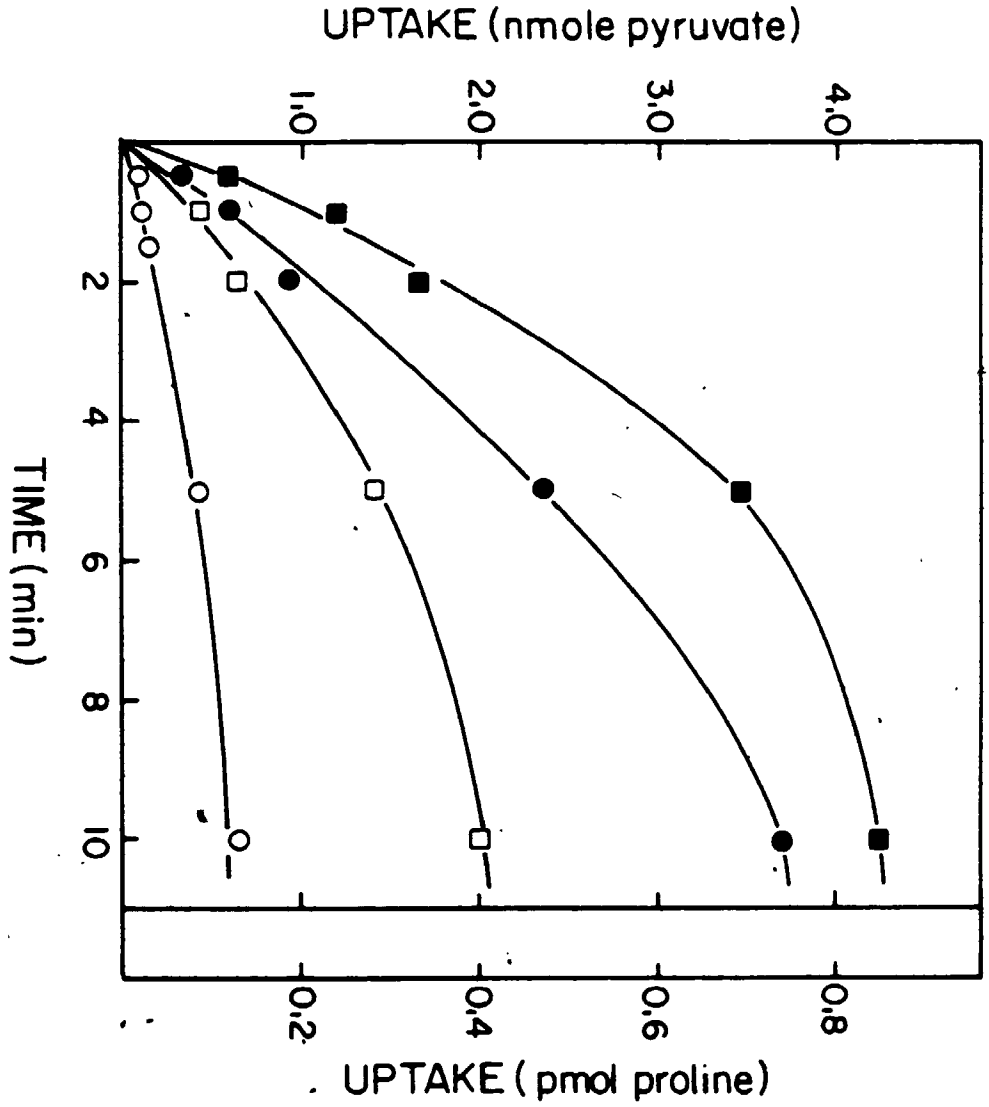
4.3. RESULTS

4.3.1. The effect of osmotic shock on proline transport and pyruvate transport

Osmotic shock of gram-negative bacteria results in the loss of many of the components normally found in the periplasmic space (Heppel, 1971). Loss of transport activity following treatment of the cells by osmotic shock, has generally been interpreted (Rosen and Heppel, 1973; Berger and Heppel, 1974) to indicate the loss of a shock-sensitive binding protein. Figure 4-1 shows the effect of osmotic shock treatment on pyruvate transport and on the shock-insensitive (Berger and Heppel, 1974) proline transport system. It is evident that both transport activities are reduced by osmotic shock, but the drop in

FIGURE 4-1. THE EFFECT OF OSMOTIC SHOCK ON PYRUVATE TRANSPORT AND PROLINE TRANSPORT.

Transport assays and the osmotic shock procedure using E. coli K12 HfrH cells were carried out as described in Materials and Methods. Pyruvate transport was determined in the presence of 20μM pyruvate and proline transport in the presence of 10μM proline to match the respective K_m's of transport. Both shocked and unshocked bacteria were diluted to the same optical density for the transport assays. (■) Proline uptake in unshocked cells; (□) proline uptake in osmotically shocked cells; (●) pyruvate transport in unshocked cells; (○) pyruvate transport in osmotically shocked cells.


UPTAKE (nmole pyruvate)
1.0
2.0
3.0
4.0
TIME (min)
2
4
6
8
10
0.2
0.4
0.6
0.8
UPTAKE (pmol proline)

