This work is dedicated to all who have attempted to teach me the real importance of learning.

A STUDY OF THE HEXOSE-6-PHOSPHATE TRANSPORT SYSTEM IN ESCHERICHIA COLI: THE PROTON TO HEXOSE-6-PHOSPHATE STOICHIOMETRY AT VARIOUS EXTERNAL pHS AND THE ION SPECIFICITY

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# PREFACE

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| | |
|---|---|
| ATP | Adenosine triphosphate |
| Å | Angstrom |
| CCCP | Carbonyl cyanide-m-chlorophenylhydrazone |
| 2-deoxy-Glc-6-P | 2-deoxyglucose-6-phosphate |
| DCCD | Dicyclohexylcarbodiimide |
| $DDA^+$ | Dibenzyldimethylammonium ion |
| DNP | Dinitrophenol |
| Gal-6-P | Galactose-6-Phosphate |
| Glc-1-P | Glucose-1-Phosphate |
| Glc-6-P | Glucose-6-Phosphate |
| HQNO | 2-n-heptyl-4-hydroxyquinoline-N-oxide |
| KSCN | Potassium thiocyanate |
| µg | Microgram |
| µl | Microliter |
| mµ | Millimicron |
| mg | Milligram |
| ml | Milliliter |
| mM | Millimolar |
| mv | Millivolt |
| NADH | Reduced nicotinamide adenine dinucleotide |
| Osm | Osmolarity |
| Pipes | Piperazine-N,N'-bis-(2-ethane sulfonic acid) |

| | |
|---|---|
| TCS | Tetrachlorosalicylanilide |
| TMG | Thiomethylgalactoside |
| $TPMP^+$ | Triphenylmethylphosphonium ion |
| Tris | Tris(hydroxymethyl)aminomethane |
| *uhp* | Gene for hexose phosphate uptake system |

# CHAPTER I

## INTRODUCTION

The major research objectives of this study are twofold: first, to directly determine the proton to glucose-6-phosphate stoichiometries at various pHs of the external medium via the physical method of potentiometry using Escherichia coli cells. These findings may be utilized to further characterize the mechanism of energy coupling to the hexose phosphate transport system in E. coli cells. Second, to further characterize the specific cation and anion effects on the activity of the hexose phosphate transport system in order that a better understanding of the mechanism(s) of the ion effects may be achieved.

# CHAPTER II

## BACKGROUND

*Escherichia coli* cells are enteric bacteria which inhabit the intestinal tract of man and warm-blooded animals. They are classified as gram-negative, nonsporulating rods with peritrichously occurring flagella. They are facultative aerobes which transport numerous ions and metabolites including glycerol, glucose, lactose, but not citrate (1). Control of metabolite transport by the cell membrane, which contains the transport proteins, is thought to be a key step in the regulation of intermediary metabolism. The actual molecular mechanisms of the regulation of transport, the transport processes, and the energy coupling to these processes still are not very well understood. Knowledge about the molecular aspects of these mechanisms is very important for a clear understanding of their function under various conditions.

It is known that *E. coli* cells transport metabolites both by diffusion and active transport. Diffusion of a metabolite can occur by simple diffusion, or by facilitated diffusion which is carrier-mediated diffusion of a substrate down its concentration gradient until equilibrium across the membrane is reached. Active transport is energy-dependent, carrier-mediated transport against a concentration gradient.

The effects of specific salts and/or ion gradients on the activity of a number of transport system in bacteria have been studied. To

date the cotransport systems in which ion gradients are coupled to substrate transport are better characterized than the effects of specific ions which stimulate substrate transport.

## Energy Utilized for Bacterial Transport

To date transport researchers have described at least four functionally distinct classes of transport systems which account for the energy-dependent accumulation of solutes in bacterial cells. The first class is represented by the systems which function by group translocation in which the transported substrate is chemically modified. A classic example is the Roseman system in E. coli (2) which transports glucose and a variety of other sugars. The sugars are phosphorylated in the transport process such that they become "entrapped" and concentrated within the cell. The second class uses the direct coupling of electron transport to substrate transport as in alanine and glycine transport in E. coli (3, 4). The third transport class is the ATP-dependent periplasmic binding-protein-mediated transport systems (5). An example of this class is the glutamine transport system in E. coli. The fourth class is those transport systems which are coupled to the electrochemical proton gradient, $\Delta p$. The E. coli systems for lactose and proline transport belong to this class (6, 7).

It was suggested by Berger and Heppel (8, 9) that transport systems belonging to the latter two categories could be distinguished on the basis of their response to osmotic shock. The ATP-dependent transport systems are shock sensitive, i.e., they lose their periplasmic binding proteins to the shock fluid. The actual role(s) of

the binding proteins in transport is somewhat obscure. It is known for certain transport systems, e.g., the β-methylgalactoside transport system in E. coli, that when the binding protein is lost a decrease in the corresponding transport activity occurs (10). The transport systems that are coupled to the Δp are supposedly osmotic-shock resistant, and do not, in general, possess a periplasmic binding protein (5).

Evidence used for the Berger and Heppel classification is based on studies in which cells were depleted of sources of endogenous energy (9). Using such cells, the effects of different exogenous energy sources on uptake, of inhibitors on stimulation by these exogenous sources, and of genetic alterations in the $Ca^{2+}$, $Mg^{2+}$ -stimulated ATPase were studied. It was shown, for instance, that glutamine transport (shock-sensitive) in the wild type strain (ML308-225) was enhanced by both glucose and D-lactate, whereas in the strain which was ATPase negative (DL54) only glucose stimulated. Glutamine transport in these two strains was inhibited by arsenate (arsenate inhibits all ATP synthesis) under all conditions studied. Also, it was inhibited by uncoupler, but only when D-lactate stimulated transport. These results suggested that only phosphate-bond energy (ATP) which was formed via oxidative or substrate-level phosphorylation could be the immediate energy source for glutamine transport. Proline transport (shock-resistant) in both strains was enhanced by both glucose and D-lactate. However, arsenate only partially inhibited proline transport in the ATPase negative mutant when stimulated by glucose. Cyanide completely inhibited this transport in both strains

stimulated by D-lactate, and uncoupler inhibited under all conditions. These results suggested that the immediate energy source was an "energy-rich membrane state" and that glucose provided energy via both the generation of glycolytic-ATP and electron transport. Also $Ca^{2+}$, $Mg^{2+}$ -ATPase was necessary for ATP driven transport, but not that driven by electron transport.

The suggestion made by Berger and Heppel that the role of ATP in the transport of proline and of glutamine is not the same in that the energized state of the membrane resulting from the $Ca^{2+}$, $Mg^{2+}$ -ATPase activity was involved only in proline transport has been challenged by Singh and Bragg (11). The latter authors using cytochrome-deficient E. coli cells with glucose or glycerol and fumarate in the presence of inhibitors of the ATPase showed inhibition of both amino acid transport systems. Moreover, under such conditions uncouplers inhibited proline and glutamine transport in these cells. These results suggest that glutamine transport is somewhat dependent on the "energized state of the membrane" as well as ATP.

The schemes presented above suggest that each class utilizes a different mechanism to couple energy to transport systems. By determining the immediate source(s) of energy coupled to transport one could further characterize the transport mechanism by performing studies based on observations related to a particular class of transport system.

A great deal of research has been carried out on various bacterial transport systems driven by the $\Delta p$, the electrochemical proton gradient. Many of these studies were designed to determine the direct source(s) of energy coupling to substrate transport. Early on

it was thought that ATP or some other high energy intermediate was always directly coupled to substrate transport (12). However, other evidence suggested that ATP was indirectly involved in driving bacterial transport systems. Harold and coworkers (13) have demonstrated amino acid and cation transport in Streptococcus faecalis that was dependent on ATP hydrolysis via the membrane-bound $Ca^{2+}$, $Mg^{2+}$ -ATPase (S. faecalis is a facultative aerobe that lacks a functional respiratory chain). Harold and Baarda had previously determined that β-galactoside transport in S. faecalis (14) and E. coli (15) under anaerobic conditions was sensitive to uncouplers (CCCP and TCS). This suggests that the ATPase is indirectly coupled to transport via the Δp. It was also shown that N,N'-dicyclohexylcarbodiimide (DCCD) and Dio-9, inhibitors of the ATPase, inhibited active transport, and that transport of $K^+$ and isoleucine in a mutant which possessed a DCCD-resistant ATPase was insensitive to DCCD inhibition (16). Kaback and coworkers have provided evidence which suggests the involvement of the electron transport chain in transport processes. They showed that E. coli vesicles in the presence of arsenate demonstrate the $O_2$-dependent active transport of proline (17) and lactose (18) which meant that electron transport did not necessarily drive substrate transport via formation of ATP. Further, they found that these transport systems were sensitive to uncouplers under such conditions.

In addition to the involvement of electron transport and ATP hydrolysis in driving substrate transport, there were other reports of substrate transport directly coupled to membrane potentials which

resulted from induced ion gradients. Kashket and Wilson (19) have demonstrated active transport of thiomethylgalactoside (TMG) by Streptococcus lactis which were incubated in a potassium-free medium in the presence of valinomycin. The valinomycin induced a $K^+$ efflux from the cells which resulted in the generation of a membrane potential, interior negative. They also showed that uncouplers eliminated this transport, but DCCD had no effect indicating that the ATPase was not involved.

Harold and coworkers (20) showed that E. coli membrane vesicles would generate a membrane potential as the result of the oxidation of D-lactate. They were able to determine the magnitude of this potential, which was about 100 mV, interior negative, as measured by the accumulation of either a lipid-soluble cation, $DDA^+$, or labeled $Rb^+$ in the presence of valinomycin. The potential was depleted by a protonophore, CCCP, or by $K^+$ influx induced by valinomycin. Nigericin, which facilitates the electroneutral exchange of $H^+$ for $K^+$, had no effect. The authors suggested that the oxidation of D-lactate by the respiratory chain caused an electrogenic (an electrogenic process is one in which the transport generates an electrical potential) extrusion of protons which resulted in a membrane potential of the appropriate magnitude to energize active transport. Thus, it appears that the "energized state of the membrane" is at least a membrane potential.

The results cited above appear consistent with the view that oxidation of substrates by the respiratory chain, the hydrolysis of ATP by the $Ca^{2+}$, $Mg^{2+}$ -ATPase, and the induced electrical potentials can lead to an energized state of the membrane. Moreover, this energized state can be utilized to drive active transport. It has become

well accepted that the energized state of the membrane is actually an electrochemical gradient of protons (21, 23, 23).

There was one report of the direct determination of a pH difference across the membranes of respiring E. coli cells, indicating that a ΔpH of 2.0 was observed at external pH 6.0. The ΔpH decreased with increasing external pH (24). pH gradients as large as 0.5 to 1.0 pH unit, interior alkaline, were determined for actively metabolizing S. faecalis cells (25). The significant findings that protons are extruded from E. coli cells (26, 27, 28, 29) and membrane vesicles (30) when substrates are oxidized, bespeaks the possibility that a pH gradient can be generated.

Experiments demonstrating that these gradients can energize transport systems are reported for β-galactosides in S. lactis (31), β-galactosides and proline in E. coli (20), neutral amino acids in S. faecalis (32), leucine in Halobacterium halobium (33), and galactose and gluconate in Clostridium pasteurianum (34). These experiments commonly employed energy-poisoned-cells or membrane vesicles in which an ion gradient was artificially induced by an acid pulse or by addition of valinomycin to $K^+$ loaded cells. The resulting transient accumulation of substrate was determined.

The findings from the experiments cited above show that cells can generate gradients, and these gradients can drive active transport. Assuming that these potentials are the immediate driving forces for transport, what mechanism couples the potentials to transport? The mechanism which appears to be the most valid is a proton-solute co-transport (or symport). This mechanism is a postulate of the chemiosmotic theory of Mitchell (35, 36). According to this theory the

oxidation of electron donors leads to the establishment of an electrochemical proton gradient, $\Delta p$, which results from the electrogenic extrusion of protons. The $\Delta p$, also called the proton motive force (PMF), consists of an electrical component and a chemical component and is mathematically expressed as,

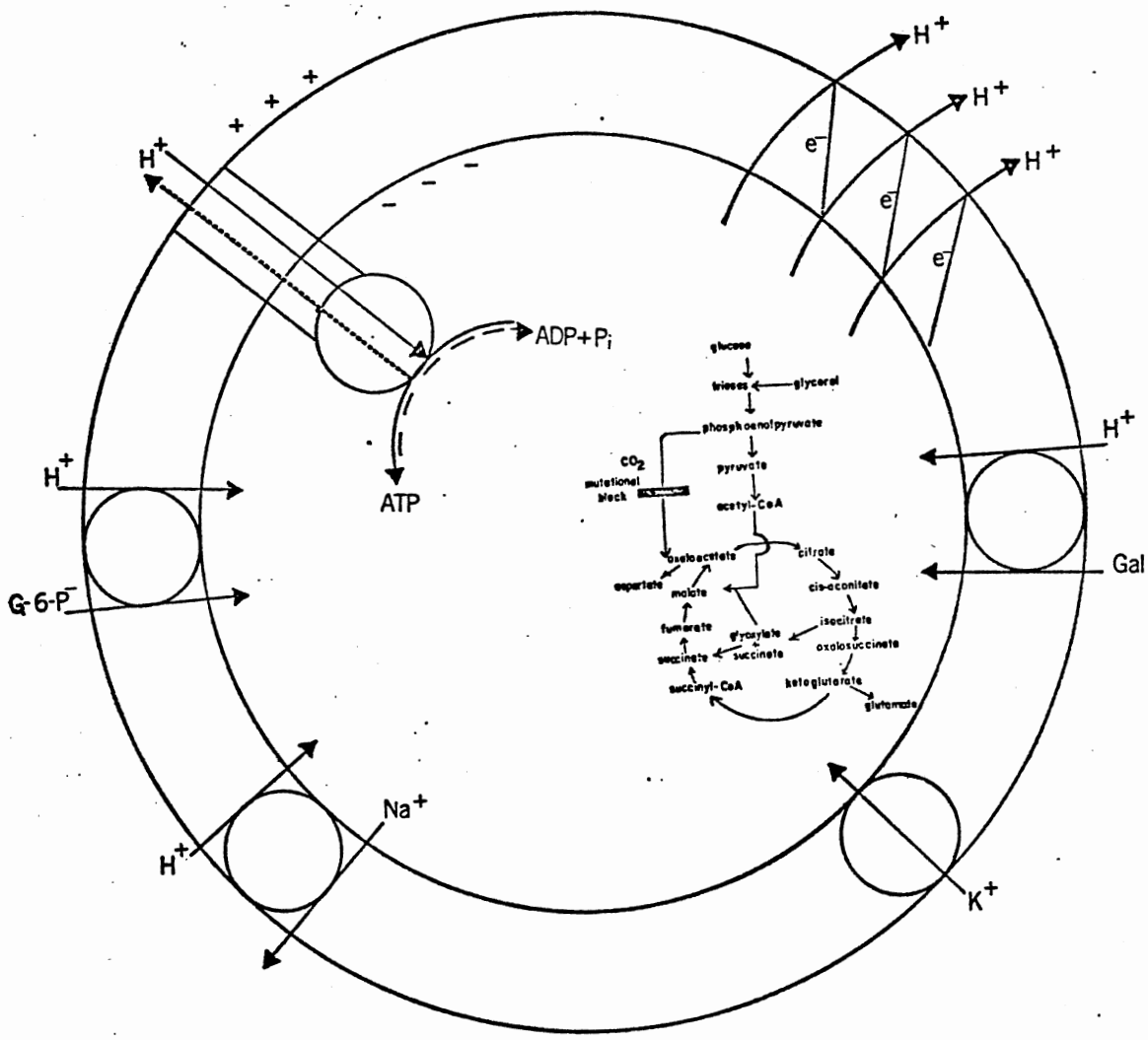
$$\Delta p = \Delta \Psi - \frac{RT}{F} \Delta pH$$

where $\Delta \Psi$ represents the electrical potential across the membrane, and $\Delta pH$ represents the chemical difference in proton concentration across the membrane. The $\Delta p$ is considered to be the primary driving force for active transport of substrates. The transport mode of a variety of substrates is different depending on the type of charge on the substrate molecule (28). Supposedly, cations are transported by a uniporter and their uptake depends only on the electrical potential gradient across the membrane. Neutral substrates and anions are transported by proton-substrate symporters with the former driven by the total proton motive force, and the latter driven only by the pH gradient across the membrane. The symporters can transport proton and substrate in either the same direction, or the opposite direction (called antiporters). A diagram of the typical chemiosmotic transport systems is shown in Figure 1.

Coupling of Energy Under Anaerobic Conditions

There is considerable evidence supporting the view that certain amino acids, sugar, and ion transport systems of bacteria under anaerobic

Figure 1. Chemiosmotic Transport Systems

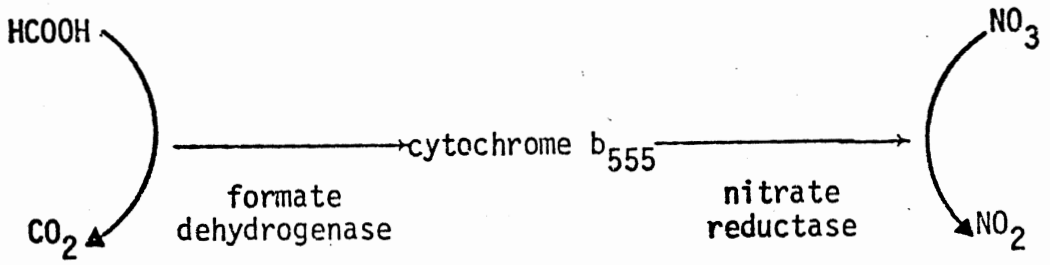

H+
H+
H+
H+
e−
e−
e−
+ + + +
− − − −
ADP+Pi
ATP
H+
G-6-P−
H+
Na+
K+
Gal
H+
glucose
trioses
glycerol
phosphoenolpyruvate
CO2
mutational block
pyruvate
acetyl-CoA
oxaloacetate
citrate
aspartate
malate
cis-aconitate
isocitrate
fumarate
glyoxylate
oxalosuccinate
succinate
succinate
succinyl-CoA
ketoglutarate
glutamate

conditions can be energized by anaerobic electron transport and/or ATP hydrolysis (37). The anaerobic electron transfer systems are thought to be coupled to active transport in a manner similar to that previously described for the aerobic electron transport chain, i.e., electron donors are oxidized, electrons are transferred down the chain via various carriers, and protons are extruded into the medium resulting in an electrochemical proton gradient which is used to drive transport and do other cellular work. A variety of different pathways can function in anaerobic respiration depending on various key enzymes. These enzymes must be induced by the presence of specific electron donors and acceptors.

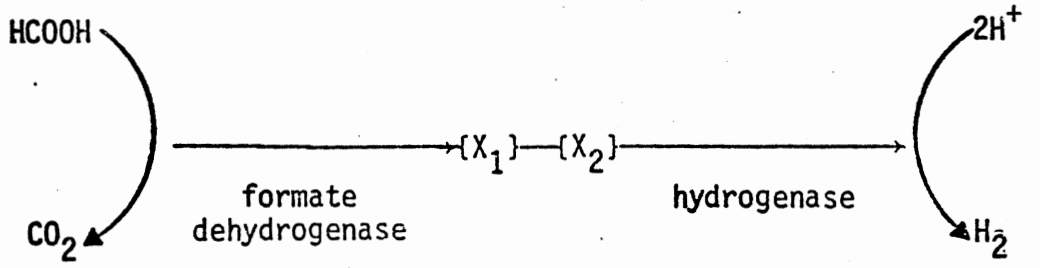
When E. coli are grown anaerobically in the presence of nitrate and formate, nitrate respiration can occur by the formate-nitrate reductase pathway (38). Under such growth conditions both formate dehydrogenase and nitrate reductase are induced. The former enzyme catalyzes the oxidation of formate to $CO_2$ and transfers electrons to oxidized cytochrome $b_{555}$. The latter enzyme transfers the electrons from the reduced cytochrome to nitrate which is reduced to nitrite. An isozyme of formate dehydrogenase is induced under anaerobic conditions in a medium of low pH that contains formate (39). This enzyme occurs as part of the formate-hydrogenlyase pathway which allows for the oxidation of formate, the transfer of electrons via some unidentified electron carriers, $\{X_1\}$, $\{X_2\}$, and the reduction of protons to form $H_2$. This last step is catalyzed by the hydrogenase. Both the formate-nitrate reductase pathway and the formate-hydrogenlyase pathway are shown in Figure 2.

Figure 2. Scheme for the Formate-Nitrate Reductase Pathway and the Formate-Hydrogenlyase Pathway of E. Coli.

Formate-Nitrate Reductase Pathway




Formate-Hydrogenlyase Pathway



Using vesicles of E. coli grown under anaerobic conditions with either nitrate or formate as an electron acceptor, Konings and Kaback (40) demonstrated the coupling of anaerobic electron flow to lactose transport.

Singh and Bragg (11) reported that the anaerobic transport of both proline and glutamine could be stimulated in cytochrome-deficient E. coli cells by the addition of glucose. Uptake of the amino acids energized by glucose was inhibited by inhibitors of the $Ca^{2+}$, $Mg^{2+}$ -ATPase (DCCD, pyrophosphate, and azide) and by uncouplers (CCCP, dinitrophenol). These findings implicate ATP hydrolysis in driving transport of the amino acids. The transport of these amino acids in wild type cells was shown to be energized by electron transfer between glycerol and fumarate via the fumarate reductase system. This system occurs in E. coli cells grown anaerobically in the presence of fumarate. Fumarate causes the induction of fumarate reductase, a key enzyme which acts to convert fumarate to succinate (37). This system is membrane-bound and consists of a dehydrogenase, quinone (menaquinone, or desmethyl-menaquinone), and fumarate reductase which is the terminal oxidase (40). Depending on the growth conditions the substrates that donate electrons to this system are L-α- glycerol phosphate, NADH, L-malate, formate, lactate, and molecular hydrogen. It was also shown that anaerobic growth of E. coli in the presence of fumarate resulted not only in the induction of the fumarate reductase system, but also the formation of the aerobic respiratory chain and the nitrate system as well. It was demonstrated that these three electron transfer systems were linked by a common dehydrogenase in

E. coli vesicles (41). Thus, it seems possible that some electron carriers are common to all three respiration chains. The findings above suggest that E. coli can couple both ATP hydrolysis and anaerobic electron transport to active transport of substrates provided that the cells are grown in the presence of the appropriate electron donor and acceptor.

However, the recent work reported by Konings and colleagues (42) suggests that E. coli K12 cells grown under anaerobic conditions with glucose as the sole carbon source contain a fumarate reductase system. This system will transfer electrons from formate or NADH via menaquinone and cytochromes to fumarate reductase. As indicated by the growth yields and maximal growth rates of mutants impaired in electron transfer or the ATPase (uncB), the fumarate reductase system is an important metabolic energy source under anaerobic conditions. Using vesicles prepared from cytochrome-sufficient and uncB cells, they demonstrated that electron transfer by the fumarate reductase system caused the formation of a membrane potential and a PMF. The former was measured by the uptake of $TPMP^+$, and the latter by the uptake of labeled amino acids. Glutamine transport was used as an indicator of the formation of ATP, and proline transport was used to indicate the formation of a PMF. Using these indicators it was demonstrated that the PMF is generated by ATP hydrolysis in cytochrome-deficient cells and by electron transfer in uncB cells. Based on growth parameters of wild type cells under anaerobic conditions it was suggested that such cells prefer to be energized by electron transfer via the fumarate reductase system, rather than by ATP hydrolysis.

Coupling of Protons and Uptakes

The major criteria for determining whether a transport system is coupled to a proton gradient are as follows: 1) a stimulation of substrate transport should occur when the $\Delta p$ across the membrane is enhanced. This phenomenon has been demonstrated in energy depleted cells as described above (refer to page 8) (31, 43); 2) the influx of substrate should occur simultaneously with the influx of protons under the appropriate conditions (44); 3) conditions resulting in the depletion of the $\Delta pH$ across the membrane should eliminate the substrate transport as well, i.e., conditions in which proton pumping is blocked by energy depletion, inhibitors, or protonophores (45). Including some of the transport systems described above (lactose, proline, etc.) there are a sizable number of systems in E. coli and other bacteria that are energized by proton gradients. Refer to Harold (1977) for a list of these systems. It has been estimated that 40% of all known E. coli transport systems are coupled to a proton gradient (46).

Transport Stoichiometries of Protons to Substrates. The proton to substrate stoichiometry studies can provide information about the proton:substrate stoichiometry and the mode(s) of energy coupling to the transport process. In general, the stoichiometry is obtained by measuring the accumulation of substrate and the corresponding accumulation of protons. The ratio of protons to substrate accumulated gives the corresponding stoichiometry value relative to the particular set of experimental conditions used to make the measurements. The differences between the stoichiometry value determined at a given external

pH and the charge on the substrate molecule at that same pH constitutes the net charge of proton-substrate cotransport. If the net charge of cotransport is positive, then this process is electrogenic and can be coupled to the $\Delta\Psi$ and the $\Delta pH$. If the net charge is neutral, then only the $\Delta pH$ can be coupled to the cotransport process. Also, a comparison of the estimates of the $\Delta p$, $\Delta pH$, and the $\Delta\Psi$ at a given set of experimental conditions to the substrate concentration gradient determined under the same conditions can be made in order to verify the possible modes of energy coupling to cotransport. One would expect the level of the particular energy parameter coupled to this process is at least as great as that of the substrate concentration gradient based on the first law of thermodynamics.

Padan et al. were the first to report that the $\Delta pH$ across the membrane of intact E. coli cells depends on the external pH (24). They show that the $\Delta pH$ is 2 units at pH 6.0, 0.0 at pH 7.65, and -0.51 at pH 9.0. Also in EDTA/valinomycin treated cells the $\Delta\Psi$ is almost constant (110-120 mV) over a range of pHs from 6.0 to 8.0.

Ramos and Kaback (49), using energized E. coli vesicles and the flow dialysis technique, determined the magnitudes of the chemiosmotic parameters ($\Delta p$, $\Delta pH$, and $\Delta\Psi$) at various pHs of the external medium. In parallel experiments they determined the concentration gradients of various substrates under the same conditions. Figure 3 illustrates the results of these studies. At pH 5.5 they found that the $\Delta p$ is approximately -200 mV and about 60% of this is due to the $\Delta pH$ component. At pH 7.5, the $\Delta p$ consists solely of the $\Delta\Psi$ component and the $\Delta pH$ is zero. The $\Delta\Psi$ remained about -75 mV at all pHs.

Figure 3. Chemiosmotic Parameters and Concentration Gradients Versus External pH

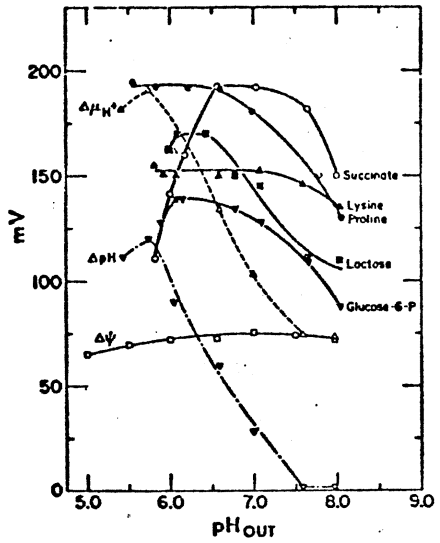



FIGURE 4: Effect of external pH on $\Delta\bar{\mu}_{H^+}$ (Δ), ΔpH (▽), and $\Delta\Psi$ (□) and on steady-state levels of accumulation of lactose (▣), proline (◎), lysine (▲), succinate (○), and glucose-6-P (▼). ΔpH and $\Delta\Psi$ were determined by flow dialysis at given external pH values as described in Figure 2 of Ramos and Kaback (1977) using [1-$^{14}$C]acetate (54 mCi/mmol) at a final concentration of 37.5 μM and [$^{3}$H]TPMP$^{+}$ (1.33 Ci/mmol) at a final concentration of 24 μM, respectively. $\Delta\bar{\mu}_{H^+}$ was calculated from these values as described in Methods. Concentration gradients for lactose, proline, lysine, succinate, and glucose-6-P were determined by flow dialysis at given external pH values as described in Figures 1 and 2 of Ramos and Kaback (1977) using sodium ascorbate (20 mM), PMS (0.1 mM), and isotopically labeled transport substrates at the following specific activities and final concentrations: [1-$^{14}$C]lactose (22 mCi/mmol) at 400 μM; [U-$^{14}$C]proline (236 mCi/mmol) at 8 μM; [U-$^{14}$C]lysine (306 mCi/mmol) at 6.5 μM; [2,3-$^{14}$C]succinate (5.18 mCi/mmol) at 200 μM; and [U-$^{14}$C]glucose-6-P (38.7 mCi/mmol) at 70 μM. Calculations were made as described in Methods. Vesicles prepared from *E. coli* ML 308-225 grown on succinate (Methods) were used for the studies with acetate, TPMP$^{+}$, lactose, proline, lysine, and succinate; and vesicles prepared from *E. coli* GN-2 grown on glucose-6-P (Methods) were used for the studies with glucose-6-P. Although not shown, it is important that $\Delta\Psi$ with GN-2 membranes is −45 mV at pH 5.5 and −100 mV at pH 7.5 (see insets in Figure 3, sections IIA and IIB in Ramos and Kaback, 1977).

Ramos,S., and Kaback,H.R.,(1977) Biochemistry 16, 854

The results of the studies cited above (24, 49) show that E. coli cells and vesicles possess a $\Delta pH$ near 0.0 at external pHs 7.5 and above. Also, they observed that the magnitude of the $\Delta p$ at pH 7.5 was not sufficient to account for the anion (e.g., Glc-6-P) concentration gradients based on a mechanism in which there is one proton taken up per anion molecule (49). Thus, the above findings are not in agreement with the original chemiosmotic model for anion transport.

Rottenberg (50) has provided models for proton-metabolite cotransport in bacterial cells which indicate that the level of anion concentration gradients can be accounted for by increases in the proton:anion stoichiometry at high external pHs. One major assumption concerning the mechanism of transport in all cases is that a ternary complex between the proton, carrier, and substrate is formed such that its overall charge is neutral. The models are illustrated in Figure 4.

The following formulation shows the relationship between the magnitude of the $\Delta p$ and the number of protons cotransported: under steady state conditions,

$$S_{out} + nH^+_{out} \rightleftarrows S_{in} + nH^+_{in}$$

where S is the substrate, n is the number of protons, $H^+$, cotransported with S.

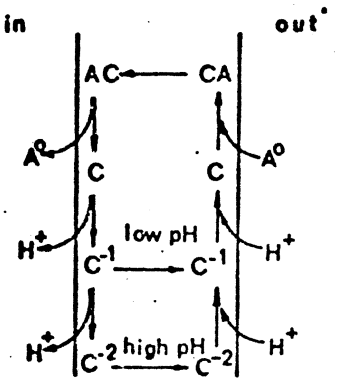
Under non-equilibrium conditions the free energy, $\Delta G$, represented by the proton and substrate gradients are,

$$\Delta G_S = RT\ln(S_{in}/S_{out})$$

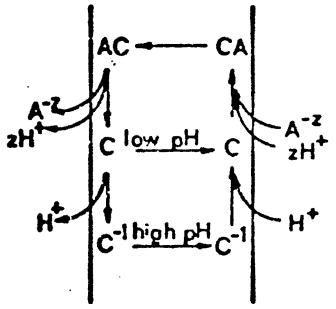
$$\Delta G_{H^+} = RT\ln(H^+_{in}/H^+_{out}) + zF\Delta\Psi$$

Figure 4. Rottenberg Models for Cotransport in Bacteria

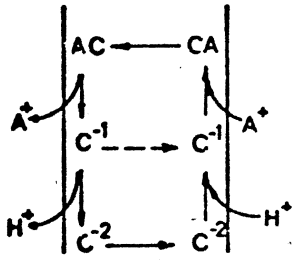
**A** neutral substrates




**B** acidic substrates




**C** basic substrates



where R, T, and F have their usual meanings, and z is the number of charges on the transported complex.

According to the first law of thermodynamics the following relationship must exist for a tightly coupled proton-substrate cotransport system:

$$\Delta G_s = -n\Delta G_{H^+}$$

From this relationship the following relation can be derived:

$$\omega\log(S_{in}/S_{out}) = -n\{\omega\log(H^+_{in}/H^+_{out}) + z\Delta\Psi\}$$

where $\omega$ is 2.3 RT/F and $z = 1^+$

or

$$\omega\log\ (S_{in}/S_{out}) = -n(\Delta\Psi - \omega\Delta pH) = -n\Delta p \qquad \text{Equation 1}$$

Thus, for n = 1, $\omega\log\ (S_{in}/S_{out}) = -\Delta p$

for n = 2, $\omega\log(S_{in}/S_{out}) = -2\Delta p$.

Using a similar approach, equations for the cotransport of protons with charged substrates can be derived (cf. Rottenberg, 1976). The following equations are applicable to the transport of anionic substrates at low or high pHs:

at low pH ($\sim$5.5) with z = 0

$$\log\ (S^{-1}_{in}/S^{-1}_{out}) = \Delta pH \qquad \text{Equation 2}$$

at high pH ($\sim$7.5) assuming a charged carrier with a neutral $pK_a$ with n=3 and z=1

$$\omega \log (S^{-2}_{in}/S^{-2}_{out}) = -\Delta\Psi + 3\omega\Delta pH \qquad \text{Equation 3}$$

Equation 2 indicates that the substrate concentration gradient at low pH is equivalent to the magnitude of the $\Delta pH$. Equation 3 shows that the substrate concentration gradient at high pHs is equivalent to the sum of the magnitudes of both the $\Delta\Psi$ and the $\Delta pH$.

Essentially two methods have been used to determine proton-substrate stoichiometries. The first method directly determines the effective proton accumulation that occurs concomitantly with the accumulation of labeled substrate into nonmetabolizing cells that are under anaerobic conditions. The $H^+$ flux is determined potentiometrically by monitoring the change in pH of the external medium. The labeled substrate accumulation is measured by the classical filtration method. The cells must be nonmetabolizing since other proton fluxes resulting from substrate metabolism would interfere with the measurement of substrate-induced proton uptake. For exactly the same reason, the cells must be kept under anaerobic conditions such that endogenous energy stores cannot be utilized. Also, thiocyanate, a lipid-soluble anion, is added to the cells for the purpose of depleting the charge gradient which could result from proton-substrate influx. The addition of thiocyanate may enhance proton-substrate uptake several fold. This approach gives a measure of the facilitated diffusion of protons and substrate into cells under such conditions. The proton-substrate stoichiometry is obtained by extrapolating

to zero time a curve obtained from the plot of the ratio of proton uptake to substrate uptake at time intervals throughout the period of transport. For a more detailed description of this method refer to West and Mitchell (1973).

These investigators, using the above described method, have determined a one-to-one proton to lactose stoichiometry for lactose transport in E. coli cells blocked in lactose metabolism at pH 7.0 (44, 51). They obtained a similar stoichiometry ratio value using a nonmetabolizable lactose analog, TMG. This stoichiometry indicates that transport of lactose probably occurs in an electrogenic manner, assuming no compensating ion fluxes. Thus, lactose transport could be energized by both the pH gradient and the electrical potential across the membrane.

The second method of determining proton-substrate stoichiometries uses the flow dialysis technique on energized membrane vesicles under aerobic conditions (44, 47-49). By this method the active transport of substrates is indirectly monitored and quantitative estimates of the chemiosmotic parameters $\Delta p$, $\Delta pH$, and $\Delta\Psi$ can be obtained at various pHs of the external medium. The $\Delta\Psi$ is measured by determining the distribution of a labeled lipophilic cation such as $TPMP^+$ across the vesicle membranes. This technique was developed by Liberman and Skulachev (52). The $\Delta pH$ is measured by determining the distribution of a labeled permeant weak acid such as $\{^{14}C\}$ acetate across the membrane. These measured values can be used to determine the internal pH by application of the method of Waddel and Butler (53). The corresponding $\Delta pH$ is then calculated by determining the difference between

the internal and external pHs. By use of the chemiosmotic equation the corresponding $\Delta p$ can be calculated. In parallel experiments with energized vesicles treated similarly, except that labeled substrate is used, the substrate concentration gradients are obtained at given external pHs. Using the appropriate equations with the experimentally determined values for the substrate gradients and the external pHs, the theoretical proton-substrate stoichiometry values can be calculated. For a more detailed description of this method refer to Ramos and Kaback (1977).

These investigators have used this method to study energized E. coli vesicles throughout a pH range of 5.5 to 8.0. Coupling this method with the use of ionophores (valinomycin and nigericin) they carried out titration studies which showed that there are essentially two classes of transport systems: first, systems that are preferentially coupled to the $\Delta p$, e.g., lactose, proline, etc. These systems are sensitive to both ionophores at pH 5.5, but only valinomycin at pH 7.5. Second, systems that are preferentially coupled to the $\Delta pH$, e.g., lactate, Glc-6-P, etc., are sensitive only to nigericin at pH 5.5, but only valinomycin at pH 7.5. A plot of the estimated magnitudes of the chemiosmotic parameters and the concentration gradients of the transported substrates versus the corresponding pHs of the external medium is illustrated in Figure 3.

As described above for anion accumulations at pH 7.5, an increase in the proton-substrate stoichiometry may cause the transport of lactate and Glc-6-P to become electrogenic. This transport could then be coupled to the $\Delta\Psi$ at the higher pHs. This suggestion is supported

by evidence obtained from similar experiments, as described above, in which the steady-state levels of the various substrates were compared to the magnitudes of the $\Delta p$, $\Delta pH$, and the $\Delta\Psi$ determined under identical conditions. The accumulation of lactate and Glc-6-P shows a direct linear relationship to the $\Delta pH$ at pH 5.5, but only to the $\Delta\Psi$ at pH 7.5. The slope derived from a plot of these variables is indicative of the proton-substrate stoichiometry. The slopes obtained for the data at pH 5.5 show a proton-substrate stoichiometry of one for lactate and greater than one but less than two for Glc-6-P. The latter value agrees well with the calculated theoretical value of 1.7 for Glc-6-P at this pH. The slope at pH 7.5 indicates the proton-substrate stoichiometries increase to one greater than the number of charges on the molecule transported (which is two for Glc-6-P at pH 7.5). Thus, the stoichiometry increases to three for Glc-6-P and two for lactate. These values suggest that transport of these substrates becomes electrogenic at pH 7.5. Similar experiments, with lactose and proline transport, gave results showing the substrate accumulation proportional to the $\Delta p$ at pH 5.5 and to the $\Delta\Psi$ at pH 7.5. Also, similar increases in the proton-substrate stoichiometries were determined for both neutral substrates as well. At pH 5.5 the stoichiometry value of 1.0 was obtained, and at pH 7.5 the value increased to 2.0.

The Rottenberg models are well supported by stoichiometry results reported by Ramos and Kaback (49). As shown above, the ionophore titration studies determined that the transport of the neutral substrates is preferentially coupled to the $\Delta p$ at pH 5.5 and to the $\Delta\Psi$ at pH 7.5 and above. These results agree well with that indicated by

Equation 1. The relationships expressed in Equation 2 and Equation 3 are also supported by Kaback's anion studies, since the anions are preferentially coupled to the $\Delta pH$ at low external pHs, but to the $\Delta\Psi$ at high pHs. Kaback's results show that the $\Delta pH$ is zero at pH 7.5 and above. Thus, the $\Delta pH$ term in Equation 3 will be zero at high pHs, leaving only the $\Delta\Psi$ component to energize anion transport. This conclusion is supported by the titration results, since only the $\Delta\Psi$ energized transport at pH 7.5 and above.

Also, the Rottenberg formulations which show that the proton-substrate stoichiometry can increase at higher external pHs are supported by two separate findings: first, Figure 3 shows that the $\Delta p$ is insufficient thermodynamically to account for the substrate concentration gradients at external pH values greater than 6.0-6.5, based on a 1:1 stoichiometry as postulated by Mitchell (44). This observation implies that the stoichiometry must increase to account for the substrate concentration gradients. Second, the indirectly determined proton-substrate stoichiometries show an increase in stoichiometry at pH 7.5 for all substrates studied. These increased stoichiometry values appear to account for the experimentally determined substrate concentration gradients based on the magnitude of the $\Delta p$, $\Delta pH$, and the $\Delta\Psi$ as indicated by Equations 1 and 2. These two equations indicate that an increase in proton-substrate stoichiometry will allow for an increase in the substrate concentration gradient.

Stoichiometries for the cotransport of protons with lactate and alanine in non-metabolizing E. coli cells were ascertained via the first method (54). Based on the magnitude of the proton fluxes and

lactate uptake at pH 6.5 and the electroneutral character of lactate uptake, the investigators concluded that the stoichiometry of this process was one proton per one lactate anion. The electroneutral character of lactate uptake was determined by the absence of $K^+$ efflux on the addition of $Ca^{2+}$ -lactate to cells in the presence of valinomycin. A similar determination was made for the proton-alanine transport under the same conditions and the same transport stoichiometry value was obtained. However, when cells were grown in a chemostat with alanine as the only carbon source and limiting nutrient, this stoichiometry was found to change to two protons per alanine, and then to four protons per alanine. The authors conclude that the increased stoichiometries are due to the selection of mutants which occur as the result of selective pressures operating in the chemostat. The mutant apparently has the capacity to increase its growth rate by changing the transport system giving rise to an increased intracellular alanine concentration.

The uptake of dicarboxylic acids in E. coli was shown to be obligatorily linked to proton uptake (55). Using essentially the first method, the stoichiometry value of two to one was determined for the transport of protons and succinate, malate, fumarate, or aspartate. This finding led the authors to suggest that succinate, fumarate, and malate are transported in electroneutral form, whereas aspartate is transported as a cation. They concluded that the uptake of the dicarboxylic acids, except for aspartate, is energized by the $\Delta pH$ across the membrane. Aspartate uptake by this system apparently is energized by the electrical potential.

The stoichiometry values for the transport of protons with phosphate and L-glutamate in various strains of Saccharomyces yeast cells has been reported (56). Sacc. carlsbergensis cells were grown in a chemostat with a limiting supply of phosphate in order to stimulate the subsequent rate of phosphate transport. Using the direct potentiometric method they determined a stoichiometry value of three equivalents of protons per mole of phosphate taken up. Using flame photometry they determined that the amount of $K^+$ efflux was two equivalents of potassium ion during the uptake process. These results suggested that phosphate transport was electrogenic. Similar experiments were performed with Sacc. cerevisiae cells which resulted in proton-glutamate transport stoichiometry of three equivalents of protons per mole of glutamate with the efflux of 1-2 equivalents of $K^+$. All of their experiments were done at pH 4.8, and based on the $pK_a$s at this pH both phosphate and glutamate would possess a single negative charge. A proton stoichiometry of three means that the net charge per anion transported is +2. Thus, the charge balance for the process is maintained by the efflux of the two positive ions. Apparently, the potassium ion gradient is also involved in the transport of these anions. The authors suggest that the sodium ion gradient may play a similar role.

## Coupling of Other Ion Flows and Uptakes

Ion Gradients and Uptakes. Presently, there is an abundance of evidence, as described above, which is consistent with the notion that an ion gradient can be coupled to active transport of a substrate via

an ion-substrate cotransport mechanism. Also, the evidence is consistent with the idea that the potential energy that can be coupled to transport is in the form of an electrical potential and a chemical potential depending, among other things, on the charge of the substrate. Cations experiencing the influence of the electrical potential attempt to move inward due to the potential interior negative. The uptake of negatively charged species would be opposed by this potential. Neutral species would not be affected by the potential, unless they are transported as a positively charged complex with other cations. In the latter case, they would experience the same potential influence as that of cations. It is possible that the uptake process may be electrogenic, and as a result, the net uptake of negatively charged species would be inhibited. The net uptake of positively charged species would be stimulated. Also, the electrogenic transport must lead to ion fluxes by secondary processes not directly involved in substrate transport. Many transport studies on a variety of systems have provided evidence which is consistent with the scheme described above (57, 58). Besides the reports on proton substrate cotransport, there are few reports concerning the measurement of stoichiometries of other ions cotransported with substrates in bacteria.

It has been known for quite some time that E. coli grown in log phase cultures will establish potassium and sodium ion gradients. The intracellular $K^+$ concentration is greater than the $K^+$ concentration in the medium, and the intracellular $Na^+$ concentration is less than the $Na^+$ concentration in the medium (59). Thus, these cells maintain oppositely directed gradients of $Na^+$ and $K^+$ which may be coupled to substrate transport. Rhoades and Epstein (60) have provided evidence for

the existence of three different $K^+$ transport systems which can function to maintain the $K^+$ gradient. Also, West and Mitchell (44) have demonstrated the presence of a $Na^+/H^+$ antiport system which functions to extrude $Na^+$ from within the cells.

The fact that the $K^+$ and $Na^+$ ion gradients exist under normal conditions would lead one to suggest that they may be coupled to cotransport of substrates in like manner to the proton gradient. However, the lack of a report on the involvement of $K^+$ ion gradients in metabolite cotransport in E. coli may imply another role for $K^+$ besides that of direct coupling to cotransport systems (7). On the other hand, there are a few reports of the direct involvement of $K^+$ in cotransport systems of other bacteria and yeast. Potassium fluxes appear to play a role in the amino acid-proton-$K^+$ cotransport of acidic amino acids in Staphyloccus aureus (61). As stated above, $K^+$ fluxes are apparently involved in the cotransport of amino acids and protons in various strains of Sacc. yeast. The $K^+$ movement in this case is thought to be due to charge imbalance resulting from the electrogenic cotransport process, and probably not due to countertransport by the amino acid carrier.

Considering the importance of $Na^+$ gradients in energizing a variety of $Na^+$-substrate cotransport systems in eukaryotes, it is somewhat surprising to find only a few reports on this kind of cotransport in prokaryotes (46, 58). There are only two reports of $Na^+$ gradients coupled to $Na^+$ cotransport systems in E. coli. Glutamate transport experiments with both cells (62, 63) and vesicles (64, 65, 66) indicate that this amino acid is accumulated by a $Na^+$ -substrate

cotransport process. In these studies the effect of various inhibitors, uncouplers, and ionophores was examined. The agents which depleted either the $H^+$ gradient or the $Na^+$ gradient caused a decrease in transport. Recent studies with vesicles derived from E. coli B cells show that glutamate uptake is both stimulated by $Na^+$ and driven by a $Na^+$ -cotransport system (67). Experiments on the melibiose transport system in E. coli cells and vesicles provided evidence which is consistent with a $Na^+$ (or $Li^+$) -substrate cotransport mechanism. In the latter study TMG and cells which were lactose transport-negative were used to eliminate both substrate metabolism and transport of the TMG by the lactose system (68).