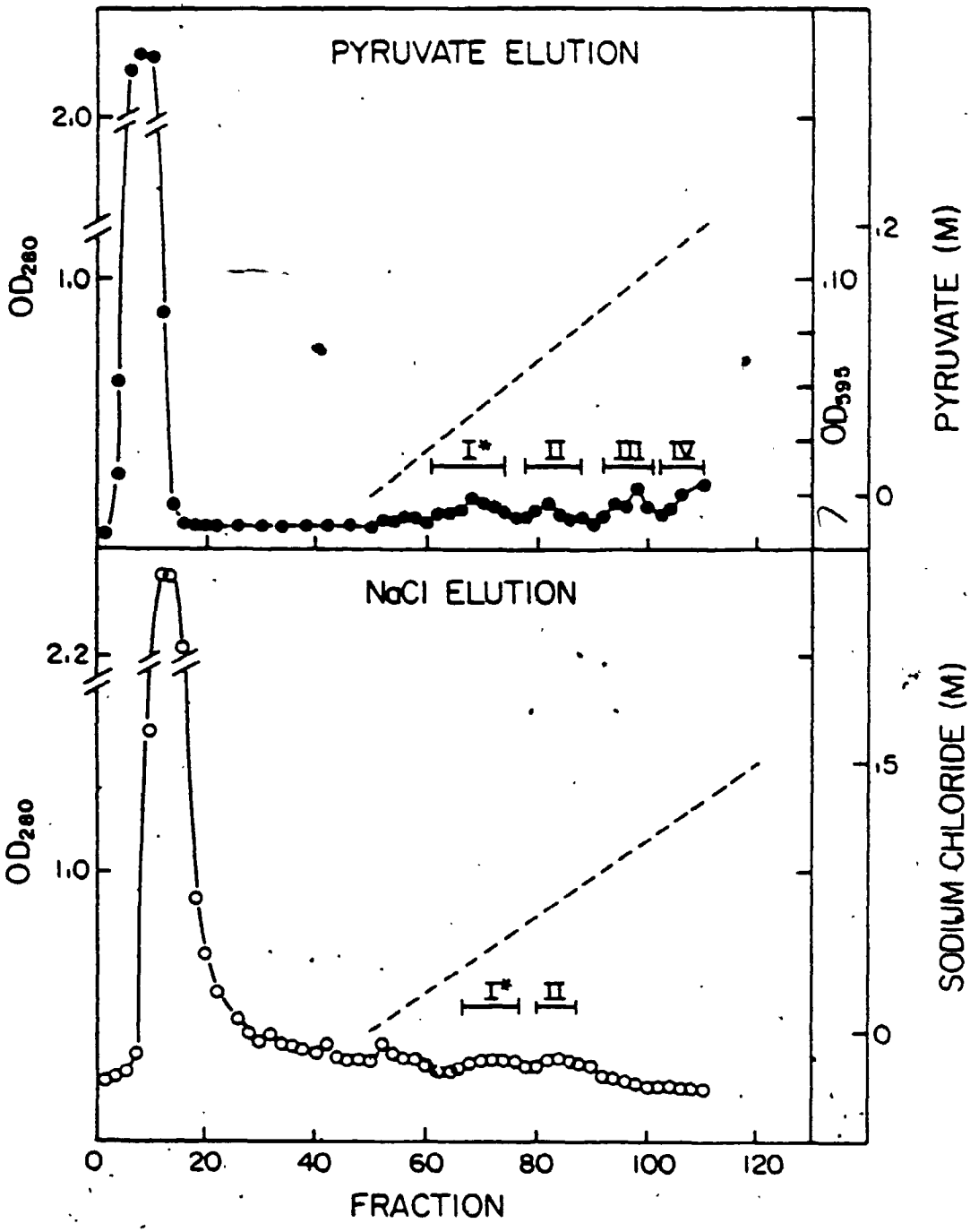
pyruvate transport is proportionally much greater than the reduction in proline uptake. Proline transport was used as a control to indicate the effect of osmostic shock on cellular metabolism, since osmotic shock, as well as releasing the periplasmic contents, has been shown to affect other aspects of metabolism (Heppel, 1969). Transport of pyruvate has been reduced by roughly 90% in shocked cells, whereas transport of proline suffered a 50% loss of activity. Clearly, the results indicate that the drop in pyruvate transport is not due to disruption of metabolic processes alone. Attempts to purify the periplasmic component responsible for the drop in transport activity proceeded based on this result.

4.3.2. Affinity column purification of the periplasmic component of pyruvate transport

Preparation of the shock fluid, as outlined in Methods, achieved an immediate partial purification, since shock releasable proteins constitute only about 15% of the cell protein (Ames and Higgins, 1983). However, many enzymes and binding proteins are released into the shock fluid during the osmotic shocking procedure (Beacham, 1979; Heppel et al. 1972). In order to purify the proteins in the shock fluid capable of binding to pyruvate, an affinity column with a covalently linked pyruvate analogue, 3-hydroxypyruvate, was prepared as outlined in Methods. Figure 4-2 shows the elution profile of affinity columns using column buffer to

FIGURE 4-2. PROFILES OF AFFINITY COLUMNS ELUTED WITH PYRUVATE AND SODIUM CHLORIDE GRADIENTS.

The hydroxypyruvate affinity columns were prepared as outlined in the Materials and Methods section. Protein not bound to the affinity column was eluted with column buffer and the optical density at 280 nm was used to follow this protein elution. Pyruvate gradient elution (●) was initiated at the beginning of the dashed line and was continued to the termination of the gradient at 0.2M pyruvate. The protein content of tnese fractions was determined by the method of Bradford (1976) at OD_{595}. The indicated peaks were pooled and subjected to binding analysis. The peak indicated with the asterisk bound the greatest quantity of pyruvate per milligram protein. The sodium chloride gradient (○) was initiated at the beginning of the dashed line and terminated at 0.5M NaCl. Protein content of the fractions was determined by OD_{280}. The indicated peaks were pooled and the peak with the highest pyruvate binding activity is marked by the asterisk. Fraction volume was 2.7 milliliters.


PYRUVATE ELUTION
2.0
1.0
OD280
OD595
.2
.10
0
PYRUVATE (M)
I*
II
III
IV
NaCl ELUTION
2.2
1.0
OD280
.5
0
SODIUM CHLORIDE (M)
I*
II
0
20
40
60
80
100
120
FRACTION

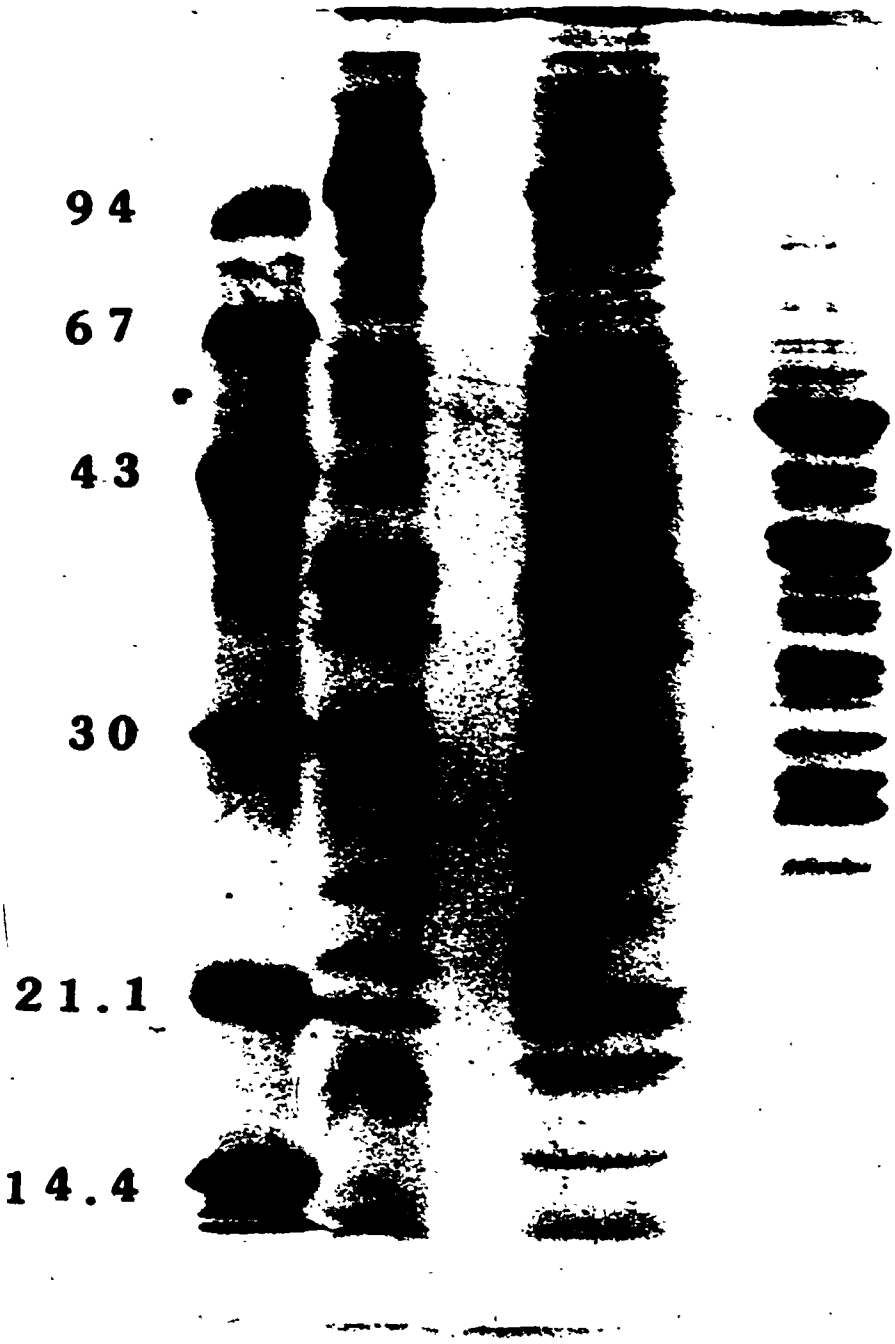
elute unbound protein and pyruvate and sodium chloride gradients to elute the bound protein fractions. The most obvious feature of both column profiles is the large peak of unbound protein which comes off the column almost immediately. This unbound protein peak produces a protein band pattern virtually identical to the shock fluid pattern shown in the SDS PAGE gel in Figure 4-3.

The protein peaks which bound the most pyruvate per milligram protein (according to the simple dialysis binding study as outlined in Methods) are marked with an asterisk in Figure 4-2. Pyruvate elution draws this binding peak off the column at a lower molarity compared to the sodium chloride elution. The pyruvate binding fractions eluted by both sodium chloride and pyruvic acid show similar patterns on SDS PAGE gels. A typical pattern is shown in lane AFF1 in Figure 4-3.

Since the affinity column did not appear capable of resolving one discrete pyruvate binding protein from the mix of proteins loaded, the column was tested for non-specific binding of protein to the diglycidyl ether spacer arm of the epoxy-activated Sepharose. This was accomplished by simply loading shock fluid on epoxy-activated Sepharose without the hydroxypyruvate coupled to the spacer arm. This procedure resulted in no protein being detected after elution with NaCl, indicating that the spacer arm is not responsible for binding any of the proteins eluted off the affinity column.

FIGURE 4-3. SDS-POLYACRYLAMIDE GEL OF THE AFFINITY COLUMN PURIFICATION STEP.

A 12% SDS-polyacrylamide gel was poured and electrophoresis of protein carried out according to the procedure of Laemmli (1970). The lanes were loaded as follows: (LMW) - low molecular weight protein standards (Pharmacia), 94K = phosphorylase b, 67K = bovine serum albumin, 43K = ovalbumin, 30K = carbonic anhydrase, 20.1K = soybean trypsin inhibitor, 14.4K = α-lactalbumin; (AFF II) - protein bound to the hydroxypyruvate affinity column and eluted with NaCl. The protein loaded had been previously separated on an affinity column, constituting the second pass of bound protein through the affinity column step; (AFF I) - bound protein eluted from a hydroxypyruvate affinity column; (SF) - the protein contained in the osmotic shock fluid.


94
67
43
30
21.1
14.4
LMW
AFFII
AFFI
SF

However, 20mM NaCl was incorporated into the column buffer at this stage to discourage any non-specific binding to the affinity column that may have occurred.