Evidence supporting the role of $Na^+$-substrate cotransport in bacteria was first reported by Stock and Roseman for the melibiose (permease II) transport system of Salmonella typhimurium (69, 70). They determined that TMG transport was markedly stimulated by $Na^+$ concentrations ranging up to 5 mM. Increased concentration of TMG stimulated $Na^+$ accumulation in these cells. Also, a $Na^+$ gradient is required for glutamate transport in H. halobium indicating a $Na^+$-glutamate cotransport process (71). Phosphate transport in yeast is thought to be coupled to a $Na^+$ cotransport system as well (72).

Ion Specificity and Uptakes. Sodium ions as well as other specific ions have the capacity to modulate (stimulate or inhibit) the activity of particular transport systems. In most cases the mechanism(s) by which the transport activity is altered is not at all well understood. Thompson and MacLeod (73, 74) suggest that $Na^+$ and $K^+$ ions, rather than their ion gradients, stimulate α-aminoisobutyric

acid (α-AIB) transport activity in marine Pseudomonads. They were able to show that $Na^+$ and $K^+$ gradients were not involved, since the $K_m$ and $V_{max}$ for α-AIB transport was unchanged when the standard suspension medium was replaced by one containing $Na^+$ and $K^+$ at the same concentrations as that present within the cells. Subsequent studies show that internal $K^+$ is required for α-AIB uptake, and that there is a relationship between $K^+$ uptake and α-AIB transport (75).

Using aconitase mutants of S. typhimurium, it was demonstrated that $Na^+$ stimulated the high affinity citrate transport system (76). Using $K^+$ depleted E. coli cells, it was shown that glutamate uptake via a glutamate transport system was greatly reduced. Preincubation of such cells in the presence of $K^+$ fully restores their capacity for glutamate uptake when $Na^+$ ions are also present in the uptake medium. However, addition of either $K^+$ or $Na^+$ alone restores glutamate uptake to only 20% of its maximum capacity in the presence of both cations. Kinetic studies show that changes in $K^+$ concentration affect the capacity for glutamate uptake, but have no effect on the $K_m$ for glutamate (63). Harold and Spitz (77) have determined that $K^+$ stimulates the accumulation of $PO_4^{2-}$ and glutamate in S. faecalis. They suggest that $K^+$ is involved indirectly in the transport processes. It was reported that $PO_4^{2-}$ transport in E. coli membrane vesicles is best stimulated when $K^+$ and $Mg^{2+}$ are present at concentrations of 40 mM and 2.5 mM, respectively (78).

Besides the effect of $H^+$, $Na^+$, and $K^+$ on the activity of various transport systems, it was demonstrated that 10 mM $Li^+$ stimulates the rate of proline transport in E. coli cells as compared to the control

which was run in the absence of monovalent cations. $Na^+$ and $K^+$ show slight stimulation of this activity. In experiments with cells that were treated with various carbon sources, KSCN, p-chloromercuribenzoate, uncouplers, or lipid soluble cations, the levels of $Li^+$ stimulation of proline uptake were determined. The results of these studies and kinetic studies show a saturable $Li^+$ stimulation effect. This fact led the authors to suggest that $Li^+$ stimulates proline transport by interacting with a membrane component other than the proline carrier. Also, proline uptake in E. coli membrane vesicles is inhibited by $Na^+$ (7). Cuppoletti and Segel (79) have shown that sulfate transport in Penicillium notatium is stimulated by both protons and metal ions. $Mg^{2+}$, $Ca^{2+}$, and other divalent cations at micromolar concentrations are the most effective. However, higher concentrations ($\sim$ 10 mM) of monovalent cations, e.g., $Na^+$, can produce a similar level of stimulation. It is suggested that $SO_4^{2-}$ transport may occur by an anion exchange mechanism with $HPO_4^{2-}$, or $OH^-$. This suggestion is based on indirect evidence obtained from studies using labeled $Ca^{2+}$ which show that only 0.2 equivalents of $Ca^{2+}$ is accumulated with 1.0 equivalent of $SO_4^{2-}$.

The effect of anions on substrate transport has received little attention by investigators based on the lack of reports. One report claims that intracellular $SO_4^{2-}$ and $HPO_4^{2-}$ strongly stimulates succinate transport in Bacillus subtilis (80). Apparently succinate is not transported by an anion exchange diffusion mechanism, because phosphate efflux is low or absent during succinate transport.

## Hexose Phosphate Transport System in E. coli

The hexose-6-phosphate transport system has been reviewed by Dietz (81). The first report which strongly suggested that Glc-6-P was transported by a hexose phosphate transport system (called the uhp transport system) was provided by Fraenkel et al. (82). They demonstrated that a glucokinase negative mutant which was unable to phosphorylate glucose could, in fact, transport and grow on Glc-6-P, but could not grow on glucose. Pogell and coworkers (83) discovered that the Glc-6-P transport system could be induced in E. coli B cells by the addition of Glc-6-P to the suspension. This finding was noted from experiments in which the transport rate of labeled Glc-6-P was compared to that of cells treated with protein synthesis inhibitors. These results suggest that the induction process requires de novo protein synthesis. Further, they showed in studies with energy starved cells that glucose or succinate could stimulate 2-deoxyglucose-6-P uptake by this transport system. This finding indicates that hexose-6-P transport is energy dependent. Besides these important findings, they obtained evidence from substrate specificity studies which demonstrated that this transport system was truly a hexose phosphate system, i.e., other sugar phosphates such as 2-deoxy-Glc-6-P, Glc-1-P, fructose-1-P, fructose-6-P, and mannose-6-P also could be transported by this system. α-methyl-glucoside-6-P, Gal-6-P, and Glc-6-sulfate were not transported indicating that this system requires specific groups at the one, four, and six positions of the hexose phosphate molecule. Winkler (84) also provided evidence that indicated that Glc-6-P and 2-deoxy-Glc-6-P could induce the formation of the

hexose-6-phosphate transport system. Later studies show that only external Glc-6-P and 2-deoxy-Glc-6-P induce this system (85). The findings cited above provided not only a new insight into the induction process of the uhp transport system, but also a stimulus that elicited many subsequent investigations concerning its unique properties of substrate specificity, energy coupling, and ion specificity.

## Substrate Specificity

Besides the hexose phosphates cited above, this transport system also interacts with numerous other sugar phosphates, such as pentose-P, amino-hexose-P, and heptulose-P, indicating a broad range of substrate specificity (81). This conclusion is based on results obtained from studies carried out either directly by measuring the uptake of a labeled substrate, or indirectly by determining whether a solute would show significant competitive inhibition of labeled Glc-6-P uptake (86). Using progressively larger concentrations of Glc-6-P it was demonstrated that the uhp system exhibited substrate saturation. The $K_m$ value for Glc-6-P transport reported for this system is $5 \times 10^{-4}$ M. It was also possible to measure efflux of labeled Glc-6-P, called counterflow, after the addition of unlabeled Glc-6-P to labeled Glc-6-P-loaded cells. These observations provided good evidence for a substrate specific, saturable, mobile carrier operative in active transport of Glc-6-P.

## Coupling of Metabolic Energy to Transport

It is stated above that Pogell et al. (83) were the first to demonstrate the energy dependence of the uhp system. Their

observations implied the involvement of both glycolysis and electron transport in providing energy for hexose phosphate transport, since substrates for both energy producing systems stimulated hexose-P uptake. Thus, subsequent research aimed at determining if both of these energy producing pathways were involved in supplying metabolic energy to the uhp transport system.

Dietz (87), after demonstrating that the uhp transport system functioned normally in membrane vesicles, determined that this system was coupled to electron transport. This determination was based on experiments showing stimulation of transport activity by the addition of oxidizable substrates (D-lactate, succinate, and α-glycerol phosphate) to the vesicles. However, ATP appears not to stimulate and the addition of arsenate to D-lactate-stimulated cells did not inhibit the stimulation, indicating that oxidative phosphorylation reactions were not coupled to hexose phosphate transport. Experiments with inhibitors and uncouplers were performed with whole cells showing that agents which uncouple, or inhibit electron transport cause the inhibition of hexose phosphate transport (86). Also, it was reported that energy-poisoned cells transported hexose phosphate, but with a greatly lowered affinity for influx. The $K_t$ of efflux, however, did not change, indicating that energy was coupled to the transport mechanism at the membrane exterior. This phenomenon appears to be a somewhat unique property of the uhp transport system. In contrast, the β-galactoside system on energization of the membrane lowered the affinity of the carrier for the efflux reaction (88, 89).

Essenberg and Kornberg (29), using whole cells, have demonstrated that both ATP utilization inhibitors (arsenate, DCCD) and electron transport inhibitors (cyanide, HQNO, anaerobiosis) partially inhibit uhp transport, whereas combinations of the inhibitors (ATP inhibitor and ETC inhibitor) produce a greater inhibition than the sum of that produced by each inhibitor alone. These findings suggest that ATP or electron transport could serve to directly energize the transport of hexose phosphates. Further, they showed that this transport was sensitive to uncouplers (DNP, CCP, TCS) both in the absence and presence of oxygen. This observation indicates indirect coupling of these energy-producing systems to hexose-P transport.

By use of direct potentiometry and filtration methods as described previously, Essenberg and Kornberg (29) have demonstrated that the uptake of Glc-6-P in cells of strain RE-48 under anaerobic conditions occurs concomitantly with a measurably alkalinization of the external medium. In similar experiments they determined that the transport stoichiometry of proton to Glc-6-P was one-to-one at pH 6.6 of the external medium. These findings and the finding that the uhp transport system was sensitive to uncouplers are consistent with a proton-Glc-6-P cotransport mechanism.

Based on the evidence above, the hexose phosphate system in E. coli might be classified, according to the Berger and Heppel scheme (refer to page 4), as an osmotic-shock-resistant, $\Delta p$ dependent system. However, it has been demonstrated that the uhp transport system in E. coli is shock-sensitive (90), but appears not to contain a periplasmic binding protein (81). This indicates another unique characteristic of this system.

As previously cited, Ramos and Kaback (49) have obtained estimates of the transport stoichiometries of protons with lactose, proline, and Glc-6-P via an indirect method with energized membrane vesicles. They claim that Glc-6-P transport at external pH 5.5 is electroneutral and coupled primarily to the $\Delta pH$. However, at pH 7.5 this transport is electrogenic and is coupled solely to the $\Delta\Psi$. Moreover, they provide evidence for a proton to Glc-6-P stoichiometry of 1.7 to 1.0 at pH 5.5, and 3.0 to 1.0 at pH 7.5. These results agree with the fact that more than one proton would be required to neutralize the negative charge on the phosphate moiety of Glc-6-P at both pHs (Glc-6-P has two pKs; $pK_1$=0.94, $pk_2$=6.11). In order for this transport to occur in an electrogenic manner at pH 7.5, at least the uptake of three protons per Glc-6-P transported would be required; two protons required to neutralize the two negative charges per Glc-6-P, and one additional proton was needed to make the process electrogenic.

Evidence reported by Tokuda and Konisky (91) obtained from experiments with colicin Ia treated cells and membrane vesicles of *E. coli* indicates a change in mode of energy coupling to the *uhp* transport system at low and high pH. They demonstrate that the colicin Ia treatment causes membrane depolarization to occur. This effect induces the collapse of the $\Delta\Psi$ under all conditions tested, but actually produces an increase in the magnitude of the $\Delta pH$ at pH 5.5. As a result, Glc-6-P transport is stimulated at pH 5.5, but inhibited at pH 7.5. These results confirm those of Ramos and Kaback, which suggest that Glc-6-P transport is preferentially coupled to the $\Delta pH$ 5.5, but to the $\Delta\Psi$ at pH 7.5.

## The Involvement of Other Ions in Hexose Phosphate Transport

As stated above, protons and other ions are involved in the transport of a variety of substrates. Besides their role in cotransport systems, certain cations are required for normal transport activity, and still others greatly stimulate transport activity. The mechanism of coupling of ion gradients to cotransport systems is thought to be well understood, but the mechanism(s) by which ions stimulate transport systems has yet to be elucidated.

Essenberg and Kornberg (29) have demonstrated that 140 mM KCl stimulates 12 fold the uptake of Glc-6-P in *E. coli* cells constitutive for the *uhp* system. Also, $Na^+$ and choline stimulated this transport, but not as well.

The only other report of ion effects on the *uhp* system showed that 50 mM fluoride ion had little effect on Glc-6-P transport; however, the uninduced level of *uhp* transport was inhibited (86).

This study was carried out for the purpose of determining the validity of the Kaback hypothesis in whole *E. coli* cells using the direct method of potentiometry. This hypothesis is based on results obtained by the indirect method of flow dialysis on membrane vesicles. It states that the mechanism of energy coupling to the proton-Glc-6-P cotransport system changes as a result of increasing the external pH. At low pHs ($\sim$ 5.5) this cotransport system is electroneutral and coupled only to the $\Delta$pH. At high pHs ($\sim$ 7.5) the Glc-6-P cotransport becomes electrogenic as a result of an increase in proton:Glc-6-P stoichiometry which is greater in value than the charge on the Glc-6-P

molecule. Thus, Glc-6-P cotransport at the higher pHs is coupled only to the $\Delta\Psi$. The proton and Glc-6-P uptakes were determined in cells under anaerobic conditions with and without KSCN at external pHs from 6.0 to 8.0. The ratio of the proton to Glc-6-P uptakes at a given pH provides the proton to Glc-6-P stoichiometry value at a given pH. Also, the effects of varying concentrations of cations and anions on the activity of Glc-6-P transport were examined. The results of the various specific ion studies provide information about the mechanism(s) which effects the stimulation of Glc-6-P transport.

# CHAPTER III

# MATERIALS AND METHODS

## Materials

Sodium {U-$^{14}$C} glucose-6- phosphate (specific activity 250 μCi per mMole) and {U-$^{14}$C} 2-deoxy-D-glucose (specific activity 10 μCi per mMole) were obtained from ICN. Nitrocellulose filters of pore size 0.45 μm and glass-fiber prefilters of type A-E were supplied by Gelman Instrument Company. Creatine phosphokinase, yeast hexokinase, adenosine triphosphate, creatine phosphate, tris (hydroxymethyl) aminomethane, piperazine-N-N'bis(2-ethane sulfonic acid), p-bis-O-(methyl styryl)-benzene, 2,5-diphenyloxazole, monosodium glucose-6-phosphate, 2-deoxy-Glc-6-P, barium fructose-1-phosphate, sodium azide, α-methylglucoside, and Dowex-1-chloride (X8, 200-400 mesh) were purchased from Sigma Company. Salts used in the uptake assays were of highest available purity. All other chemicals were reagent grade and purchased from commercial sources.

The barium salt of fructose-1-phosphate was converted to the sodium salt by the use of equimole amounts of sodium sulfate to precipitate the barium.

### Preparation of 2-deoxyglucose-6-Phosphate

The synthesis of {U-$^{14}$C}-2-deoxyglucose-6-phosphate was performed

exactly as described by the procedure of Lowenstein (92), except that the reaction mixture was incubated with the enzymes for three hours, rather than 45 minutes, which resulted in a higher product yield. One major peak of radioactivity was obtained from the Dowex-1 X 8 column and these radioactive fractions were neutralized and concentrated by rotary evaporation at 35-40°C using vacuum from a water aspirator. Analysis of the radioactive peak by thin layer chromatography, using an MN 300 cellulose plate (Analtech, Inc.), developed in t-butanol:HCL (4:1), and autoradiography showed only one spot of radioactivity with an $R_f$ value identical to that of the standard 2-deoxy-Glc-6-P. The unlabeled standards were visualized by acid molybdate treatment according to the procedure of Hanes and Isherwood (93).

## Strains Used

The various strains of *E. coli* used in these experiments are listed in Table I. Refer to Bachman, Low, and Taylor (94) for the definitions of various gene abbreviations.

## Selection of *uhp*$^C$ Mutants

All *uhp*$^C$ mutants were obtained by selection for growth on plates containing minimal 56 media (95) supplemented with various amino acids, 10mM fructose-1-P, and solidified with 2% agar (96). Colonies that appeared were plated on minimal 56 plates supplemented with various amino acids for the purpose of identifying cells with the proper phenotypic properties. The appropriate mutants were selected and grown in glycerol minimal salt media as described under "Growth

TABLE I

STRAINS USED

| Strain | Markers | Parent Strain | Reference to Parent Strain |
|---|---|---|---|
| RE-48 | *uhp*$^C$, *pgi*, *zwf* | K10-7-15 | (29) |
| RE-114 | *uhp*$^C$, *lacI*, *lacZ*, *uncA* | DL-54 | (97) |
| RE-115 | *uhp*$^C$, *uncA* | NR-70 | (98) |
| RE-157 | *uhp*$^C$, *his*, *pro*, *uncA* | NI-44 | (99) |
| RE-90 | *uhp*$^C$, *his*-1, *pgi*-7, *eda*-1, Δ*eda*-*zwf*, *str*-115, *pgi*::Mu 1 | DF-214 | (100) |

All of the strains were derived by selection for growth on F-1-P (96).

of Bacteria." Using these mutants, rates of uptake of labeled Glc-6-P with and without 1 mM glucose were compared. Mutants which showed high rates of Glc-6-P uptake compared to the parent that were unaffected by the presence of glucose were selected for subsequent transport studies. Uhp constitutive derivatives of the uncA strains were tested for the presence of the uncA lesion by testing growth on succinate and glucose. UncA strains only grow on glucose.

## Growth of Bacteria

Batch cultures which were to be used in transport studies were inoculated with 1/100 volume of overnight glycerol-grown cultures, an grown overnight at 37°C on half-strength minimal salts medium 56 (95). This medium was supplemented with vitamin $B_1$ at 1 µg per ml. Glycerol at 20 mM and other carbon sources at 10 mM were added to the medium. RE-157 cells were grown on the same minimal medium except that it was supplemented with 10 mM glucose, histidine (50 mg/L), and proline (50 mg/L). Cells were harvested in exponential growth phase (0.2 to 0.3 mg dry weight/ml) by centrifugation at 5000 x g.

## Measurement of Labeled Hexose Phosphate Uptakes

In a typical assay, RE-48 cells were suspended in the desired medium at a density of 0.5 to 1.00 mg dry weight per ml, buffered to pH 6.6 with 1 mM Pipes-tris buffer. In cases where the salt might change the pH, it was checked and adjusted as necessary with dilute HCl or dilute KOH. Assays of total volume 0.25 - 1.0 ml were carried

out at room temperature in 15 ml scintillation vials. To initiate the assay 1/20 volume of 2 mM {U-$^{14}$C} Glc-6-P (specific activity 1 mCi per mMole) was added. Using Eppendorf pipettes, samples of 0.1 or 0.2 ml were taken from the assay mixture at intervals and immediately filtered through nitrocellulose filters of 0.45 μm pore size. The filters were washed with 2.0 ml of the salt solution of interest. The filters were dried overnight at room temperature. By use of a Beckman LS3150 Liquid Scintillation Counter set with counting error less than 2.0%, the amount of radioactivity of each filter was determined in toluene containing 0.4% 2,5-diphenyloxazole and 0.02% p-bis-O-(methylstyryl)-benzene. A standard amount of the labeled Glc-6-P was counted in the same manner in order that the counting efficiency (typically about 80%) could be determined. The blank determinations (controls) were carried out with cells which had been treated with either 50 μl toluene per ml or 1 mM $HgCl_2$. The toluene supposedly destroys the membrane permeability barrier for small molecules and ions such that Glc-6-P might enter or exit via nonspecific routes (101). The $HgCl_2$ is thought to specifically inhibit the hexose phosphate transport system, if it is added prior to the addition of the hexose-6-phosphate (102). Averages of the nmoles Glc-6-P uptakes per mg dry weight were calculated for at least three determinations in most cases. Alterations in this procedure are noted in the figure and table legends.

Stoichiometry Experiments

The apparatus used for this study consisted of a water-jacketed 6.0 ml capacity sample cell (Bolabs) which was capped with a rubber

stopper, fitted with a magnetic stirrer and two combination pH electrodes (Markson - 1885, Corning - 476050), whose potentials were amplified by an electrometer (Johnson Foundation, University of Pennsylvania). The pH and ΔpH output from this latter device was recorded on an oscillographic recorder (Bell and Howell). Concentrated cells were added to the sample cell maintained at 25°C which contained 140 mM KCl at the desired pH. This cell suspension also contained, where indicated, 25 mM KSCN and 0.5 mM buffer. Pipes were used for studies at pHs 6.0 and 7.0, and tris buffer was used at pHs 7.5 and 8.0. This cell suspension had a total volume of 4.0 ml and a cell density of 1.65 mg dry weight per ml. The mixture was bubbled with water-saturated, oxygen-free nitrogen and stirred magnetically during a 20 minute equilibration period and throughout the entire assay period. All solutions that were added to the cell suspension were bubbled with nitrogen as well. The pH of the cell suspension was adjusted by the addition of small aliquots of HCl or KOH. The final pH was adjusted similarly to a value which was approximately 0.03 pH units more alkaline than that of the five μmoles of {U-$^{14}$C} Glc-6-P or {U-$^{14}$C} 2-deoxy-Glc-6-P solution which was added to initiate that assay. At time intervals of 15, 30, 45, 60, 90, and 120 seconds, 0.5 ml aliquots of the assay mixture were removed and diluted in 5.0 ml of the 140 mM KCl solutions. This diluted mixture was immediately filtered through two type A-E glass fiber prefilters and one nitrocellulose filter of 0.45 μm pore size and washed with 2.0 ml of the same salt solution. The filters were dried and counted as described above.

## Measurement of Proton Uptakes

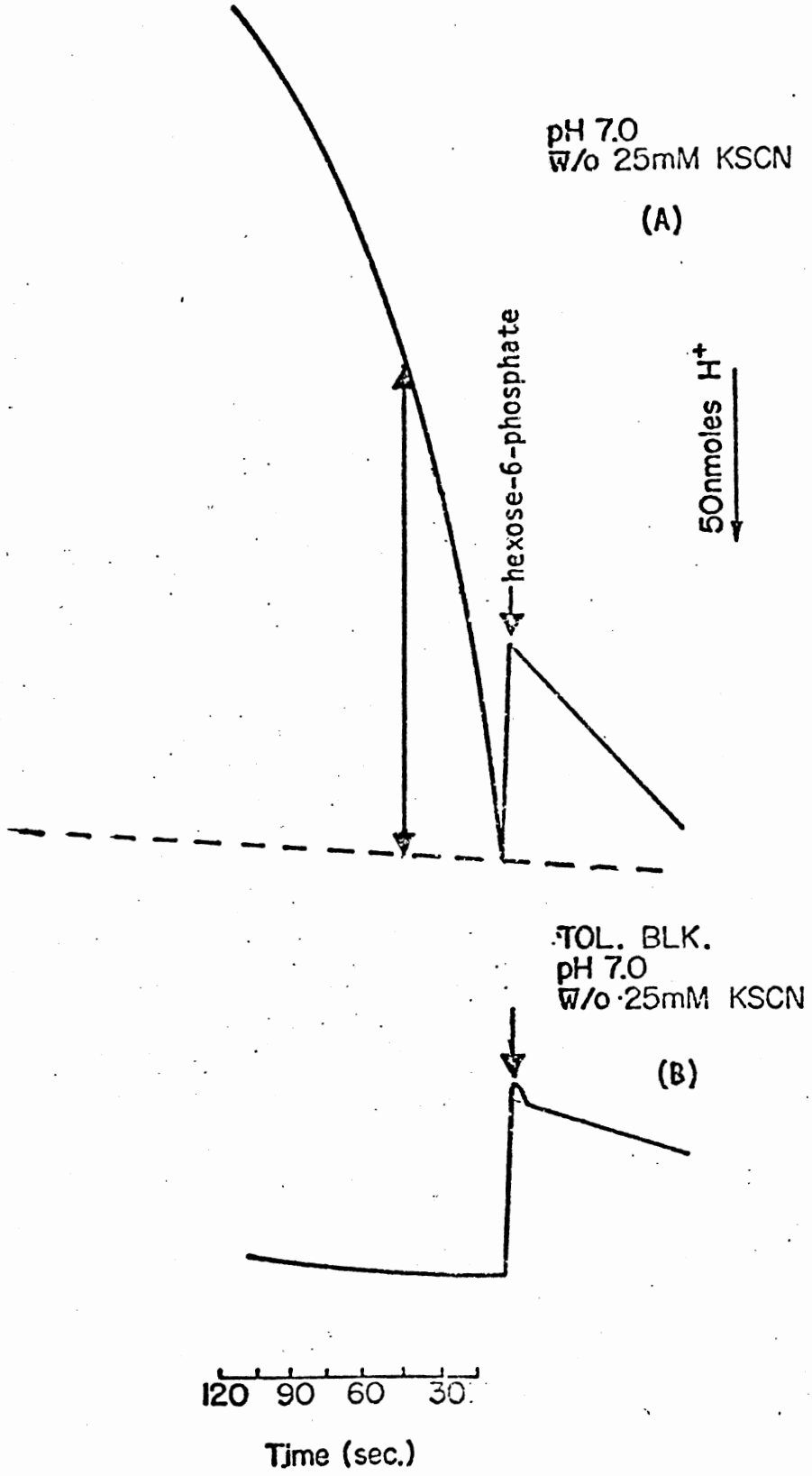
The apparatus used in these measurements is the same as that described above for Glc-6-P uptakes in the stoichiometry studies. In order that proton uptakes could be measured without interference from manipulation of the system which occurred during the measurement of the Glc-6-P uptakes, parallel experiments were run under the same conditions in which changes in pH and ΔpH were continuously monitored. Blank experiments (controls) using toluene-treated cells were carried out with each set of experiments for the purpose of determining if the alkaline shift in pH, which was induced by the addition of the hexose-6-phosphate, was due to pH mismatch between the cell suspension and the hexose-6-phosphate solution. The toluene destroys the membrane permeability such that Glc-6-P might enter or exit the cells via non-specific routes and induced proton flux might be minimal, if any. Calibration of the instrument was carried out by the addition of a known known amount of standard NaOH.