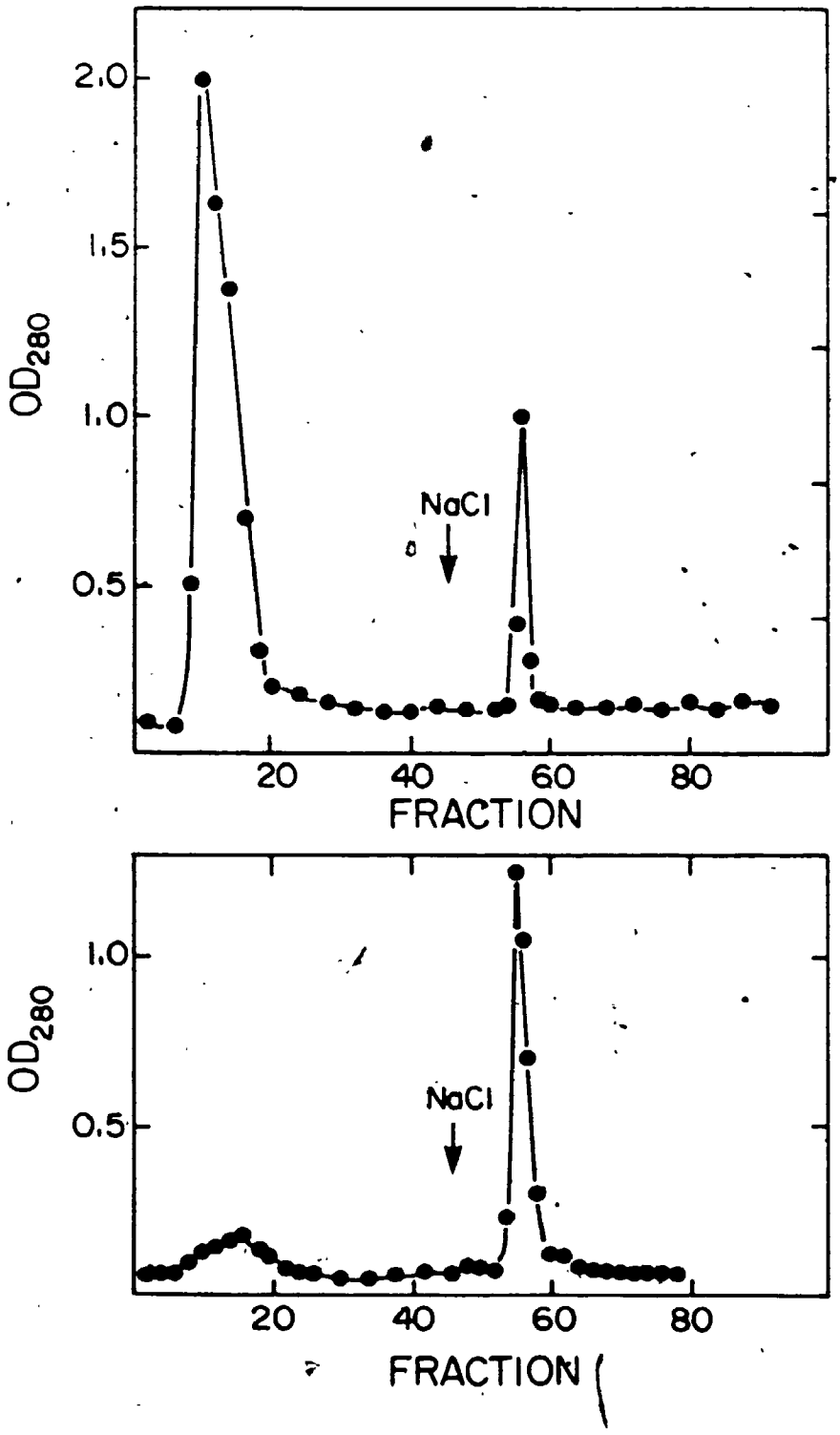
Another possible explanation for the inability of the affinity column to resolve a discrete pyruvate binding protein was overloading of the affinity gel. To test this possibility, the bound protein was eluted from the affinity column with 0.5M NaCl and reapplied to the affinity gel. The column profile from this double affinity run is shown in Figure 4-4. It is evident that some overloading of the column did occur, since a small amount of protein came off in the initial unbound peak. Of the approximately 7 milligrams of protein applied to the column, only a little over 3 milligrams were eluted off in the bound fraction. However, when appled to a SDS PAGE gel, as shown in Figure 4-3, no greater resolution of a single binding protein was achieved, although several bands, notably at 95,000, 36,000, 25,000 and 22,000 molecular weight, appear to be enhanced by this second pass and some minor bands were reduced or disappeared. Due to this inability of the affinity column to purify a discrete binding protein, other column chromatography procedures were applied to this problem.

4.3.3. Purification of the pyruvate binding protein by other chromatography methods

Other column chromatograhy methods were applied to the partially purified affinity column preparation in attempts

FIGURE 4-4. THE EFFECT OF REAPPLICATION OF THE BOUND PROTEIN TO AN AFFINITY COLUMN.

Shock fluid was loaded on an affinity column, as described in Methods, and the elution profile is represented in the top panel. Bound protein was eluted by the addition of 0.5M NaCl to the phosphate-arsenate column buffer. Elution was started at the fraction indicated by the arrow. The protein peak eluted with NaCl was pooled, dialyzed to remove the salt, and reapplied to the washed affinity column. The bottom panel shows the elution profile of the second application of bound protein to the column. Bound protein was again eluted with 0.5M NaCl in column buffer. Fraction volume = 2.7 milliliters.


2.0
1.5
1.0
0.5
OD_{280}
NaCl
20
40
60
80
FRACTION
1.0
0.5
OD_{280}
NaCl
20
40
60
80
FRACTION

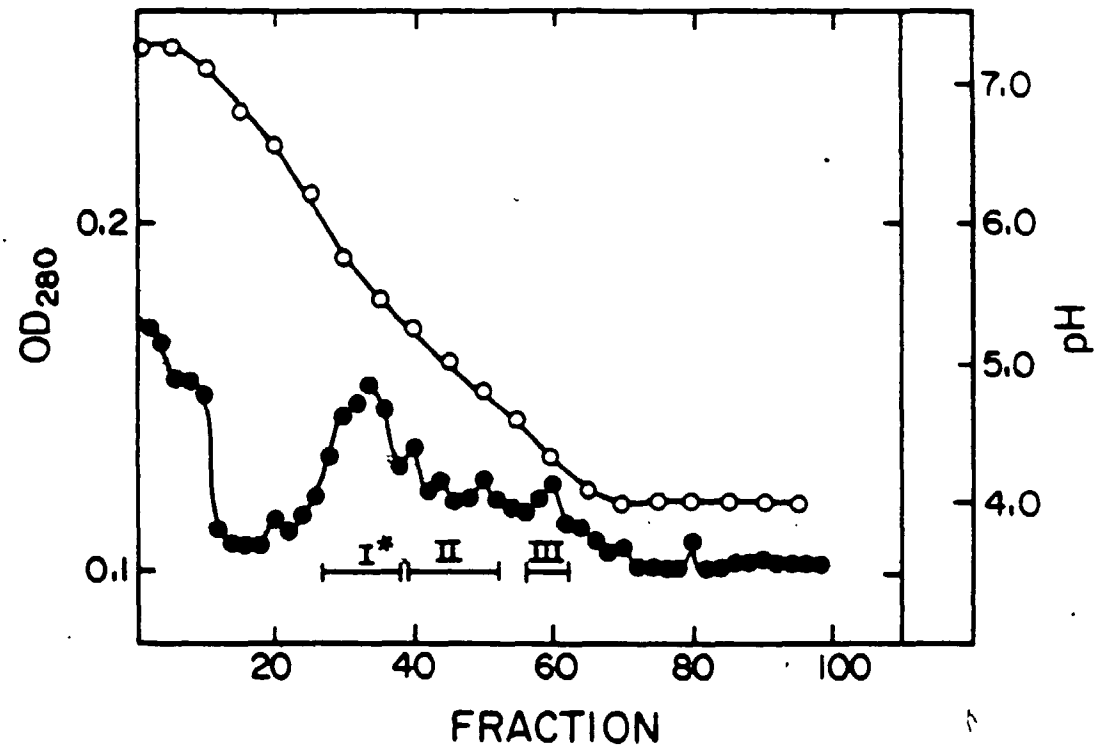
to purify the protein to homogeneity. One of the first methods tried was chromatofocusing, a chromatography method marketed by Pharmacia which separates proteins on the basis of their isoelectric point. Figure 4-5 shows an elution profile of a chromatofocusing run with 3.7 milligrams of protein from an affinity column run. The eluted peak which bound the most pyruvate (marked with an asterisk) corresponded to an isoelectric point of roughly 5.5. Unfortunately, the amount of protein contained in the pooled fractions was insufficient, even after ammonium sulfate precipitation, to apply to a polyacrylamide gel. Subsequent separations with the chromatofocusing column also produced disappointing recoveries of the protein loaded, frequently with a 50% or greater loss of total protein loaded. For this reason, chromatofocusing was abandoned in order to try other methods of separation.

Ion-exchange chromatography was also tried as a method of resolving the proteins eluting from the affinity column. DEAE-cellulose and DEAE-Sephadex, the former an anion exchanger and the latter an anion exchanger and molecular sieve, were both incapable of resolving the binding protein to homogeneity. Although some resolution took place, the yield of protein was too low for any procedure beyond electrophoresis and binding studies.

Since the hydroxypyruvate attached to the affinity column had a free carboxyl group which could be ionized to a

FIGURE 4-5. ELUTION PROFILE OF A CHROMATOFOCUSING COLUMN.

Chromatofocusing (Pharmacia) is a method af separating proteins based on their isoelectric point. Details of the resin and buffer systems are protected by patent. Shock fluid protein was partially purified by the affinity column procedure and applied to the chromatofocusing column. Elution was carried out by a decreasing pH gradient (o) and the protein was determined by optical density (280 nm) readings. The initial large peak (above pH 7) was due to the imidazole buffer used to equilibrate the column prior to loading. The indicated peaks were pooled and binding activity determined. The peak labelled with the asterisk had the highest specific activity of binding. Fraction volume = 2.5 milliliters.


OD280
0.2
0.1
7.0
6.0
5.0
4.0
pH
I*
II
III
20
40
60
80
100
FRACTION

degree on the column, it occurred to us that the affinity column might also be acting as a cation exchanger. To test this possibility, a CM-cellulose column was poured and the proteins which had bound to the affinity column were loaded. Unfortunately, only a few minor proteins were retained on the CM-cellulose, indicating that the contaminating proteins were not due to the affinity column behaving as a cation exchanger.

Gel-filtration, with G-100-Sephadex and G-75-Sephadex, was also applied to the affinity column proteins. Figure 4-6 shows a G-75 elution profile, with two molecular weight standards superimposed. Figure 4-7 shows the SDS PAGE gel of the indicated pooled fractions. Separation has taken place but not to homogeneity. Unfortunately, binding activity was also absent in this particular separation. Comparison of this gel pattern to previously run gels revealed that a major band that normally runs at about 36,000 molecular weight in the affinity fraction is absent from this preparation. This can be easily seen in the comparison to Figure 4-3. A check of previous gels in which pyruvate binding was low or absent also showed a decrease or an absence of this particular band. In an early experiment in which a tube gel was sliced and the protein extracted by homogenization into buffer, the greatest pyruvate binding activity was detected in the middle of the gel, which corresponds to the 30-40,000 molecular weight range. This

FIGURE 4-6. ELUTION PROFILE OF A G-75 SEPHADEX COLUMN.

The G-75 sephadex column was prepared according to the procedure outlined by Pharmacia. Protein bound to the affinity column was pooled, dialyzed, and applied to the G-75 column. Elution was carried out with the phosphate-arsenate column buffer described in Methods. Protein eluted from the G-75 column was followed by optical density readings at 280 nm (o). The indicated peaks were pooled and binding activity assayed. The column had previously been tested for resolution of molecular weight standards by the application of 2.5 millgrams of aldolase (40K molecular weight peak), and 2.5 milligrams of trypsin (23.3K molecular weight peak). This standard elution profile (●) is superimposed above the binding protein elution profile to give a rough molecular weight estimate of the eluted peaks.