## Method of Determining Proton-Hexose-6-Phosphate Stoichiometry Ratios

The hexose-6-phosphate uptake results obtained from counting the radioactive experiment and blank samples were in terms of counts per minute and were converted to nmoles hexose-6-phosphate uptake per mg dry weight.

A typical pH trace for the experimental and blank runs is illustrated in Figure 5. Upon addition of the hexose-6-phosphate to the cells there occurs an acid shift in pH of the external medium. The

Figure 5. Typical pH Traces


pH 7.0
W/o 25mM KSCN
(A)
hexose-6-phosphate
50nmoles H+
TOL. BLK.
pH 7.0
W/o 25mM KSCN
(B)
120 90 60 30
Time (sec.)

curved portion of trace A (experimental) represents an alkaline shift which is probably due to the uptake of protons concomitant with the uptake of hexose-6-phosphate.

An example of the method used to determine the amount of proton uptake (nequivalents/mg dry wt.) at a given time is as follows: 1) To determine the point at which zero proton uptake occurs (i.e., the y intercept), a straight line constituting the x-axis is drawn at a right angle at the minimum point of the line corresponding to the acid shift. At points on the trace corresponding to the alkaline shift at times of 2.5, 5.0, 7.5, 15, 30, 45, 60, 75, and 90 seconds of proton uptake perpendiculars are drawn to the x-axis. The length of these perpendiculars (called the y values) is measured. The x-values in terms of seconds of uptake and the corresponding y-values are treated with the least squares polynomial in order to determine the best fit via an orthogonal polynomial method (103). The zero time point of the proton uptake is determined from an extrapolation of the calculated values to time zero (the y intercept). 2) The baseline of the experimental trace (dashed line in (A)) is constructed by drawing a line through the point corresponding to zero proton uptake. The slope of this line corresponds to that of the blank trace from zero time to 120 seconds. 3) The amount of proton uptake at a given time is determined by measuring the distance between the experimental trace and the baseline at each time point. The double-headed arrow illustrated in Figure 5 represents the amount of proton uptake at 45 seconds. These measured values are converted to nanoequivalents of $H^+$ uptake by comparison to the deflection resulting from the base calibration. By dividing these values by the mg dry weight, a measure of

the nanoequivalents of $H^+$ uptake/mg dry weight is obtained. The mg dry weight is obtained by determining the turbidity of a sample of the cell suspension via a Klett-Summerson colorimeter with #42 filter. This value is compared to standards measured similarly and the mg dry weight/ml determined.

The stoichiometry ratio values are obtained for each interval by taking the ratio of the corresponding nanoequivalents proton uptake per mg dry weight to the nmoles hexose-6-phosphate uptake per mg dry weight. In all cases stoichiometry ratio values are averages from at least two determinations. The best estimate of the proton to hexose-6-phosphate stoichiometry at a given external pH is obtained by extrapolating to zero time the best fit line of a plot of the average stoichiometry ratio values versus time of uptake. Variations of the procedure described above will be discussed in the figure legends.

## Method of Testing Anaerobic Respiration in RE-48 Cells

The apparatus and experimental conditions were the same as those described above for the proton and Glc-6-P stoichiometry studies. Tests were performed with RE-48 cells in the presence of KSCN at pHs of 6.0 and 7.5. When the cell suspension became anaerobic as indicated by the steady baseline of the pH trace, Glc-6-P was added to initiate the assay. After the pH trace became steady again, fumarate and glycerol were added to a concentration of 5 mM. The resulting pH trace was continuously monitored throughout a three minute period.

# CHAPTER IV

# RESULTS

## Proton to Hexose-6-Phosphate Stoichiometry Ratios Determined at Various pHs of the External Medium

The results described in this section are from experiments which were designed to determine the stoichiometry ratios over a broader range of experimental conditions than was previous studied; namely, the values were determined at time intervals throughout a two minute period with cells with and without 25 mM KSCN at external pHs of 6.0, 7.0, 7.5, and 8.0.

### Stoichiometry Determinations at External pH 6.0

The results of an experiment with RE-48 cells carried out at external pH 6.0 are shown in Figure 6. The uptake of protons and Glc-6-P appear to be almost linear within the first 10 seconds. Then the rate of uptake starts to gradually decrease. The overall uptakes are quite similar. However, the proton uptakes may be slightly less than the Glc-6-P uptakes beyond 30 seconds, perhaps due to proton leakage from the cells. Uptakes in cells with and without 25 mM KSCN appear to be similar. The average stoichiometry ratios determined from these data are illustrated in Figure 7. In general, the

**Figure 6.** Proton and Glc-6-P Uptakes at External pH 6.0

Refer to "Materials and Methods" for experimental procedure.

Cells with KSCN-Δ-Δ
Cells without KSCN-o-o

The error bars indicate the magnitude of the standard error of the mean and each point is the average of 4-6 determinations.

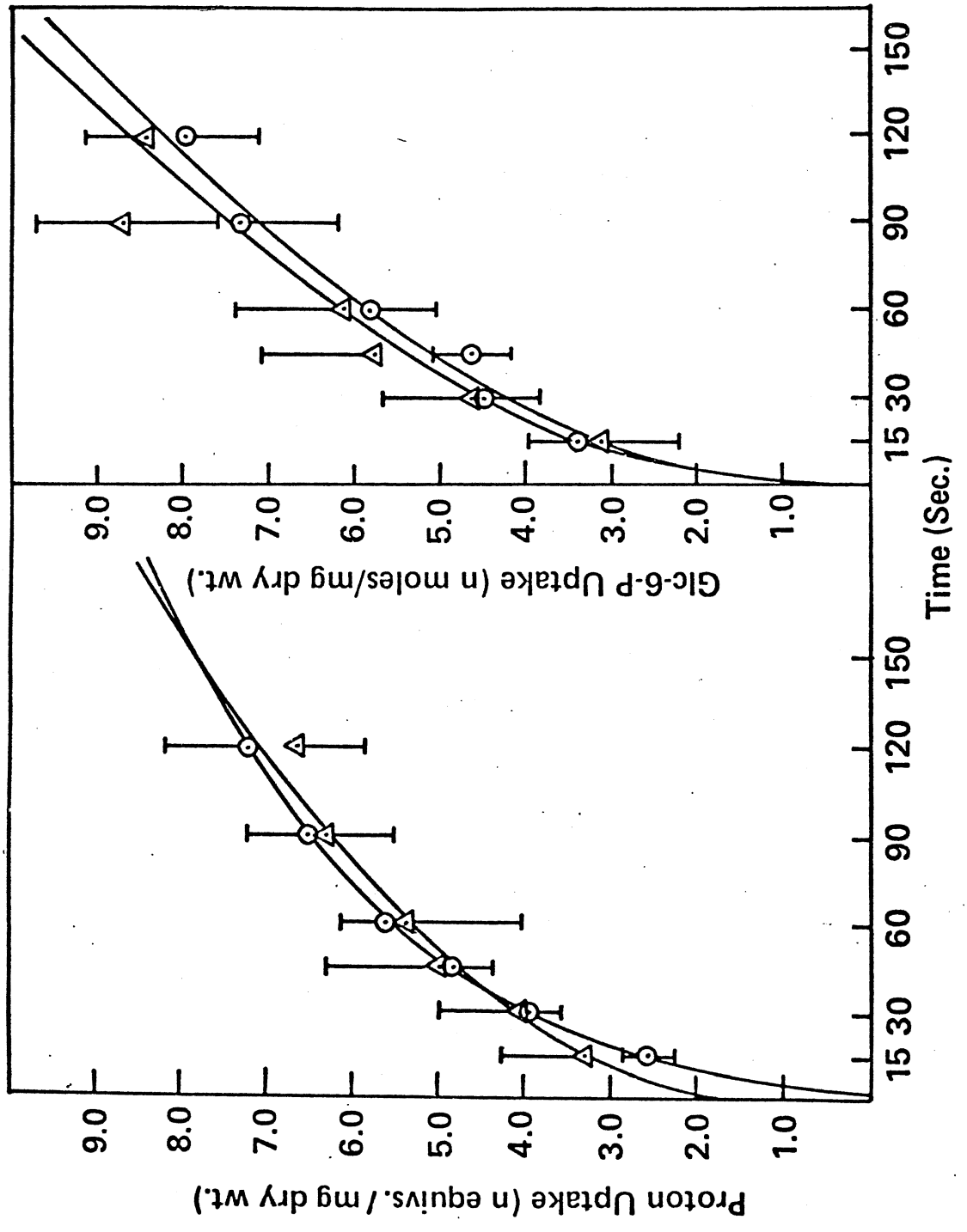
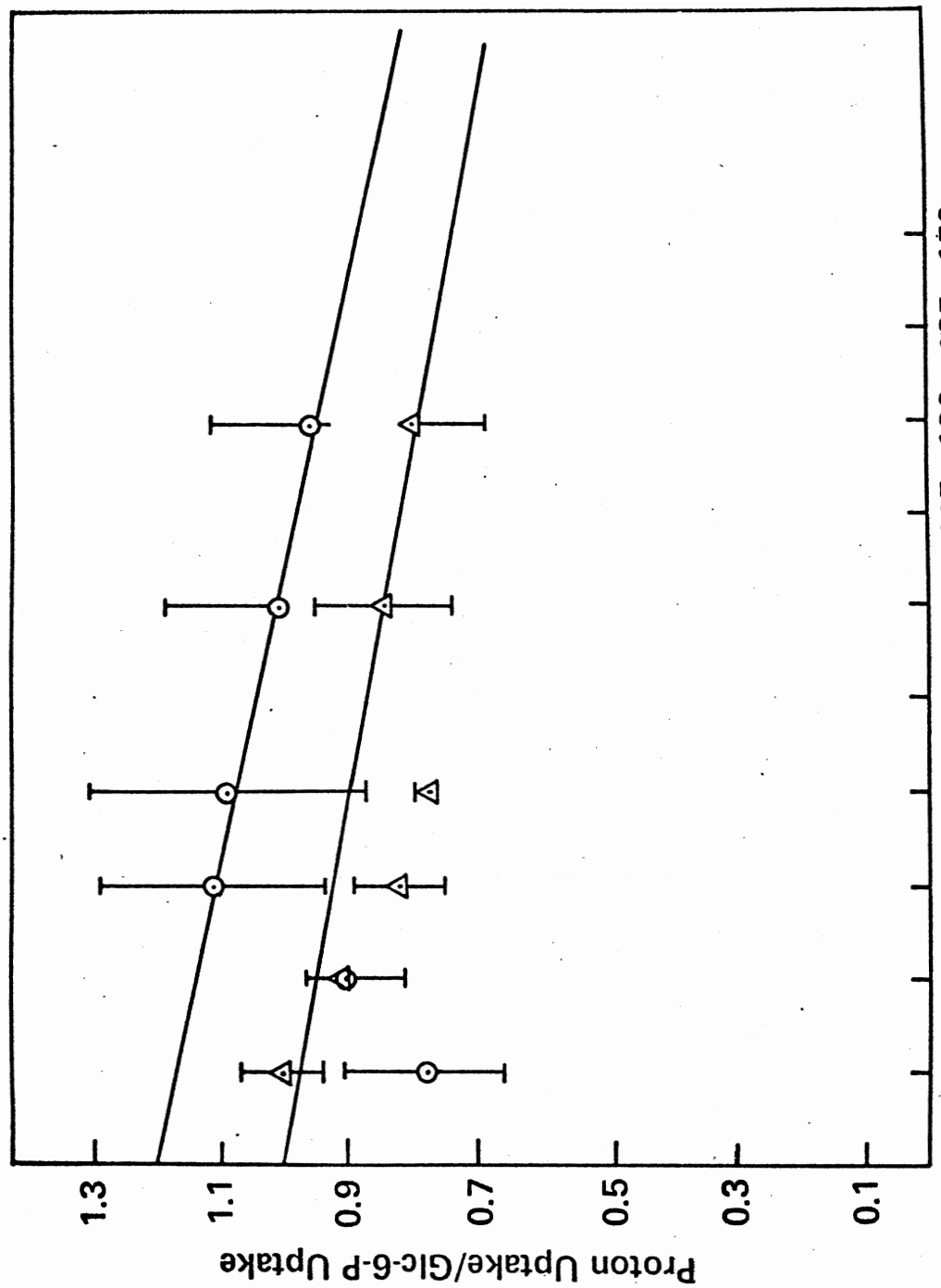

Proton Uptake (n equivs. / mg dry wt.)
Glc-6-P Uptake (n moles/mg dry wt.)
Time (Sec.)
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
15 30 60 90 120 150

Figure 7. Stoichiometry Ratio Values at External pH 6.0

Cells with KSCN-Δ-Δ
Cells without KSCN-o-o


Proton Uptake/Glc-6-P Uptake
1.3
1.1
0.9
0.7
0.5
0.3
0.1
15
30
45
60
75
90
105
120
135
150
Time (Sec.)

ratio values decrease with increasing time for cells, both with and without KSCN. This decrease is thought to result from proton leakage. Thus, the best estimate of the proton-Glc-6-P stoichiometry is obtained from an extrapolation to zero time, provided that protons are merely leaking back to equilibrium. These extrapolations from the best linear least-squares fit to the data provide proton to Glc-6-P values of 1.21±.01 for cells without KSCN and 0.92±.06 for cells with KSCN. The fit for the values without KSCN excludes values at 15 and 30 seconds since they are clearly aberrant. The aberrant values may have resulted from proton leakage which is more apparent at the earlier times of uptake.

The results from proton and Glc-6-P uptake studies with RE-90 cells treated with KSCN at pH 6.0 are similar to those obtained with RE-48 cells under the same conditions.

No acid production was observed upon addition of fumarate and glycerol to the cell suspension with KSCN, indicating no anaerobic respiration.

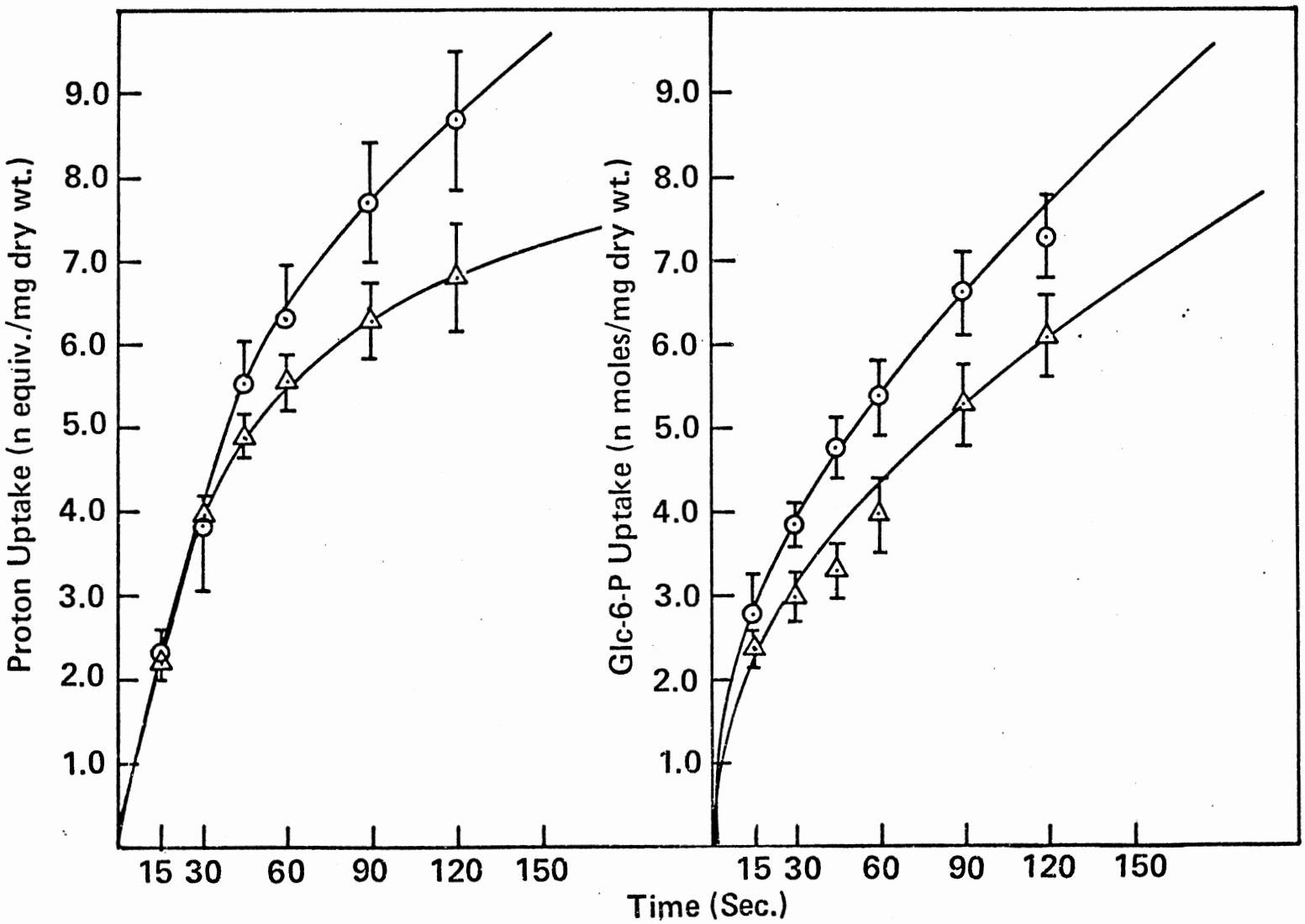
Stoichiometry Determinations at External pH 7.0

The results from the proton and Glc-6-P uptake experiments with RE-48 cells at external pH 7.0 are shown in Figure 8. Proton and Glc-6-P uptakes similar to those obtained at pH 6.0 are observed. However, both uptakes are less after 60 seconds for cells in the presence of KSCN. This effect is important since the proton uptake is slightly more diminished than the Glc-6-P uptake. As a result the

**Figure 8.** Proton and Glc-6-P Uptakes at External pH 7.0

Cells with KSCN-Δ-Δ
Cells without KSCN-o-o

The error bars indicate the magnitude of the standard error of the mean and each point is the average of 5 determinations.


Proton Uptake (n equiv./mg dry wt.)
Glc-6-P Uptake (n moles/mg dry wt.)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
15 30 60 90 120 150
Time (Sec.)

slope of the best fit line to the ratio values will be increased. Thus, the extrapolated value will be greater than the value shown from the experiments with cells not treated with KSCN as observed in Figure 9. The best estimates of the proton to Glc-6-P stoichiometry ratios for cells with or without KSCN at an external pH 7.0 are 1.83±.05 and 1.17±.04, respectively. The best fit line excludes aberrantly low values at 15 and 30 seconds. The stoichiometry ratio value for cells with KSCN at this pH is about twice that at external pH 6.0, i.e., at pH 7.0 almost twice as many protons are taken up per Glc-6-P transported as compared to that which was taken up at pH 6.0.

Stoichiometry Determinations at External pH 7.5

Figure 10 shows that the proton uptakes at pH 7.5 are quite different overall from those observed at pHs 6.0 and 7.0, whereas the Glc-6-P uptakes appear similar to those at the two lower pHs. Proton uptakes plateau at approximately 30 seconds and beyond 30 seconds they appear to reverse. This behavior is characteristic of proton leakage. The proton uptakes with KSCN were more perturbed than those without KSCN by the apparent proton leakage. This leakage was observed at both lower pHs but to a lesser extent. The fact that the proton leakage occurs to a greater extent than at lower pHs may be indicative of an active proton pumping process. However, it was observed that the rapid apparent proton leakage for cells without KSCN stopped with time and did not extend below the baseline, suggesting that the leakage occurs by a passive process in this case. The proton-Glc-6-P stoichiometry values are depicted in Figure 11. The best estimates of the

Figure 9. Stoichiometry Ratio Values at External pH 7.0

Cells with KSCN-Δ-Δ
Cells without KSCN-o-o

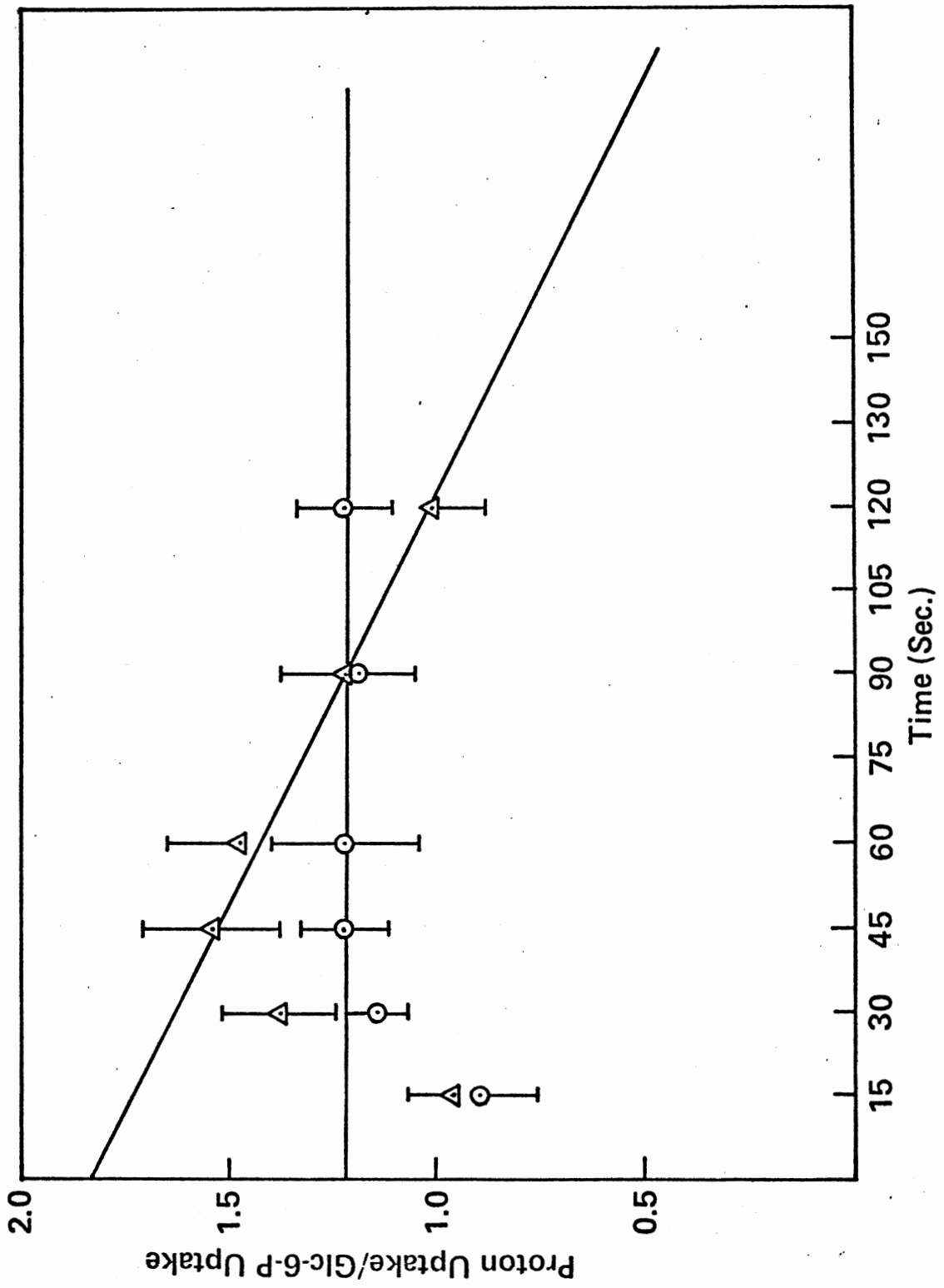

Proton Uptake/Glc-6-P Uptake
2.0
1.5
1.0
0.5
15
30
45
60
75
90
105
120
130
150
Time (Sec.)