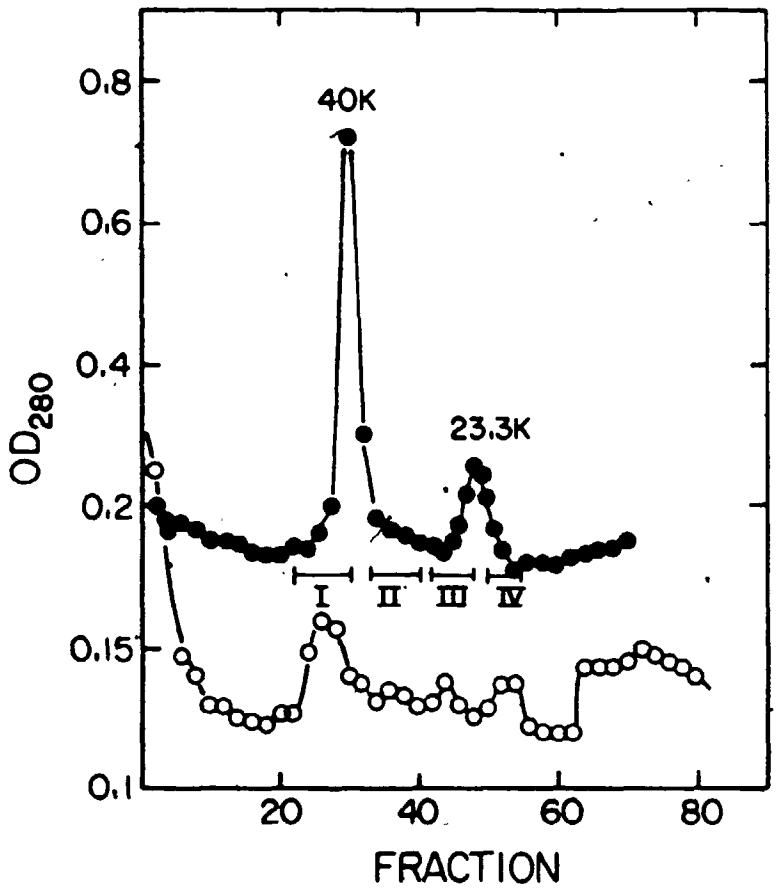
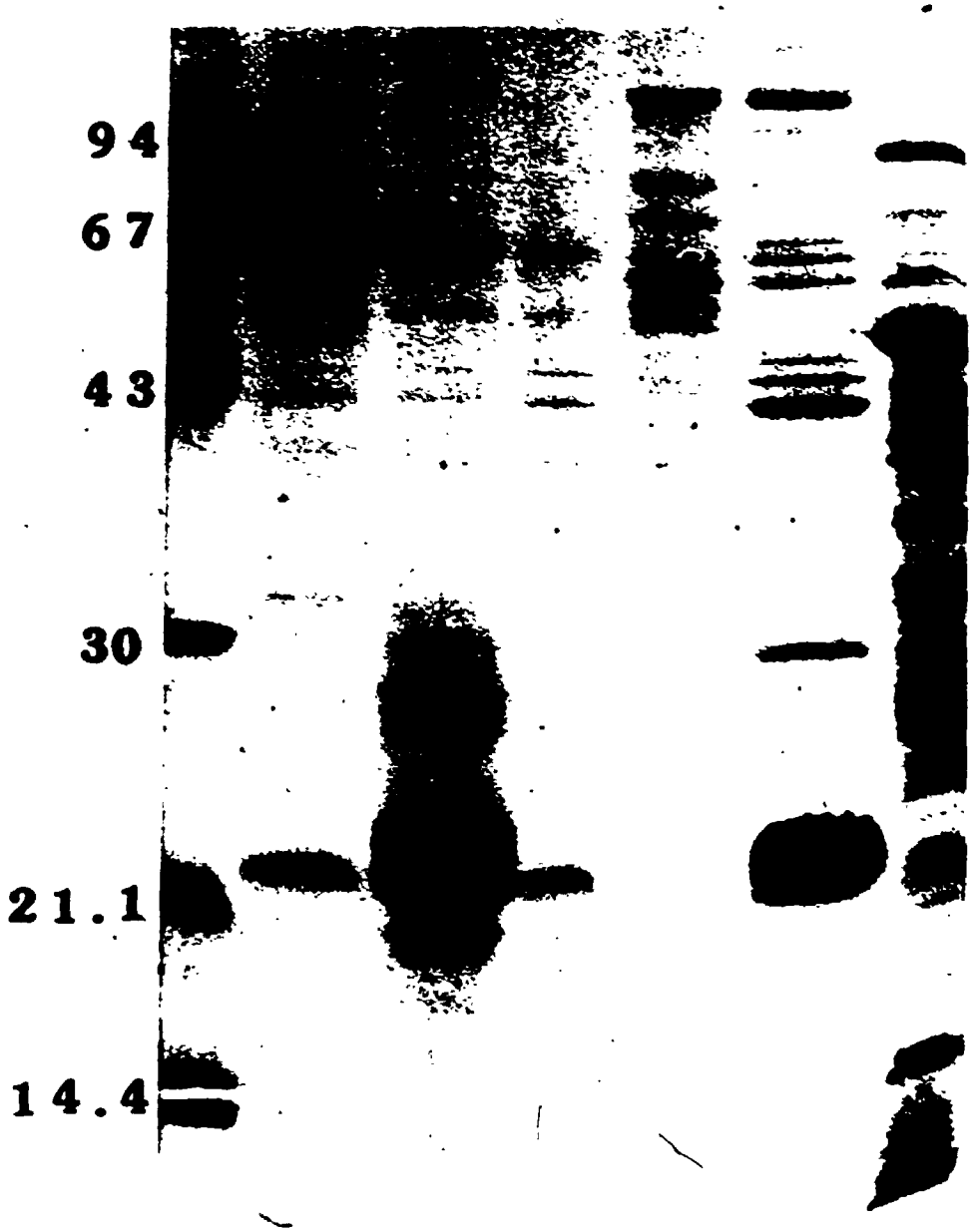

0.8
0.6
0.4
0.2
0.15
0.1
OD280
40K
23.3K
I
II
III
IV
20
40
60
80
FRACTION

FIGURE 4-7. SDS-POLYACRYLAMIDE GEL OF THE G-75 SEPHADEX POOLED FRACTIONS.

The pooled fractions of the G-75 sephadex column elution profile (Figure 4-6) were applied to a 12% polyacrylamide gel prepared and electrophoresed by the method of Laemmli (1970). The lane designations are as follows: (LMW) - low molecular weight protein standards (Pharmacia), 94K = phosphorylase b, 67K = bovine serum albumin, 43K = ovalbumin, 30K = carbonic anhydrase, 20.1K = soybean trypsin inhibitor, 14.4K = α-lactalbumin; (IV) - Peak IV, G-75; (III) - Peak III, G-75; (II) - Peak II, G-75; (I) - Peak I, G-75; (AFF) - pooled protein bound to the affinity column and loaded on the G-75 column; (SF) - shock fluid protein which was loaded on the affinity column.


94
67
43
30
21.1
14.4
LMW IV III II I AFF SF

information, plus the fact that most binding proteins occur as monomers with molecular weights ranging from 22,000 to 42,000 (Boos, 1975), led us to tentatively identify the pyruvate-binding protein as this 36,000 molecular weight monomer. However, this identification must still be verified by purification to homogeneity and determination of a pyruvate binding constant. There is also a possibility that several binding proteins may be isolated for a given substrate, as Sweet et al. (1984) confirmed for citrate transport in Salmonella typhimurium .

The greatest problem found with these methods of column chromatography was resolution of sufficient quantities of the binding protein to carry out characterization. Since a very sensitive method, comparable to an enzyme assay, was not available for detecting the binding protein in the column fractions, column resolution was limited by the need to detect protein peaks by methods such as optical density. If greater quantities of the binding protein were available in the initial shock fluid, then columns such as the gel filtration resins could be lengthened to provide better resolution and still ensure that sufficient binding protein could be detected. Towards this end, attempts were made to induce the pyruvate-binding protein in E. coli .

4.3.4. Methods used to induce pyruvate-binding protein in strains JRG 596 and HfrH

Induction of a binding protein by exposure of the

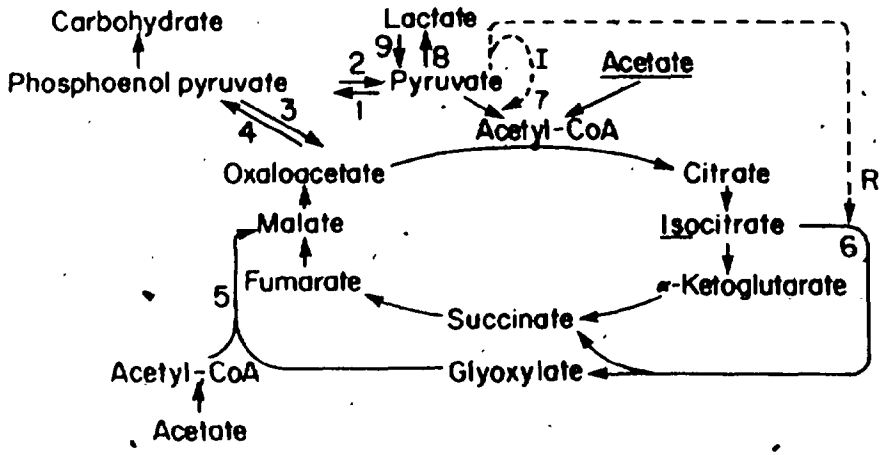
bacteria to the transported substrate has been successfully achieved by many researchers (Sweet et al., 1984; Ito, 1982). The main obstacle associated with the induction of pyruvate-binding protein by the use of pyruvate in the growing medium was the fact that mutants, such as JRG 596, that lack phosphoenol pyruvate synthetase are inhibited in their growth by pyruvate (Brice and Kornberg, 1967). The reason for this growth inhibition according to Flatgaard et al.(1971) is that the glyoxylate cycle is required in these phosphoenol pyruvate mutants to replenish the citric acid cycle, but pyruvate represses these glyoxylate cycle enzymes. The key role that pyruvate plays in these metabolic routes can be easily seen in Figure 4-8.

Supplementation of the growth medium with pyruvate and various citric acid cycle intermediates was tried in a attempt to induce pyruvate binding protein. Addition of each of the Krebs cycle intermediates tested resulted in successful growth of the mutant strain even in the presence of pyruvate. However, no increase in pyruvate transport activity over normal activity could be detected in these cells.

The wild type E. coli HfrH was then grown on pyruvate as the sole carbon source in order to eliminate the possibility that only JRG 596 could not be induced for pyruvate binding protein. However, exposure of HfrH to pyruvate resulted in no noticable increase in the quantity

FIGURE 4-8. METABOLIC ROUTES OF ESCHERICHIA COLI CELLS GROWN ON PYRUVATE.

Schematic of pyruvate metabolism taken from Flatgaard et al (1971). Enzymes are represented by number as follows: (1) phosphoenol pyruvate synthase; (2) pyruvate kinase; (3) phosphoenol pyruvate carboxylase; (4) phosphoenol pyruvate carboxykinase; (5) malate synthase; (6) isocitrate lyase; (7) pyruvate dehydrogenase complex; (8) lactate dehydrogenase (NAD linked or soluble); (9) D- and L-lactate dehydrogenases (not NAD linked or membrane bound); (I) induction of enzyme; (R) repression of enzyme by pyruvate.


Carbohydrate
Lactate
Phosphoenol pyruvate
Pyruvate
Acetate
Acetyl-CoA
Oxaloacetate
Citrate
Malate
Isocitrate
Fumarate
α-Ketoglutarate
Succinate
Glyoxylate
Acetyl-CoA
Acetate
1
2
3
4
5
6
7
8
9
I
R

of protein binding to the affinity column and no perceivable change in band intensities in PAGE gels.