Figure 10. Proton and Glc-6-P Uptakes at External pH 7.5

Cells with KSCN-Δ-Δ
Cells without KSCN-o-o

The error bars indicate the magnitude of the standard error of the mean and each point is the average of 2 determinations.

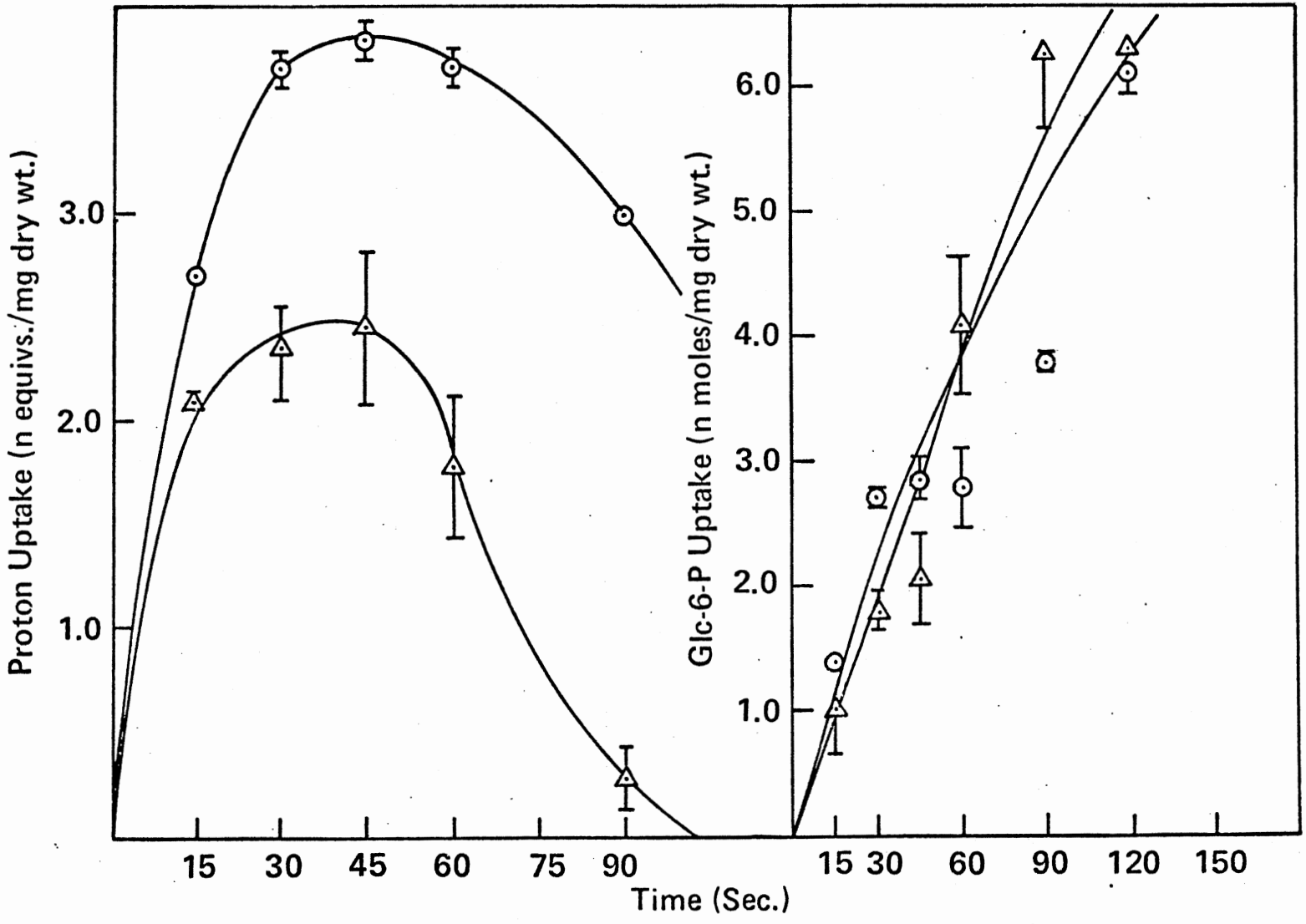
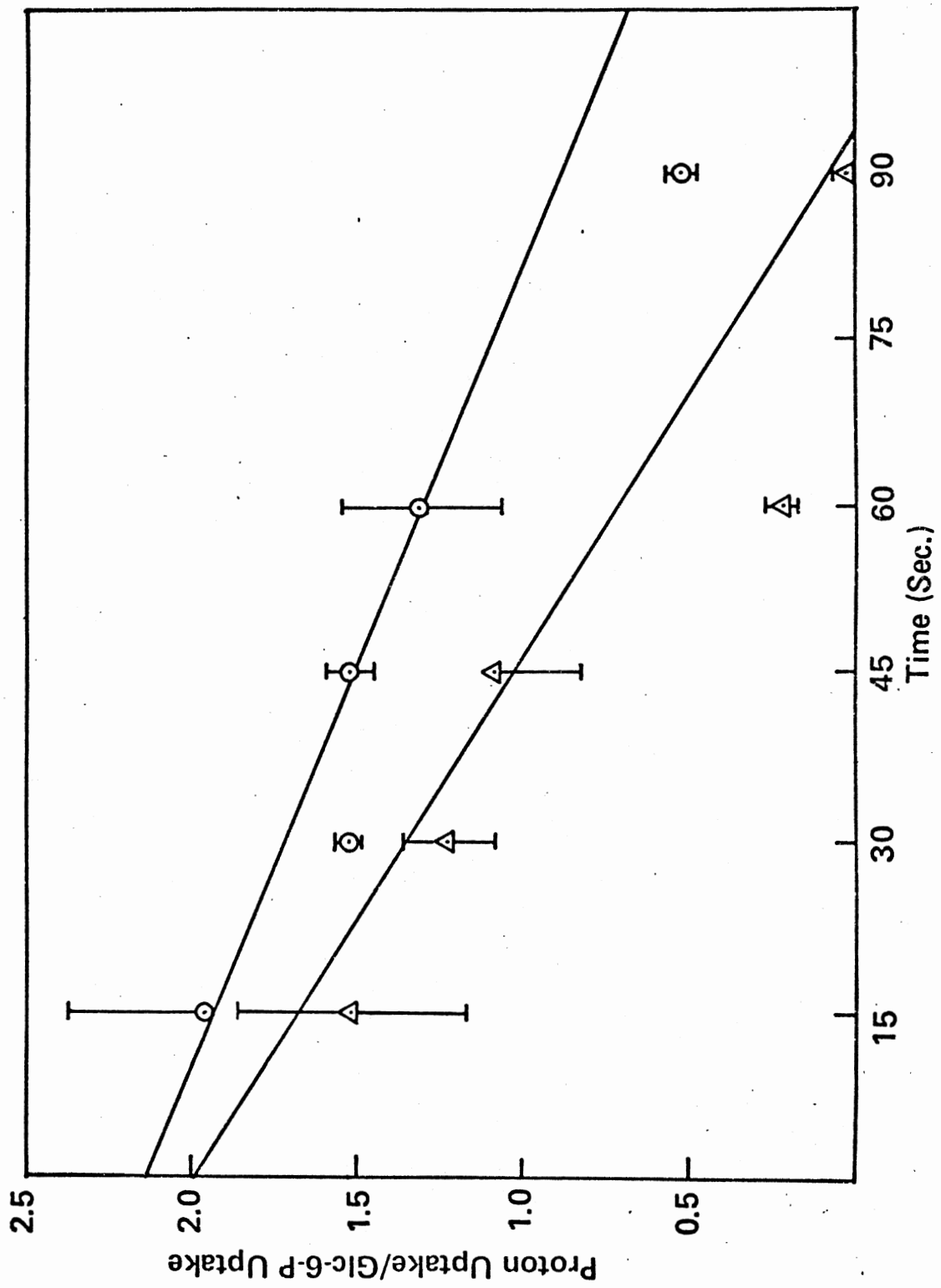

Proton Uptake (n equivs./mg dry wt.)
3.0
2.0
1.0
15
30
45
60
75
90
Time (Sec.)
Glc-6-P Uptake (n moles/mg dry wt.)
6.0
5.0
4.0
3.0
2.0
1.0
15
30
60
90
120
150

Figure 11. Stoichiometry Ratio Values at External pH 7.5

Cells with KSCN-Δ-Δ
Cells without KSCN-o-o


Proton Uptake/Glc-6-P Uptake
2.5
2.0
1.5
1.0
0.5
15
30
45
60
75
90
Time (Sec.)

proton-Glc-6-P stoichiometry ratio values at external pH 7.5 are 2.20±.15 for cells without KSCN and 1.96±.18 for cells with KSCN. These values are slightly greater than those obtained at pH 7.0, and at least two times greater than those determined at pH 6.0. No acid production was observed upon addition of fumarate and glycerol to the cell suspension containing KSCN, again demonstrating no anaerobic respiration.

Stoichiometry Determinations at External pH 8.0

The proton and Glc-6-P uptakes determined as described above with RE-48 cells at external pH 8.0 are shown in Figure 12. The Glc-6-P uptakes occur as expected, but the proton uptakes with cells in the presence of KSCN are strongly perturbed by the rapid proton efflux which occurs at times greater than 15 seconds. In this case the proton efflux continues below the baseline, indicating that the phenomenon may result from active proton pumping. It is not clear why cells not treated with KSCN show no proton uptake, but do exhibit a measurable Glc-6-P uptake at this external pH. A plot of the stoichiometry ratio values versus time is shown in Figure 13. Because of the apparent nonlinearity of the plot of the ratio values, a least squares polynomial best fit was made to the data using an orthogonal polynomial method (103). A linear extrapolation using the slope of this curve at 15 seconds was made to zero time from the 15 second stoichiometry ratio value (2.38). Using this approach, the best estimate of the proton to Glc-6-P stoichiometry value at external pH 8.0

Figure 12. Proton and Glc-6-P Uptakes at External pH 8.0

Cells with KSCN-Δ-Δ
Cells without KSCN-o-o

The error bars indicate the magnitude of the standard error of the mean and each point is the average of 4 determinations.

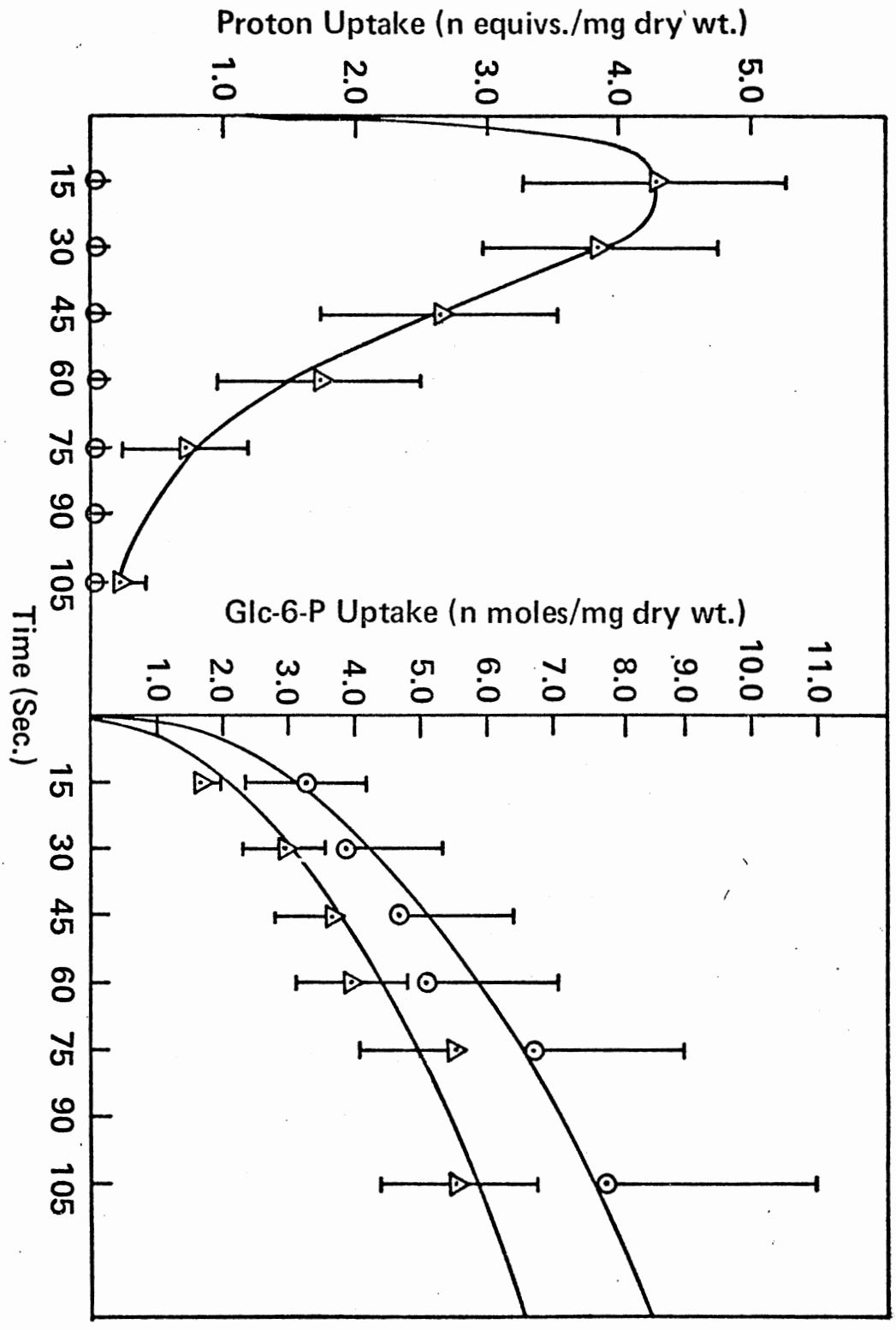
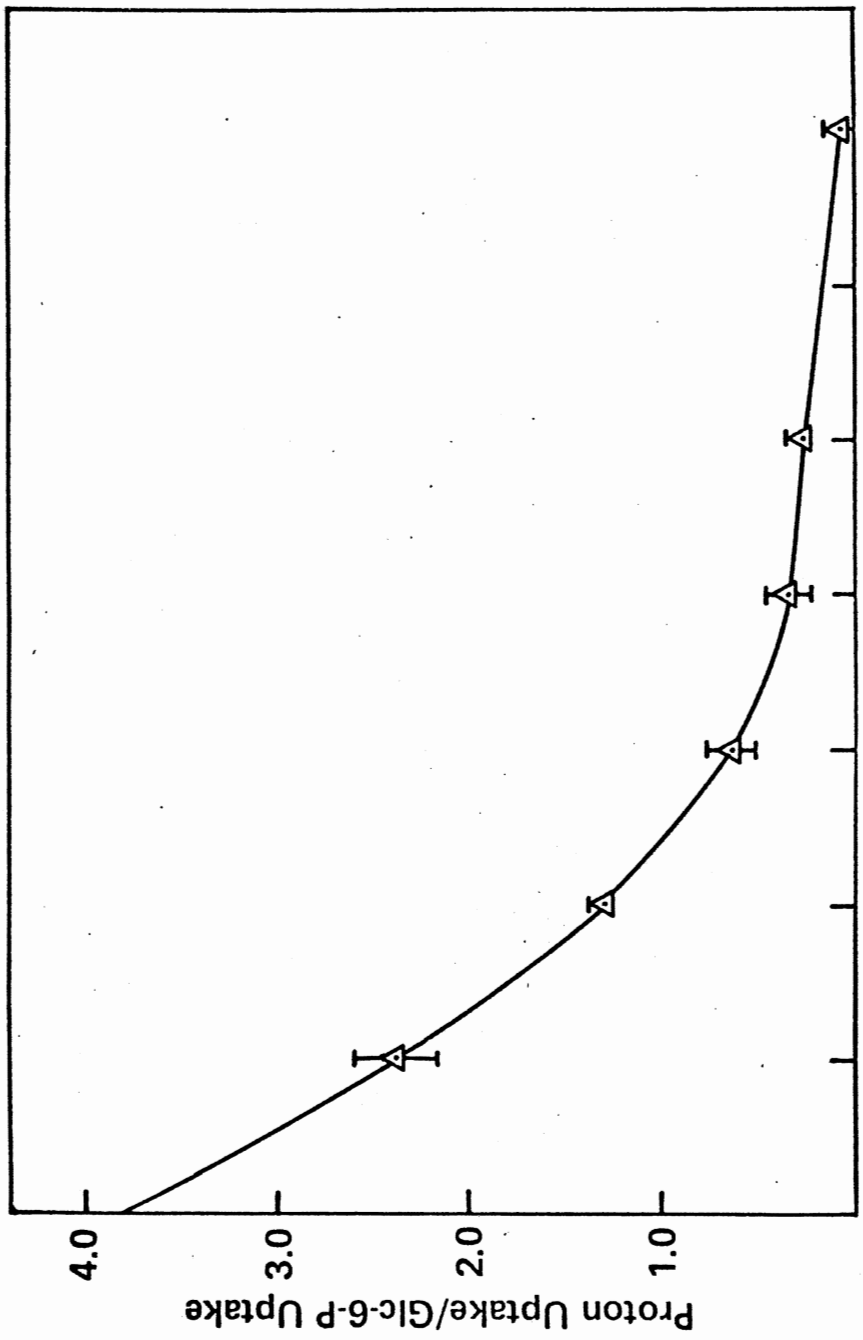

Proton Uptake (n equivs./mg dry wt.)
1.0
2.0
3.0
4.0
5.0
15
30
45
60
75
90
105
Time (Sec.)
Glc-6-P Uptake (n moles/mg dry wt.)
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
15
30
45
60
75
90
105

Figure 13. Stoichiometry Ratio Values at External pH 8.0

Cells with KSCN-Δ-Δ
Cells without KSCN-o-o


Proton Uptake/Glc-6-P Uptake
4.0
3.0
2.0
1.0
15
30
45
60
75
90
105
Time (Sec.)

is 3.7. This value could be an overestimate of the true value, since the ratio value at 15 seconds is only 2.38, and active proton pumping appears to have occurred. The latter phenomenon would result in decreased stoichiometry ratio values beyond 15 seconds and an overestimate of the stoichiometry ratio value extrapolated to zero time.

In order to determine whether the best estimate of the proton-Glc-6-P at pH 8.0 was an overestimate, proton and hexose-6-phosphate uptake experiments were carried out at pH 8.0 with the uncA mutants RE-114, RE-115, and RE-157 using 2-deoxy-Glc-6-P, a nonmetabolizable substrate analog. The genetic characteristics of uncA mutants are described in Table I. The RE-114 and RE-115 cells were treated with 0.01 mM DCCD according to the procedure of Essenberg and Kornberg for the purpose of eliminating proton leakage through the ATPase (29). These attempts with RE-114 and RE-115 cells gave proton uptakes which were only about 0.2 of the corresponding 2-deoxy-Glc-6-P uptakes. In studies with RE-157 cells treated with KSCN at pH 8.0 resulted in proton uptakes were only .75 of the corresponding 2-deoxy-Glc-6-P uptake. The best estimate of the proton to 2-deoxy-Glc-6-P stoichiometry is 0.59±.14 for this strain. Similar low stoichiometry values were obtained with 2-deoxy-Glc-6-P in RE-48 cells with KSCN at external pH 6.0. One interesting result of the 2-deoxy-Glc-6-P induced proton uptakes with RE-48 cells is that the apparent active proton efflux occurs with cells in the presence of KSCN.

A summary of the best estimate of the proton to hexose-6-phosphate stoichiometries at the various external pHs is illustrated in Table II.

TABLE II

BEST ESTIMATE OF THE PROTON TO HEXOSE-6-PHOSPHATE STOICHIOMETRY RATIOS AT VARIOUS EXTERNAL pHs

| Strain | External pHs | Stoichiometry w/o KSCN | Ratio Values* w/KSCN |
|---|---|---|---|
| RE-48 | 6.00 | 1.21±.01 | 0.92±.06 |
| RE-48 | 7.00 | 1.17±.04 | 1.83±.05 |
| RE-48 | 7.50 | 2.20±.15 | 1.96±.18 |
| RE-48 | 8.00 | -- | 3.8 |
| RE-157 | 8.00 | 0.59±.14 | -- |

*Stoichiometry ratio value ± standard error of the mean for 2 to 6 determinations.

A plot of the proton to Glc-6-P stoichiometries and the average net charge on the Glc-6-P molecule at various external pHs is shown in Figure 14.

## Ion Specificity of the Hexose-6-Phosphate Transport System

### Effect of Varying Cations on Glc-6-P Transport

Effect of KCl and NaCl at Various Concentrations. Previous studies by Essenberg and Kornberg (29) have shown that concentrations of KCl up to 140 mM stimulate the rate of uptake of Glc-6-P thirteenfold. Thus, it was of interest to characterize further the stimulation throughout a larger concentration range than previously studied. Figure 15 illustrates the results of this experiment. KCl appears to stimulate better than NaCl throughout. The maximum range of stimulation occurs between 150 and 500 mM. Beyond 500 mM the stimulation drops off markedly with increasing concentrations of the chloride salts. Inhibition of uptakes occurs at salt concentrations greater than about 1.0 M. The maximum stimulation of KCl is greater than two times that of the control, which is 1 mM PIPES-tris buffer.

Effect of Additional Monovalent Chloride Salts. In order to determine whether the salt stimulation of the Glc-6-P uptake was caused by either the cation or the anion, a series of chloride salts of monovalent cations was studied at various concentrations. Figure 16 shows that most alkali metals cause a 3- to 5-fold stimulation in uptake rate over the control containing 1 mM PIPES-tris buffer.