4.4. DISCUSSION

There has been sufficient evidence presented here to make a case for the presence of a periplasmic component of the pyruvate transport system. Treatment of the whole cells by osmotic shock leads to a reduction in pyruvate transport activity that cannot be accounted for strictly on the basis of impairment of metabolism. Transport of shock-insensitive proline was lowered by the osmotic shock procedure, but not to the degree found for pyruvate transport (see Figure 4-1). Protein released by osmotic shock was also able to recognize and bind to an affinity column bearing 3-hydroxypyruvate as the ligand, but did not bind to a similar column using alanine, coupled through the amino group, as a pyruvate analogue. Although the carbon backbones of the two ligands are identical, the alanine lacks the keto group on the second carbon. This result would indicate a specific recognition of the intact pyruvate molecule by the protein that bound to the column. Elution of the bound protein also takes place at a lower concentration of pyruvate than sodium chloride (Figure 4-2), indicating that recognition of pyruvate rather than simple charge is responsible for the effectiveness of pyruvate elution. The partially purified protein was also able to bind to pyruvate in equilibrium dialysis studies, indicating specific recognition of free

pyruvate by one or more of the proteins present.

Failure of the pyruvate affinity column to resolve only pyruvate-binding protein is inexplicable at this point in time. Strong protein-protein interactions may explain the heterogeneity of proteins eluted from the column, but the possibility remains that each of these proteins is capable of binding to pyruvate by specific recognition of substrate or a substrate analogue.

This failure to achieve a one-step purification of the pyruvate-binding protein in E. coli , added to our inability to induce the binding protein, makes the task of purifying the binding protein to homogeneity by conventional column chromatography techniques a difficult if not impossible task. The problems encountered in resolving the small quantities of protein without a more sensitive method of detecting the binding protein were discussed in the Results section. However, even if detection of small quantities of pyruvate binding protein was possible, the problem of preparing sufficient (milligram) quantities of binding protein to do adequate characterization studies would still have to be solved.

Although purification of large quantities of citrate binding protein has been successfully achieved (Sweet et al., 1984) by conventional chromatography using large quantities of cells as the starting material, simple scaling up of our preparation will probably not achieve the same

results. The citrate binding protein was inducible and constituted a major protein in the shock-released starting material, a condition that has yet to be realized for the pyruvate-binding protein.

In order to purify the pyruvate-binding protein to homogeneity, it is likely that the genetic characteristics of the transport system will have to be determined and the techniques of cloning or immunological methods applied to enhance the quantity of binding protein available. The binding protein can then be purified and thoroughly characterized by determination of molecular weight, amino acid sequence, binding constants and specificities.

Ideally, reconstitution of pyruvate transport by addition of purified binding protein into a mutant which lacks or produces inactive binding protein is the best test of the physiological role of the binding protein in the cell. A similar reconstitution of transport activity has been acheived in the well characterized maltose transport system (Brass et al., 1981). The purification of sufficient quantities of pyruvate-binding protein will hopefully allow successful reconstitition studies to take place and erase any doubts about the physiological function of the periplasmic component of the pyruvate transport system.

4.5. CONCLUSIONS

There is ample evidence presented here to substantiate

the conclusion that transport of pyruvate in E. coli takes place via a specific active transport system with a K_m of roughly 20μM. Evidence accumulated from the whole cell and vesicles transport studies indicates that driving force for transport is the electrochemical proton gradient generated by the respiratory chain (see Hellingwerf and Konings, 1985 for an overview of energy flow in bacteria). Although a periplasmic component appears to be involved in pyruvate transport, hydrolysis of ATP, which has been found to be the driving force in most periplasmic binding protein transport systems (Ames and Higgins, 1983), is not involved in transport in vesicles or the whole cells.

Another common feature of periplasmic binding protein systems is their inability to carry out transport in membrane vesicles (Wilson, 1978). This lack of activity is apparently due to the loss of the periplasmic components during the preparation of vesicles. The pyruvate-binding protein appears to be essential to whole cell transport, but membrane vesicles are able to transport pyruvate without the periplasmic component.

Pyruvate transport is not the only exception to the norm of periplasmic binding protein transport systems found in the field of carboxylic acid transport. The dicarboxylate transport system in E. coli has been well characterized by Lo (1977), and like pyruvate transport, has a periplasmic binding protein, is active in vesicles, and is

driven by an electrochemical proton gradient. Although speculation about the molecular mechanism of pyruvate transport is premature at this point in the investigation, it seems likely that because transport is active in vesicles, a membrane-bound component, containing a specific pyruvate recognition site, is responsible for this transport across the cytoplasmic membrane. The pyruvate-binding protein must also contain a specific recognition site for pyruvate and may be required for transport through the outer membrane or through the peptidoglycan layer of the periplasmic space. A mechanism such as this was proposed by Lo (1977) for dicarboxylic acid transport. Further characterization of the pyruvate transport system should determine if the dicarboxylate transport mechanism is common to other carboxylic acids.

The specificity of the pyruvate transport system, although apparently restricted to pyruvate in whole cells, is still in question in membrane vesicles. Although competitive inhibition by L-lactate in vesicles was eliminated by the addition of oxalic acid, there still remains a possibility that the membrane bound component that transports pyruvate in vesicles is capable of recognizing lactate as well. One explanation that would fit both the whole cell and the membrane vesicle response to lactate is that the periplasmic binding protein in whole cells may be the element that ultimately controls the specificity of the

system. The veracity of this explanation will have to be determined by further vesicle studies as outlined in the Discussion section in Chapter 3, and determination of the specificity of the binding site in purified pyruvate-binding protein.

It is evident that much work remains to be done on the pyruvate transport system in E. coli . Future work must concentrate on an analysis of the number of genes involved in the pyruvate transport system and creation of mutant strains that are deficient or defective in these gene products. With these tools, a determination of the molecular mechanism of transport may be eventually made and many of the questions remaining in the field of pyruvate transport answered.

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