Figure 14. Proton to Glc-6-P Stoichiometries and Net Charge on Glc-6-P at External pHs

Average Net Charge-o-o
Proton-Glc-6-P Stoichiometries
Cells with KSCN-Δ-Δ
Cells without KSCN-o-o

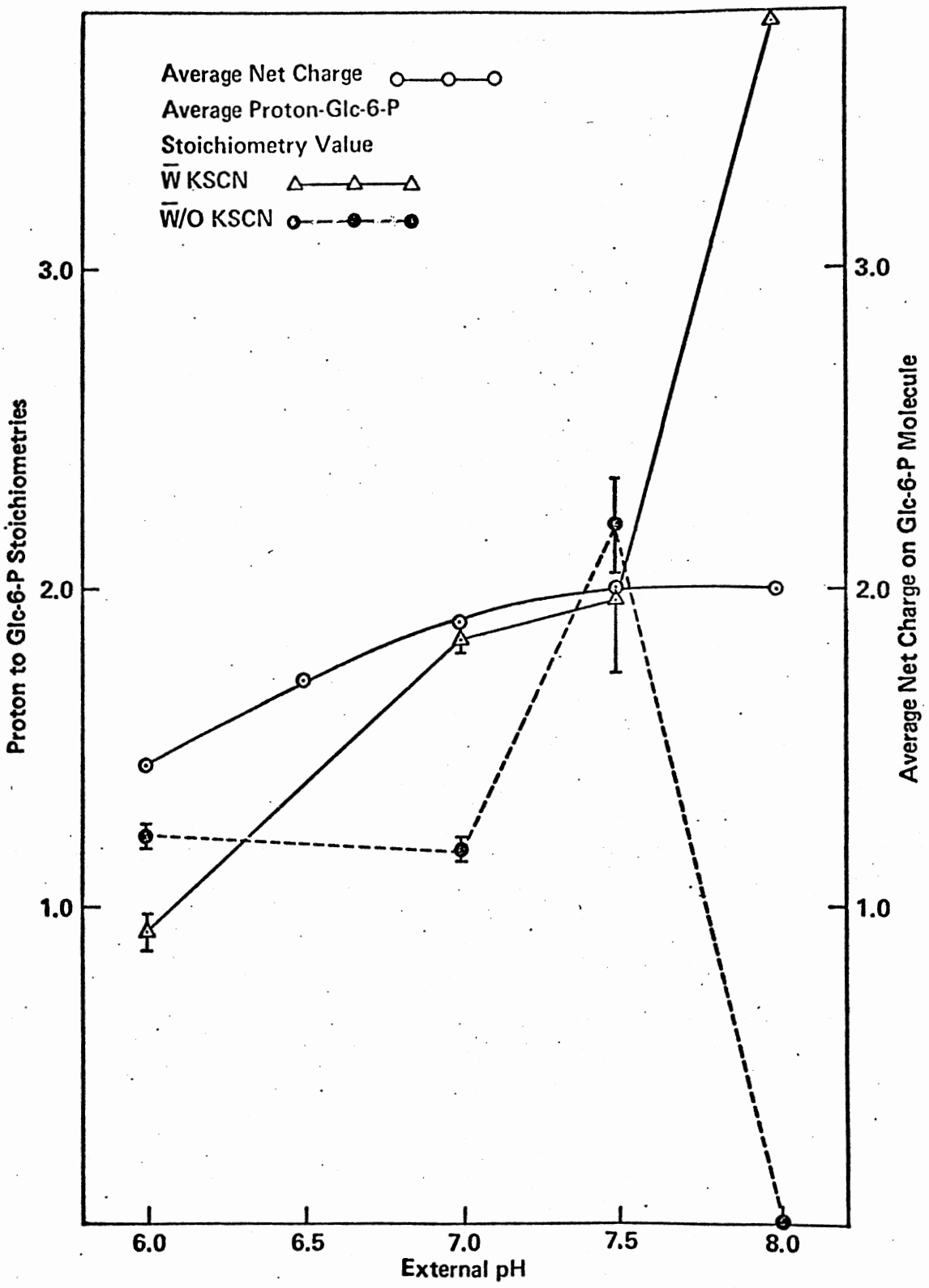

Average Net Charge
Average Proton-Glc-6-P
Stoichiometry Value
W KSCN
W/O KSCN
3.0
2.0
1.0
Proton to Glc-6-P Stoichiometries
Average Net Charge on Glc-6-P Molecule
6.0
6.5
7.0
7.5
8.0
External pH

Figure 15. Cation Effects of KCl and NaCl at Various Concentrations on Glc-6-P Uptake

The experiment was performed with RE-48 cells as described in "Materials and Methods" with a 1.0 ml total assay volume. A single 0.2 ml cell sample was taken after 70 seconds of uptake.

KCl-Δ-Δ
NaCl-o-o

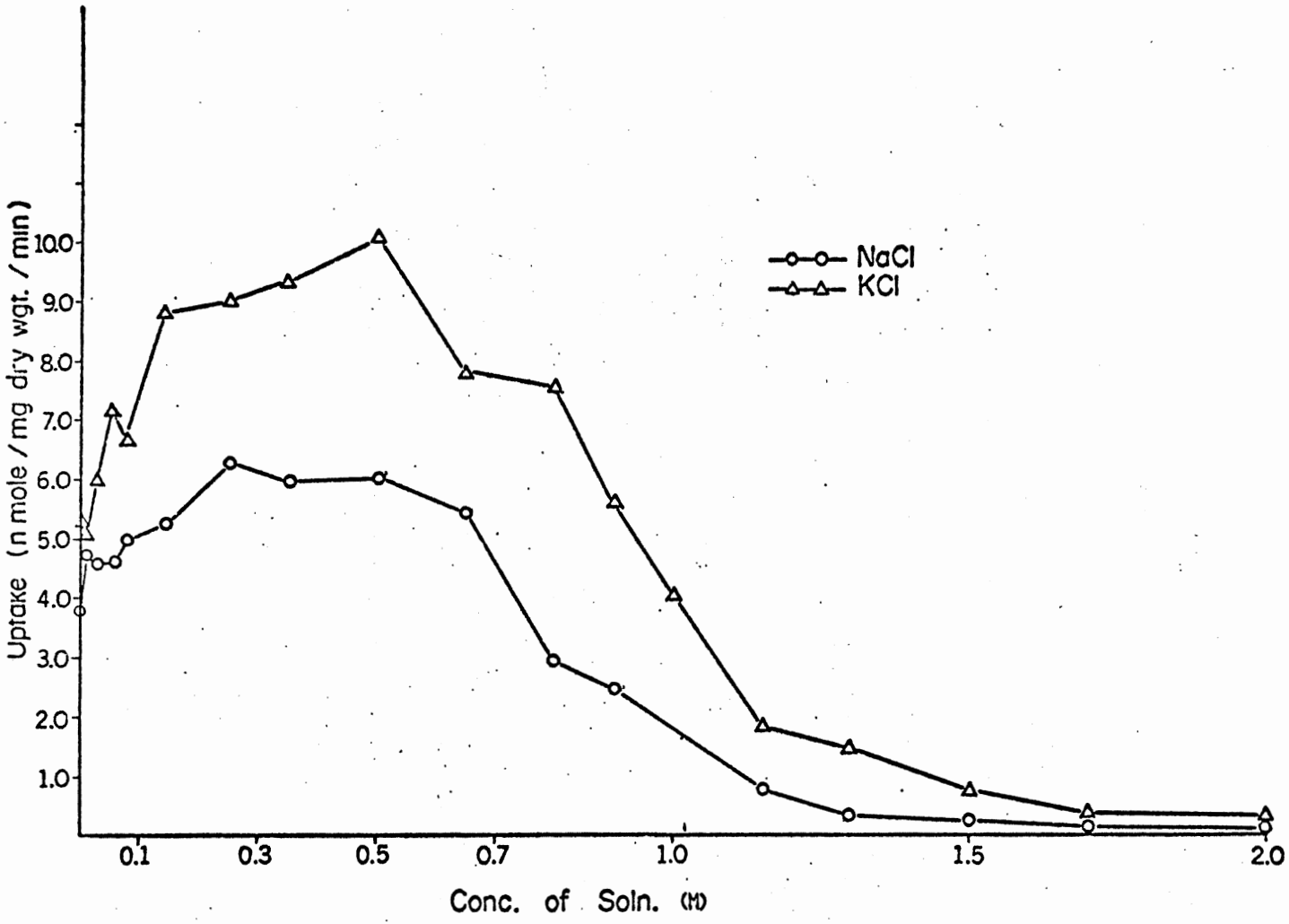
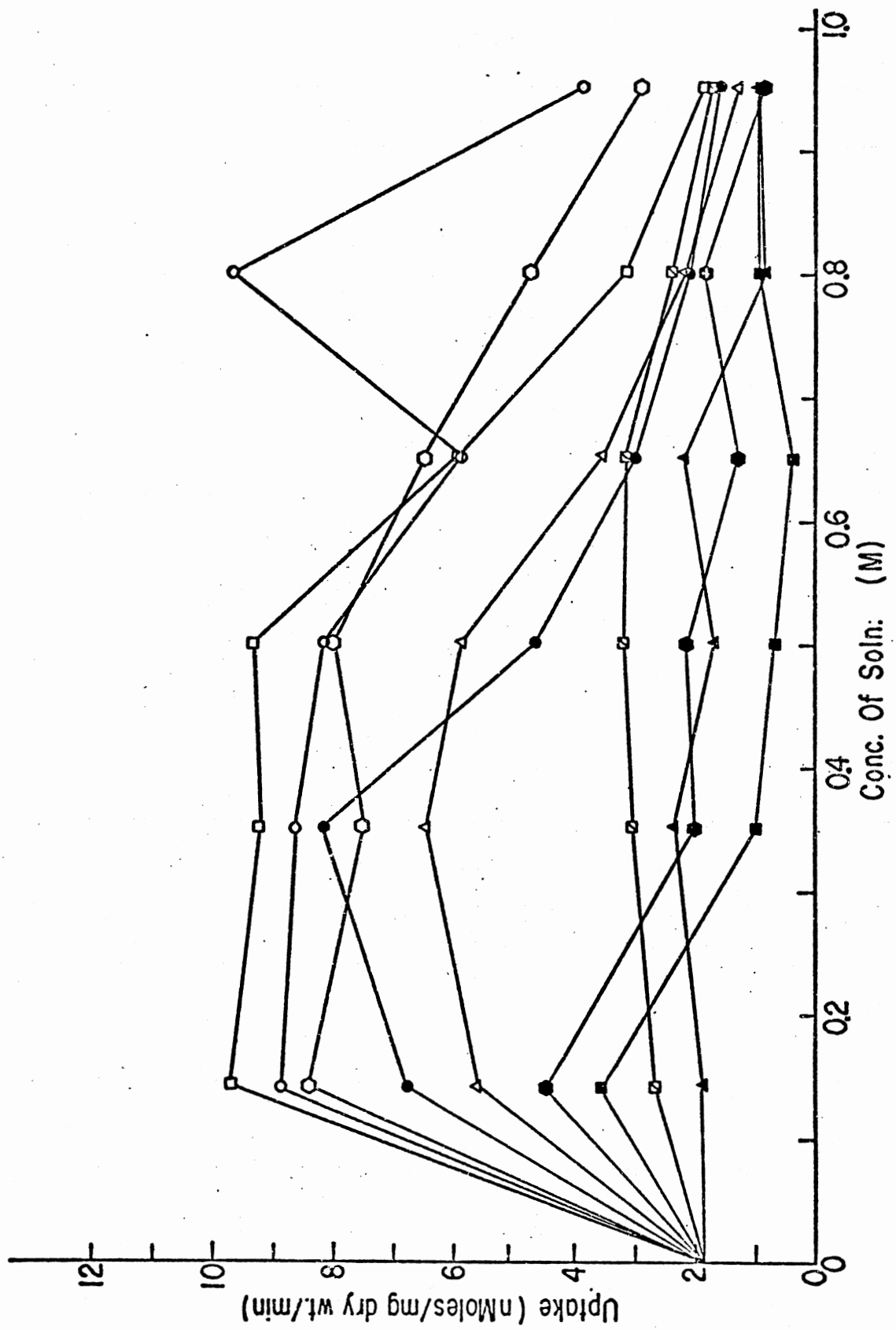

Uptake (n mole / mg dry wgt. / min)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.1
0.3
0.5
0.7
1.0
1.5
2.0
Conc. of Soln. (M)
NaCl
KCl

Figure 16. Effects of Additional Monovalent Chloride Salts on Glc-6-P Transport

RE-48 cells were washed and resuspended in 1/50 volume of a 90 mM concentration of the desired salt, containing 2 mM PIPES-tris buffer at pH 6.6. 50 µl of this concentrated suspension was diluted into 0.9 ml of the same salt at variable concentration. These concentrations and the appropriate dilution factors were used to calculate the plotted salt concentration. The transport assay was as described in "Materials and Methods," except that a volume of 0.25 mls was used and a single cell sample of 0.2 mls was filtered after 70 seconds uptake. The following salts were used in this study: CsCl, □; RuCl, o; KCl, ○; $NH_4Cl$, O; TrisHCl, ●; $Me_4NCl$, ■; LiCl, ⧄; CholineCl, ▲; and NaCl, Δ.


Uptake (nMoles/mg dry wt./min)
0
2
4
6
8
10
12
0
0.2
0.4
0.6
0.8
1.0
Conc. Of Soln. (M)

Maximal stimulation by these salts occurs between 150 and 500 mM, after which the stimulation decreases with increasing salt concentration. $Cs^+$, $Rb^+$, $K^+$, $NH_4^+$, and $Na^+$ show the greatest stimulation, while stimulation by $tris^+$, and $(Me)_4N^+$ is comparatively slight at 140 mM and drops off to zero thereafter. Choline and $Li^+$ show essentially control levels of activity.

Effect of a Series of Methylammonium Salts. The results in Figure 16 indicate that $NH_4Cl$ stimulates Glc-6-P transport well, whereas $Me_4NCl$ stimulates very little. Therefore, a study of a homologous series of methylammonium chloride salts at 140 and 350 mM was performed to characterize further the relationship between cation size and stimulation of Glc-6-P uptake. The results are illustrated in Table III. As seen before, at 140 mM salt concentration $K^+$ stimulated uptake more than $NH_4^+$, and $Me_4N^+$ stimulated only slightly as compared to the 1 mM PIPES-tris buffer (control). There was a decreasing capacity of the methyl-substituted ammonium cations to stimulate transport in going from the least to the most methyl-substituted cation. The sharpest decrease in activity occurred between the mono- and di-methylated ions. Thus, at 140 mM salt concentration it would appear that cations with radii equal to or greater than 2.47 Å did not significantly stimulate Glc-6-P uptake. However, the results obtained at 350 mM salt concentration were not as clearly indicative of this point.

Glc-6-P Uptakes as a Function of Ion Size. A more complete understanding of the relationship between crystallographic radii of

TABLE III

Glc-6-P Uptake in Methylammonium Salts

| Salt | Glc-6-P Uptake-At* | | Crystallographic Radius (Å) |
|---|---|---|---|
| | 140mM | 350mM | |
| KCl | 9.58±0.13 | 10.85±0.16 | 1.33 |
| $NH_4Cl$ | 7.44±0.20 | 9.55±0.03 | 1.48 |
| $MeNH_3Cl$ | 6.09±0.o6 | 11.42±0.02 | 2.09 |
| $Me_2NH_2Cl$ | 3.39±0.06 | 7.84±0.15 | 2.47 |
| $Me_3NHCl$ | 3.62±0.26 | 5.95±0.04 | 2.76 |
| $Me_4NCl$ | 2.75±0.25 | 3.37±0.07 | 3.00 |
| Buffer** | 2.60±0.02 | | |

*Glc-6-P uptake expressed as nmoles/mg dry wt/min ± standard deviation and each point is the average of 3 determinations.

**1 mM PIPES-tris at pH 6.6

The crystallographic radii for $K^+$ and $NH_4^+$ were obtained from Pauling (104), and the radius for $Me_4N^+$ was taken from Halliwell and Nyburg (105). The radii for other methyl-substituted ammonium cations were interpolated from $NH_4$ and $Me_4N^+$ assuming the change in volume for each methyl-substitution was one quarter of the difference in volume between $NH_4^+$ and $Me_4N^+$, and calculating the radius assuming spherical ions. Refer to Figure 15 for experimental details.

cations and stimulation of Glc-6-P transport might be obtained from the results plotted in Figure 17. This plot of the data shows good correlation between both variables, with $Cs^+$ (1.69Å) showing the most effective stimulation of transport. It would seem that the $Na^+$ (0.95Å) and $Li^+$ (0.60Å) are too small and the di-, tri-, and tetramethylammonium cations are too large to effect maximum stimulation of Glc-6-P uptake. Ammonium (1.48 Å) does not stimulate as expected for its size. A plot of the hydrodynamic cation radii versus Glc-6-P activity does not show as good a relationship.

Effect of $MgCl_2$ and KCl. Due to the physiological importance of $Mg^{2+}$ in biological systems and the previous reports that $Mg^{2+}$ stimulates Glc-6-P transport (29), a comparative study of various concentrations of chloride salts of $Mg^{2+}$ and $K^+$ on the transport activity was performed. The results illustrated in Figure 18 show that $Mg^{2+}$ stimulated as much as $K^+$. However, the maximal $Mg^{2+}$ stimulation occurred at a relatively low concentration (10 mM), while maximal $K^+$ stimulation occurred between 140 and 500 mM $K^+$ concentration. Also, the $Mg^{2+}$ stimulation was only optimal over a concentration range of 10 to 20 mM after which the degree of stimulation fell off markedly. Thus, it appears that $Mg^{2+}$ might stimulate Glc-6-P transport in a different manner from that of $K^+$ and other monovalent cations.

## Effect of Varying Anions on Glc-6-P Transport

Effect of Mono-, Di-, and Tri-valent Anions of the $K^+$ Salts. A parallel series of experiments employing potassium salts of various

Figure 17. Glc-6-P Uptakes as a Function of Ion Size

The Glc-6-P uptake rates in 350 mM solutions of chloride salts of the indicated cations were taken from data illustrated in Figure 15 and Table III. Since the absolute uptake rates were not the same for both experiments, the rates from Table III were corrected by multiplying by the average of the ratios of these rates for KCl and $NH_4Cl$. Each of these salts and $Me_4NCl$ are represented by two points because they were used in both experiments. The cation crystallographic radii were obtained as previously described in Table III.

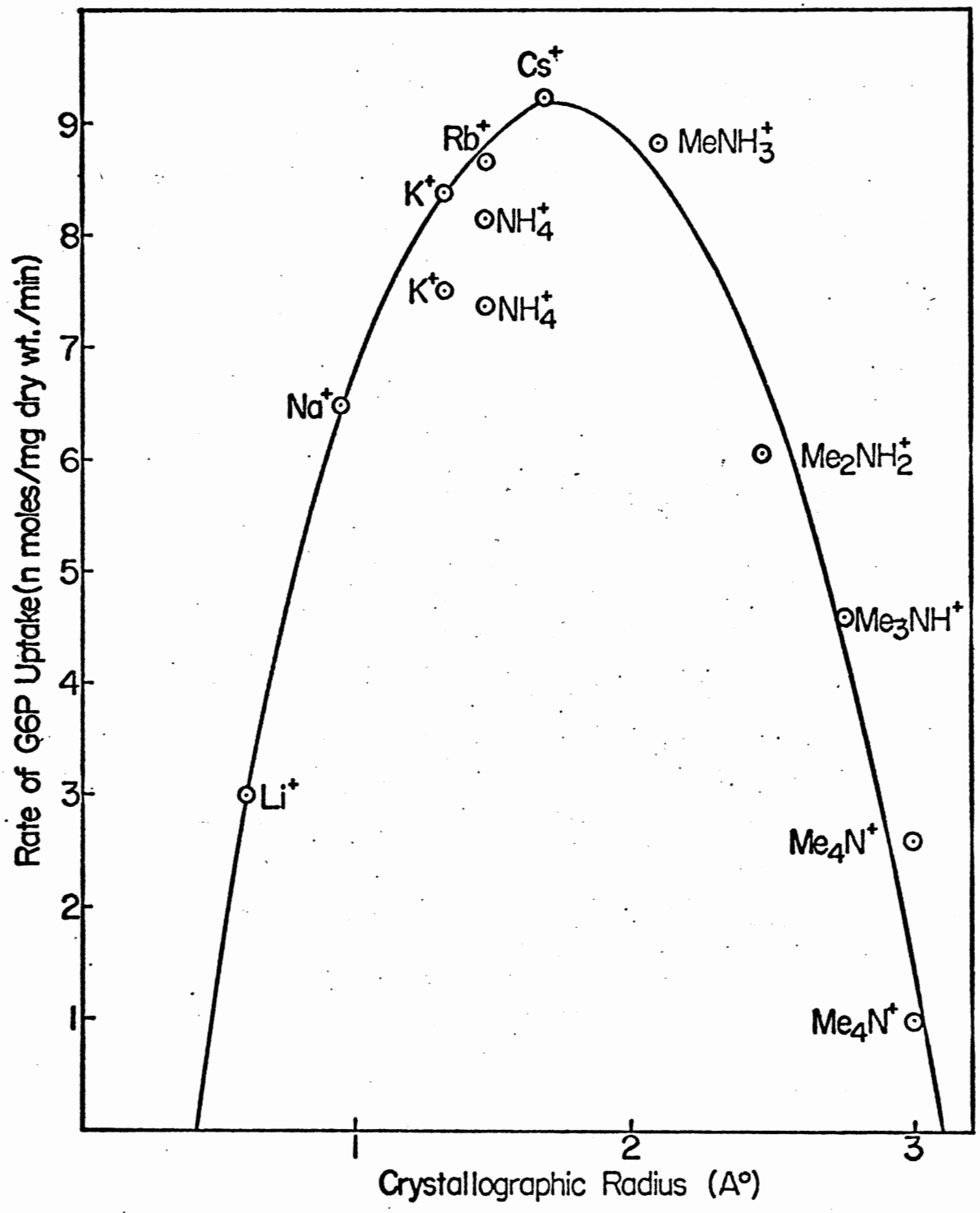
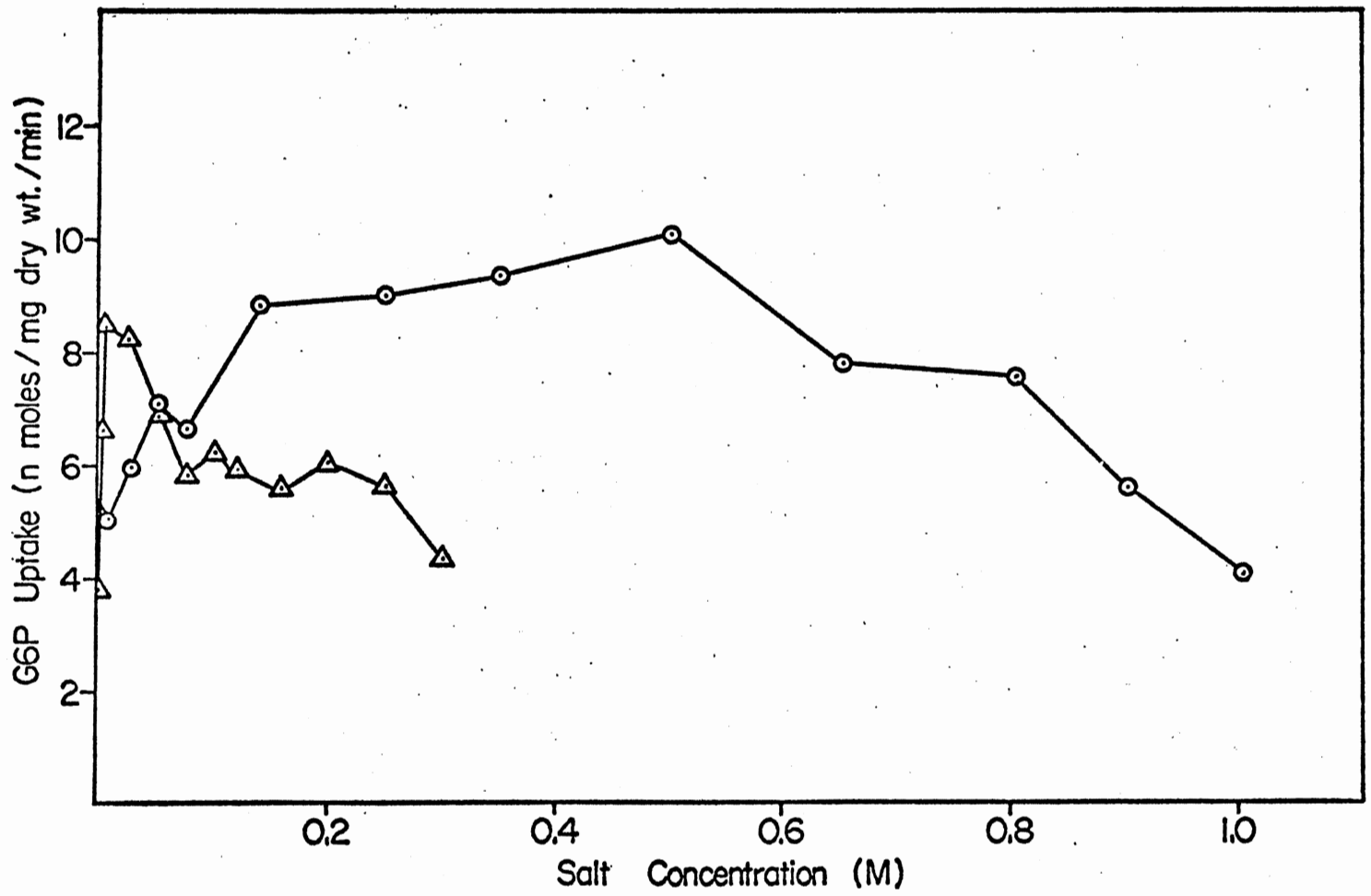

Cs+
Rb+
K+
NH4+
K+
NH4+
Na+
Li+
MeNH3+
Me2NH2+
Me3NH+
Me4N+
Me4N+
9
8
7
6
5
4
3
2
1
1
2
3
Rate of G6P Uptake(n moles/mg dry wt./min)
Crystallographic Radius (A°)

Figure 18. $MgCl_2$ and KCl Salt Effects on Glc-6-P Uptake

Stimulation in the presence of various concentrations of $MgCl_2$(Δ-Δ) and KCL (o-o) was measured as described in Figure 15.


G6P Uptake (n moles / mg dry wt. / min)
12
10
8
6
4
2
0.2
0.4
0.6
0.8
1.0
Salt Concentration (M)

anions was carried out to determine their effects on Glc-6-P uptake. As illustrated in Table IV, the anions fall into four classes in their capacity to alter the rate of Glc-6-P uptake relative to chloride ion. $Br^-$, $I^-$, $NO_3^-$, $ClO_4^-$, acetate, and possibly formate and tartrate exhibit similar activity. $HCO_3^-$, $SO_4^{2-}$, and $PO_4^{2-}$ stimulate about 50% as well as $Cl^-$. Citrate appears to stimulate the rate of uptake in Experiment 1, while $F^-$, and perhaps $HCO_3^-$, seem to be ineffective.

Effect of Anion Concentration, Metal Concentration, Osmolarity, and Ionic Strength. The effects of various concentrations of KCl, $K_2SO_4$, and $K_3$citrate on the activity of Glc-6-P transport was determined as described above for the monovalent cation studies in order to identify the parameter(s) of the salt solution (salt concentration, $K^+$ concentration, osmolarity, or ionic strength) which correlated well with Glc-6-p transport activity. The same set of transport data was plotted against each of these parameters.

Figure 19 shows no clear correlation between salt concentration and Glc-6-P transport activity. Again, citrate and $Cl^-$ elicited about the same activity, but $SO_4^{2-}$ showed significantly less. Only a fair correlation existed between $K^+$ concentration and Glc-6-P transport activity throughout the entire concentration range as depicted in Figure 20. The osmolarity did not correlate as well as the $K^+$ concentration with Glc-6-P transport as shown in Figure 21. As shown in Figure 22, the ionic strength did not correlate well with Glc-6-P transport activity.

TABLE IV

EFFECT OF ANIONS ON Glc-6-P UPTAKE

| Anion | Concentration (mM) | Experiment I* | Experiment II* |
|---|---|---|---|
| $F^-$ | 140 | 10±3 | 30±8 |
| $Cl^-$ | 140 | 100 | 100 |
| $Br^-$ | 140 | 86±8 | 89±23 |
| $I^-$ | 140 | 108±11 | 69±17 |
| $NO_3^-$ | 140 | 79±7 | 86±20 |
| $HCO_3^-$ | 140 | 0±21 | 32±7 |
| $Cl^-$ | 54 | 66±4 | 74±19 |
| $ClO_4^-$ | 54 | 73±17 | 60±15 |
| $SO_4$ | 100 | 66±11 | 40±10 |
| $PO_4$ | + | 67±6 | 45±11 |
| formate | 140 | 21±2 | 69±17 |
| acetate | 140 | 68±6 | 44±13 |
| tartrate | 100 | 119±10 | 61±14 |
| citrate | 75 | 148±21 | 59±18 |

*Values expressed as percent of rate in 140 mM KCl ± standard error of mean and each value is the average of at least 3 determinations.

+pH 6.6 phosphate contained 126 mM $PO_4$, 174 mM K

This experiment was performed as described in "Materials and Methods" with a total assay volume of 1.0 ml. At intervals of 10, 30, 50, and 70 seconds, 0.2 ml samples were filtered. The rate of Glc-6-P uptake for cells in the presence of a specific anion was determined as the slope of the best fit line to a plot of Glc-6-P uptake versus time of uptake. All salt concentrations were selected such that the osmolarity for each would be a constant, except that $KClO_4$ was used at 54 mM because of its limited solubility.

Figure 19. Effect of Anions on Glc-6-P Uptake Using KCl, $K_2SO_4$, and $K_3$citrate

Assays were performed as described in Figure 16.

KCl-o-o
$K_2SO_4$-Δ-Δ
$K_3$citrate-□-□

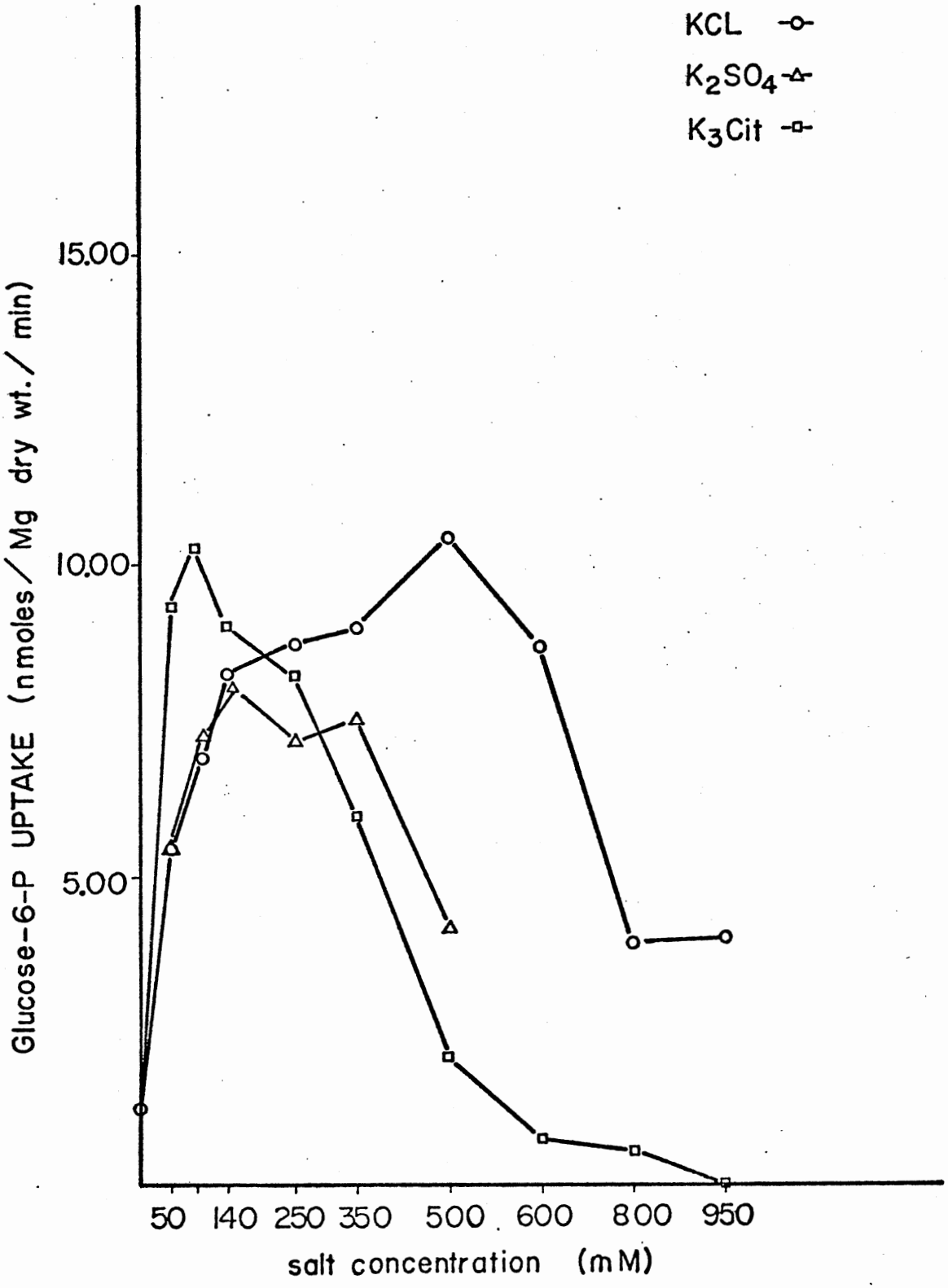

KCL
$K_2SO_4$
$K_3Cit$
15.00
10.00
5.00
Glucose-6-P UPTAKE (nmoles / Mg dry wt. / min)
50
140
250
350
500
600
800
950
salt concentration (mM)

Figure 20. Effect of $K^+$ Concentration on Glc-6-P Uptake Using KCl, $K_2SO_4$, and $K_3$citrate

Assays were performed as described in Figure 16.

KCl-o-o
$K_2SO_4$-Δ-Δ
$K_3$citrate-□-□

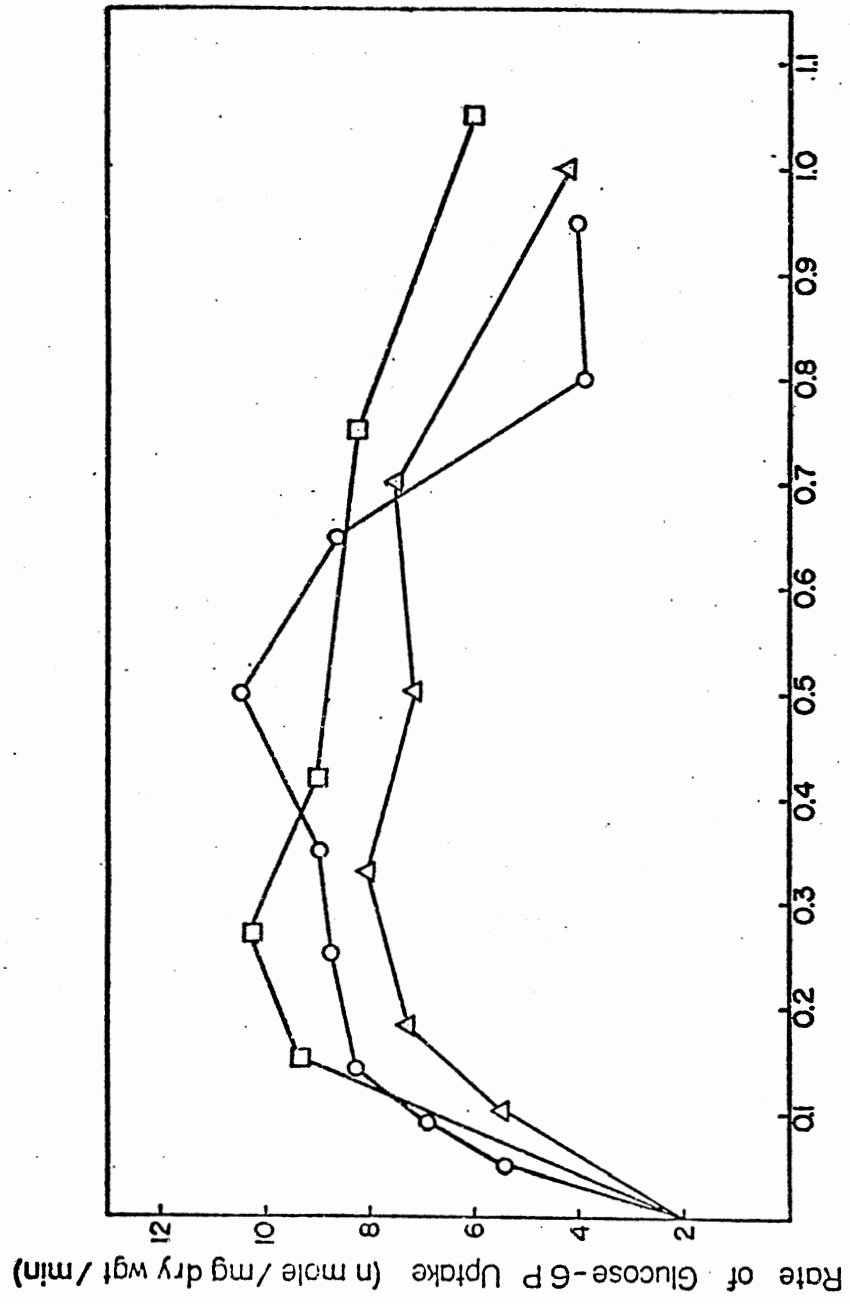

Rate of Glucose-6 P Uptake (n mole /mg dry wgt /min)
2
4
6
8
10
12
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
K+ Concentration (M)

Figure 21. Effect of Osmolarity on Glc-6-P Uptake Using KCl, $K_2SO_4$, and $K_3$citrate

Assays were performed as described in Figure 16.

KCl-o-o
$K_2SO_4$-Δ-Δ
$K_3$citrate-□-□

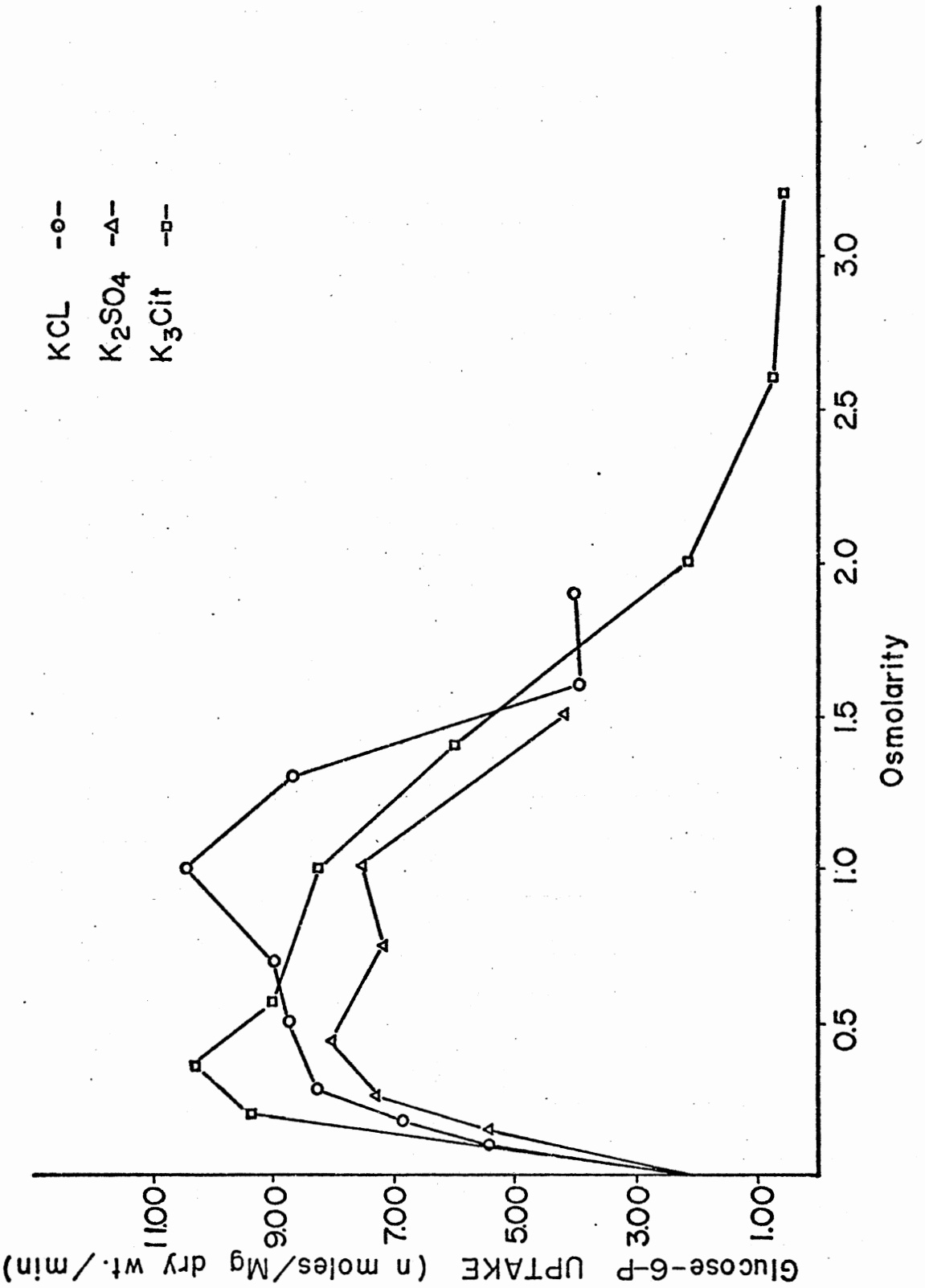
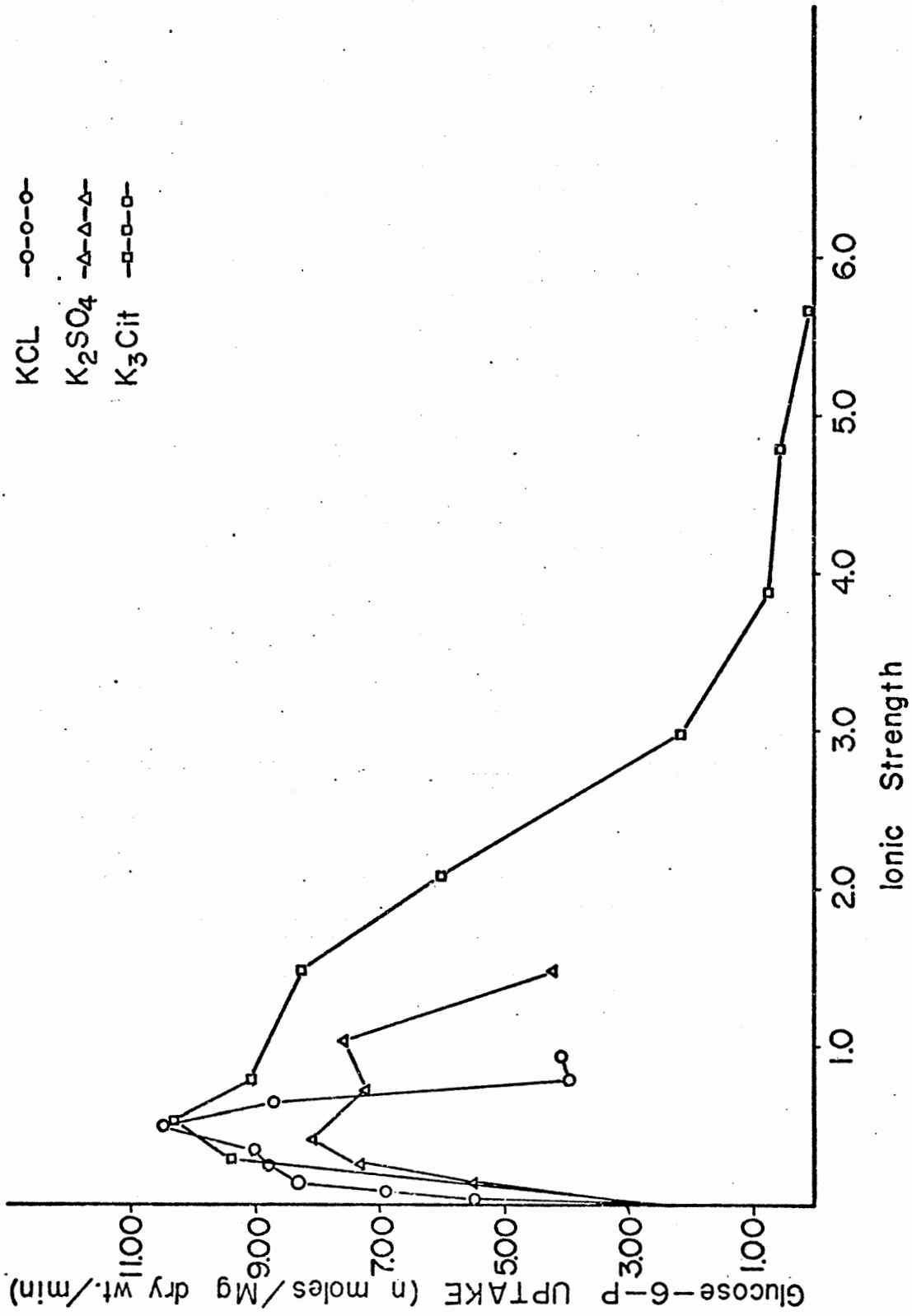

KCL -o-
$K_2SO_4$ -Δ-
$K_3Cit$ -□-
Glucose-6-P UPTAKE (n moles/Mg dry wt./min)
1.00
3.00
5.00
7.00
9.00
11.00
0.5
1.0
1.5
2.0
2.5
3.0
Osmolarity

Figure 22. Effect of Ionic Strength on Glc-6-P Uptake Using KCl, $K_2SO_4$, and $K_3$citrate

Assays were performed as described in Figure 16.

KCl-o-o
$K_2SO_4$-Δ-Δ
$K_3$citrate-□-□


KCL
$K_2SO_4$
$K_3Cit$
Glucose-6-P UPTAKE (n moles/Mg dry wt./min)
.001
.003
.005
.007
.009
.011
1.0
2.0
3.0
4.0
5.0
6.0
Ionic Strength

# CHAPTER V

# DISCUSSION

In this project both the proton to Glc-6-P stoichiometry ratios at various pHs of the external medium and the ion specificy of the hexose-6-phosphate transport system in E. coli cells have been investigated. The studies carried out have provided results which further characterize the underlying energy coupling to this transport system. Also, the results suggest other studies that might further elucidate the mechanisms of energy coupling.

As previously indicated by West and Mitchell (44), Essenberg and Kornberg (29), Ramos and Kaback (49), etc., results obtained from this type of study may be best understood when interpreted in a chemiosmotic framework. Therefore, all subsequent discussion precludes other interpretations less consistent with the data. In consideration of the findings from previous studies by Essenberg and Kornberg (29), and Ramos and Kaback (49) which strongly suggest the validity of this interpretation of the data, it is assumed that the hexose-6-phosphate system is at least a proton-Glc-6-P cotransport system.

## Proton to Hexose-6-Phosphate Stoichiometries from Studies with RE-48 Cells

The main purpose of determining the proton:Glc-6-P stoichiometries in cells by potentiometric and filtration methods was to test

the hypothesis of Ramos and Kaback (49) by a direct physical method in a whole cell system. This hypothesis is based on results obtained by the indirect method of flow dialysis on membrane vesicles. In brief, it states that the mechanism of energy coupling to the proton: Glc-6-P cotransport system changes as the result of increasing the external pH. At low pHs ($\sim$ 5.5) this cotransport system is electroneutral and coupled only to the $\Delta$pH. At high pHs ($\sim$ 7.5) this cotransport system becomes electrogenic as the result of an increase in proton:Glc-6-P stoichiometry to a value which is greater than the charge on the Glc-6-P molecule. Thus, Glc-6-P cotransport at the higher pHs is coupled only to the $\Delta\Psi$.

The stoichiometry experiments using direct potentiometric and filtration methods with RE-48 cells in the presence of KSCN have provided evidence which indicates, in general, that an increase in the proton-Glc-6-P stoichiometry occurs on increasing the external pH from 6.0 to 8.0. However, significant differences in the stoichiometry values between cells and vesicles have been observed, indicating that the energy coupling to Glc-6-P transport may be different for the two systems.

Figure 14 shows that the number of protons transported per Glc-6-P increases from about 1.0 at pH 6.0 to about 2.0 at pH 7.0 and 7.5 to about 3.8 at pH 8.0. Also, this figure shows that stoichiometry, in general, parallels the average net charge on the Glc-6-P molecule up to approximately pH 7.5. At values greater than pH 7.5, the stoichiometry values increase sharply. These values are assumed to level off at a value of about 3.8 at external pH 8.0, while the

net charge on Glc-6-P remains essentially constant at a value of approximately two. Since the proton:Glc-6-P stoichiometry does not exceed the value of the net charge on the Glc-6-P molecule until after pH 7.5, the proton:Glc-6-P cotransport is an electroneutral process throughout this range of external pHs. Thus, only the $\Delta$pH would be expected to couple to the transport in this pH range. Also, since the stoichiometry is greater than the net charge on the Glc-6-P molecule at external pHs higher than 7.5, this transport process must be electrogenic at these more alkaline pHs. Thus, only the $\Delta\Psi$ component would be expected to couple to this transport at these external pHs. Also, the fact that KSCN is required to see proton uptakes at pH 8.0, but not at lower pHs, suggests that Glc-6-P uptake occurs in an electroneutral manner at pH 7.5 and below, and in an electrogenic manner at pH 8.0. Proton uptake at pH 8.0 does not occur unless KSCN is present to break down the electrical ptoential generated by the proton-Glc-6-P cotransport with a proton:Glc-6-P stoichiometry of 3:1. At the lower pHs (6.0, 7.0, and 7.5) the Glc-6-P-induced proton uptakes occur even when the cells are not treated with KSCN, indicating that proton-Glc-6-P cotransport at these pHs is not electrogenic as indicated by the stoichiometry data at pH 7.0 and 7.5. At pH 6.0 the proton:Glc-6-P cotransport can be electroneutral, and other cations compensate for the difference between the proton-Glc-6-P stoichiometry and the net charge on the Glc-6-P molecule.

The stoichiometry value at pH 6.0 agrees well with that reported by Essenberg and Kornberg for RE-48 cells at pH 6.6 (1.0±0.1) (29). These investigators also used the West and Mitchell method of determining stoichiometry values. A similar trend in increasing protons

to Glc-6-P stoichiometry with increasing external pH as that observed in cells was previously reported to occur in E. coli membrane vesicles (49). However, the stoichiometry value determined at pH 5.5 (1.7 protons/Glc-6-P) is 0.8 greater than the value reported for cells at pH 6.0 (0.92±.06) (cf. Table II). The stoichiometry value for vesicles at pH 7.5 was reported as one more than the charge on Glc-6-P (Glc-6-P is a divalent anion at this pH). This value is 1.1 greater than the value (1.96±.18) determined for cells. In fact, the value reported for vesicles at pH 7.5 is more in line with the value (3.8) reported for cells at pH 8.0. It is certainly possible that these differences in proton-Glc-6-P stoichiometries are real ones which result from differences in the energy coupling to the two systems. Also, this conclusion can be drawn from a more significant apparent difference between these results for cells and vesicles which occur in the range of external pHs from 7.0 to 7.5. A comparison of the stoichiometry values for cells at these external pHs to the corresponding net charge on the Glc-6-P molecule indicates that the proton-Glc-6-P cotransport is electroneutral. This observation does not agree with the results of the ionophore titration results obtained with energized membrane vesicles at pH 7.5 which indicate that Glc-6-P cotransport is coupled only to the $\Delta\Psi$ at pH 7.5. It is this finding, in part, that led Ramos and Kaback to infer that the proton:Glc-6-P stoichiometry is 3:1 at this pH. For cells the values of the $\Delta$pHs alone are estimated to be about 60 mV at pH 7.0 and even less at pH 7.5 ($\sim$ 20 mV) (24). Since the steady state concentration gradients of Glc-6-P in vesicles are approximately 125 mV at these

external pHs, it is concluded that the ΔpHs alone are not large enough to account for the observed Glc-6-P concentration gradients even with a proton:Glc-6-P stoichiometry of 2:1. This implies that some other form of energy coupling is involved. The only other form available is the $\Delta\Psi$ which increases in magnitude from about 100 mV at pH 6.0 to about 150 mV at pH 8.0 (106). If the $\Delta\Psi$ could be coupled to Glc-6-P transport in cells at these external pHs, then the resulting $\Delta p$ would certainly be large enough to accommodate the observed Glc-6-P concentration gradients. The $\Delta\Psi$ can couple to Glc-6-P transport only when the net charge of Glc-6-P transport is positive. Since the observed proton-Glc-6-P stoichiometries are only 2:1 at pHs 7.0 and 7.5, other mechanisms by which this transport can become electrogenic must be involved. There are at least two possible mechanisms which are consistent with the stoichiometry data and allow for electrogenic Glc-6-P transport. In the first mechanism other cations are coupled to the proton-Glc-6-P cotransport such that the net charge becomes positive. Since in vesicles three cations are cotransported per Glc-6-P at pH 7.5, it seems likely that three cations would be cotransported with Glc-6-P in cells at this pH. For example, a $K^+$-$2H^+$-Glc-6-$P^=$ cotransport mechanism may occur with one full positive net charge. The second possible mechanism is countertransport of an anion. By this mechanism the cotransport of two protons and one Glc-6-P molecule is coupled to the efflux of an anion. The ion specificity results suggest that $HCO_3^-$ ion may be involved in this type of mechanism, since increasing concentrations of $HCO_3^-$ ion appears to inhibit Glc-6-P transport partially as compared to the

transport activity in 140 mM KCl. The net charge of $2H^+$-Glc-6-$P^=$ ($HCO_3^-$) cotransport by the countertransport mechanism at pHs 7.0 and 7.5 is one full positive charge. Since the proton-Glc-6-P stoichiometry values increase to at least a value of 3.0 at pH 8.0 for cells, the requirement of either a cation or countertransport mechanism in proton-Glc-6-P transport in cells may no longer be necessary. In order to determine which of the two possible mechanisms may be used in Glc-6-P transport, additional studies will be required.

From the change in proton:Glc-6-P stoichiometry with increasing pH, one can get some evidence concerning the specific groups on the carrier which are involved in the cotransport process. If it is assumed that the proton:Glc-6-P stoichiometry values for RE-48 cells with KSCN do not increase much above the value of 3.0 at the higher external pHs, then one gets a value of about 7.8 (cf., Figure 14) for the $pK_a$ of the group whose titration this part of the curve depends on. The validity of this assumption is indicated by the observed stoichiometry value (2.38±.22) at 15 seconds uptake (cf., Figure 13). This $pK_a$ value suggests the involvement of a sulfhydryl group(s) of cysteine ($pK_a$=8.33) in the cotransport process. This suggestion is also supported by the finding of Essenberg and Kornberg (29) that alkylating agents (iodoacetate, iodoacetamide, and N-ethylmaleimide) which readily react with sulfhydryl groups inhibit Glc-6-P transport.

In general, the stoichiometry values increase with increasing external pH as a result of both a gradual decrease in Glc-6-P uptake and a gradual increase in proton uptake. The most marked increase in proton uptake is observed when comparing the 15 and 30 second average

values at pHs 7.5 and 8.0. The difference between the 15 second values at pH 7.5 and that at pH 8.0 is approximately two nanoequivalents. The direct comparison of average uptake values may be misleading, since uptakes vary considerably from one culture to the next an any given external pH. However, major trends in the proton and Glc-6-P uptakes may be observed. This claim would appear to be valid because differences in uptakes due to the different transport capabilities of each culture appear to be of less magnitude, in general, than that which results from a particular trend.

The proton and Glc-6-P uptakes occur as might be expected at pHs 6.0 and 7.0. However, at pHs 7.5 and 8.0, the proton trace exhibits an initial uptake followed by a sharp decline at times greater than 30 seconds. This phenomenon appears to occur to a larger extent at pH 8.0. However, at both pHs the proton traces decrease below the baseline, indicating that this decrease may result from active pumping of protons from the cells. If this activity were due to a rapid leak of protons one would expect the rapid decrease to stop and level off at the baseline. If active proton pumping does occur, then extrapolation to zero time of the stoichiometry ratio values is an inaccurate method of determining the best estimate of the proton-Glc-6-P stoichiometry values. The purpose of the extrapolation is to correct the stoichiometry for any leakage of protons, which is expected to increase with time. If rapid pumping occurs the proton:Glc-6-P ratio values will be underestimated, since the proton uptakes will be less by an amount corresponding to that which is actively pumped out. Since the effect of the active proton pumping is greater with increasing

time of uptake, the underestimate of the proton-Glc-6-P stoichiometry ratios will become larger with time of uptake. Therefore, when these reduced ratio values are extrapolated to zero time, the proton: Glc-6-P ratio value obtained will be an overestimate of the true value (an example of an overestimation of the true stoichiometry value is illustrated in Figure 13). From the above, it is obvious that the extrapolation corrects only for passive proton leakage. In the cases where active proton pumping occurs, steps must be taken to eliminate this activity such that an accurate estimate of the stoichiometry values may be determined.

## Proton and Hexose-6-Phosphate Stoichiometries from Studies with uncA Mutants

One way of eliminating the possible active proton pumping which could cause an underestimate of the proton uptakes at all external pHs is to use mutants which are genetically blocked in specific metabolic processes resulting in proton pumping. The mutant of choice is the uncA mutant which is genetically uncoupled and lacks a functional ATPase. This mutant is not only blocked in the ATPase function, but also in aerobic electron transport since the assay conditions are anaerobic. Thus, the mutant can neither hydrolyze ATP by the ATPase, nor can it transfer electrons down the electron transport chain. Due to the lack of both of these functions, it cannot actively extrude protons into the medium unless it has the capacity for anaerobic respiration.

Direct tests show that anaerobic respiration does not occur under the conditions of the assay. 2-deoxyglucose-6-P, a nonmetabolizable

substrate analog, was used in transport studies with the uncA mutants, since they were not metabolically blocked for Glc-6-P utilization as was RE-48. In general, the uncA mutants, RE-114, RE-115, and RE-157 gave low proton uptakes compared to the corresponding levels of 2-deoxyglucose-6-P uptakes. Thus, it was not possible from these experiments to conclude whether the stoichiometry values were underestimated. However, two observations indicate that the effect of active pumping was minimal, if any, on the Glc-6-P-induced proton uptakes with RE-48 cells at external pHs 6.0 and 7.0, and perhaps pH 7.5. First, the proton uptakes do not exhibit a sharp decrease in uptakes. Second, the proton uptakes level off and do not go below the baseline, except at pH 7.5 for cells treated with KSCN, indicating that only passive leakage of protons occured at pH 6.0, 7.0, and 7.5.

Stoichiometry experiments with RE-114 and RE-115 cells required incubation with DCCD to prevent proton leakage which occurs because the defective $F_1$ portion of the ATPase permits leakage of protons through an exposed proton conducting pathway of $F_0$ (43, 107). This leakage must be eliminated since it would also lead to an underestimate of the stoichiometry ratio values. The DCCD is thought to react with a protein component of the $F_0$ such that the proton conducting pathway becomes sealed and the leakage is decreased to the level of the wild type cells. The low proton uptakes with RE-114 and RE-115 cells may have resulted from proton leakage through the ATPase, since only one-fourth of the concentration of DCCD used in the original reports was used (107). It is also possible that the low proton uptakes with RE-114 and RE-115 cells resulted from the DCCD reacting with the

proteins involved in proton-Glc-6-P transport and/or the energy coupling process or perhaps 2-deoxy-Glc-6-P transport was uncoupled to proton uptakes. DCCD will react and form chemically stable adducts with various functional groups of proteins including sulfhydryl groups (108).

All of the studies with the uncA mutants at pH 8.0, using 2-deoxyglucose-6-P showed proton uptakes that were unaffected by active proton pumping since they neither exhibited a sharp decrease beyond 15 seconds, nor did they descend below the baseline. This finding suggests that the ATPase of RE-48 cells may be involved in pumping protons, at least at pH 8.0.

## Possible Uncoupling of Glc-6-P Uptake from Proton Uptake

A number of features of the proton and hexose-6-P uptakes and the resulting stoichiometries indicate that the hexose-6-P-induced proton uptakes may not be tightly coupled to the hexose-6-P uptakes, and/or that the cells are leaky to protons under the conditions of the assay at various pHs: 1) The initial low Glc-6-P-induced proton uptakes at pHs 6.0 and 7.0. These could be interpreted as either a lag in uptakes, or as an initial proton leak which is more apparent at this time, because the proton uptakes are of lowest magnitude. The lag suggests that the Glc-6-P cotransport can couple to something other than protons. As indicated by the proton-Glc-6-P stoichiometries beyond 30 seconds, proton fluxes are coupled to the true coupling ion. 2) Low 2-deoxy-Glc-6-P-induced proton uptakes by typical 2-deoxy-Glc-6-P uptakes in KSCN treated RE-48 cells and RE-157 cells at pHs 6.0

and 8.0, respectively. The proton-2-deoxy-Glc-6-P stoichiometry was only 0.59±.22 for RE-157 cells at pH 7.5. Thus, other cations may have been involved in these uptakes. Proton leakage may have also contributed to the observed low proton uptakes. 3) Normal Glc-6-P uptakes without proton uptake in RE-48 cells in the absence of KSCN at pH 8.0. In contrast, both proton and Glc-6-P uptake occurs as expected in cells treated with KSCN at this pH. Thus, proton uptake is apparently uncoupled from Glc-6-P uptake without KSCN at this pH. Proton leakage does not appear likely, unless KSCN somehow stops the leak which seems improbable. Since proton uncoupling may have occurred, other cations (e.g., $K^+$) may have coupled to Glc-6-P transport. Other possible mechanisms of Glc-6-P transport are Glc-6-$P^=$ uptake followed by compensating $K^+$ ion fluxes, or countertransport of $2HCO_3^-$ per Glc-6-$P^=$. In order to distinguish between these possibilities, additional studies will be required.

This is the first report of experimentally induced uncoupling of a proton-substrate cotransport system. The uncoupling mechanism may be of biological importance, since it may be advantageous to E. coli cells in a general deenergized state (a condition of the cells under the experimental conditions) to have the capacity to couple more than one ion gradient to the cotransport of a metabolite. This is of particular importance since this metabolite can be readily metabolized to produce ATP via glycolysis and oxidative phosphorylation without the initial expenditure of ATP. Further, it seems possible that such a versatile ion coupling mechanism may offer the cells an added selective advantage during the process of evolution when both metabolite supply and internal energy stores were very limited.

## Other Potential Complications and Corrective Measures

The main advantage of using whole cells in this stoichiometry study is that cells are readily prepared, as opposed to membrane vesicles which require extensive preparation (109). Incidentally, Glc-6-P-induced proton uptakes in vesicles cannot be measured potentiometrically for reasons which are not well understood (110). Despite this advantage, cells are very complex systems. They provide a number of complications which must be effectively dealt with in order that the desired parameters can be accurately measured.

The stoichiometry experiments were designed to measure the transport of protons in response to facilitated diffusion of Glc-6-P down its concentration gradient in nonmetabolizing cells.

Certain measures were taken to eliminate or test the effect of various problems such as metabolism of the transported substrate, active proton extrusion, and proton leakage.

The problem that may interfere most with the proton and Glc-6-P uptakes is active proton extrusion due to cellular metabolism. A number of precautions were taken to reduce and, perhaps, eliminate this problem. Since E. coli cells are facultative anaerobes, their cellular metabolism can occur both aerobically and anaerobically. In order to block the active proton extrusion resulting from aerobic metabolism, studies were carried out under anaerobic conditions using a special mutant, RE-48, which is genetically blocked for the synthesis of phosphoglucoisomerase and Glc-6-P dehydrogenase to prevent Glc-6-P utilization. Since both of these enzymes are nonfunctional,

Glc-6-P could not be metabolized by either glycolysis or by pentose phosphate shunt. The Glc-6-P could possibly be converted to glycogen. This pathway is thought not to occur to any great extent in E. coli and does not generate acid. Since the uptakes were carried out under anaerobic conditions, endogenous energy stores could not serve as substrates for the aerobic electron transport chain. Thus a $\Delta p$ could not be generated by aerobic respiration. In these cells it is possible that endogenous energy stores could have generated ATP by substrate-level phosphorylation and a $\Delta p$ may have resulted from the hydrolysis of ATP by the $Ca^{2+}$, $Mg^{2+}$-ATPase. It is important that no hydrolysis of the ATP occur, since any active proton pumping would interfere with the accurate measurements of the hexose-6-P-induced proton uptakes. Active proton extrusion seemed not to have occured because the Glc-6-P was concentrated only approximately twofold in the cells used in these stoichiometry experiments indicating that a $\Delta p$ was not present. This determination assumes an internal cell volume of 2.7 µl per mg dry weight.

Since RE-48 possesses only point mutations in the pgi and zwf genes there is a possibility that it produced only partially inactive forms of the corresponding enzymes. Thus, Glc-6-P metabolism still might take place, but at a rate much slower than in the wild-type cells. Another mutant strain, RE-90, possessing a zwf deletion and a Mu phage insertion in the pgi gene was used in stoichiometry experiments at pH 8.0 to insure that the Glc-6-P was not metabolized. These blocks are expected to result in a complete loss of protein. Results similar to those with RE-48 cells were obtained, indicating that the

proton pumping was apparently not due to utilization of Glc-6-P by either glycolysis or the pentose phosphate pathway. There is always the possibility that the Glc-6-P was metabolized by some other pathway which has yet to be discovered.

In order to prevent anaerobic respiration which might have occurred the cells were grown under very aerobic conditions. The cells were grown in Erlenmeyer flasks filled to only 1/10 volume which were vigorously shaken on a reciprocal shaker. This procedure was carried out because aerobic conditions result in the repression of synthesis of many key enzymes which function in anaerobic respiration (cf., page 9). Also, anaerobic respiration via fumarate reductase activity was checked and found to be absent in cells at pH 6.0 and 7.5.

Since proton leakage could occur in aged cells, only freshly grown cells were used in the stoichiometry experiments. Cells which were kept in 140 mM KCl for as long as 6 to 8 hours showed significantly less proton uptake than that obtained with freshly prepared cells, but Glc-6-P uptakes were only slightly less than expected. This observation also indicates that Glc-6-P transport in aged cells is not well coupled to proton uptake.

## Summary of the Hexose-6-Phosphate Stoichiometry Studies

In summary, stoichiometry experiments using direct potentiometric and filtration methods with RE-48 E. coli cells in the presence of KSCN have provided evidence which indicates that an increase occurs in the proton:Glc-6-P stoichiometry on increasing the external pH

from 6.0 to 8.0. However, significant differences between the stoichiometry values for cells and membrane vesicles have been observed, indicating that the energy coupling to Glc-6-P transport may be different for the two systems.

Stoichiometry values of 0.92±.06 and 1.21±.01 were determined at external pH 6.0 for cells with and without KSCN, respectively. These values increased to 2.20±.15 and 1.96±.18 at external pH 7.5. At external pH 8.0 a value of 3.8 was determined for cells in the presence of KSCN. Comparison of the proton:Glc-6-P stoichiometry values at a given external pH to the corresponding net charge of the Glc-6-P molecule suggests that this cotransport may be electroneutral, and thus coupled to the $\Delta pH$ at external pHs from 6.0 to 7.5. At external pH 8.0 the cotransport system is electrogenic and should be coupled to the $\Delta\Psi$. The increase in stoichiometry between pH 7.5 and 8.0 indicates that a functional group responsible for the increase in proton:Glc-6-P stoichiometry has a $pK_a$ of about 7.8. A sulfhydryl group(s) of cysteine may be involved in Glc-6-P transport.

The stoichiometry value determined with RE-48 cells treated with KSCN at pH 8.0 may be an overestimate, since apparent active proton pumping occurred. Stoichiometry studies with uncA mutants at pH 8.0 gave 2-deoxy-Glc-6-P-induced proton uptakes which did not show the sharp reversal, suggesting that the ATPase may be involved in this reversal with RE-48 cells at pH 8.0.

That hexose-6-phosphate uptakes may be uncoupled from proton uptakes and that other cations may be involved in this transport is suggested by the lag in proton uptakes at pHs 6.0 and 7.0; the absence

of proton uptakes, but typical Glc-6-P uptakes in RE-48 cells untreated with KSCN at pH 8.0; and low 2-deoxy-Glc-6-P-induced proton uptakes with RE-48 cells and RE-157 cells treated with KSCN at pHs 6.0 and 8.0, respectively.

A number of other studies could be performed to further characterize ion coupling to Glc-6-P uptake. Direct ion flux measurements (e.g., $Na^+$, $K^+$), along with measurement of proton and Glc-6-P uptakes would show whether fluxes of these other ions are induced. Labeled cations and/or ion selective electrodes would probably be the most desirable methods for these studies since both are commercially available. Indirect studies with cells under specific cation-free conditions may provide results showing the cation requirements, e.g., studies carried out in choline may indicate a $K^+$ requirement. However, obtaining cation-free conditions may be difficult due to cation leakage from the cells. Studies with RE-48 cells treated with aresenate may provide Glc-6-P-iduced proton uptakes which are not perturbed by active proton pumping. Therefore, the question whether or not the stoichiometry ratio values are underestimated at all pHs might possibly be answered.

## Ion Specificity of the Hexose Phosphate Transport System

### The Effect of Cations on Glc-6-P Uptake

From the observed pattern of stimulation the monovalent cations exhibit a higher degree of specificity for stimulating Glc-6-P uptakes than do the anions, since there are smaller differences exhibited

between the anions and no clear correlation with any property of the anions.

The studies with monovalent cations confirm and extent the work reported by Essenberg and Kornberg (29), who first demonstrated that KCl at various concentrations up to 140 mM stimulated Glc-6-P uptake. The results in Figures 15 and 16 extend the original observations, since they illustrate that $K^+$, $Na^+$, and other alkali ions ($Rb^+$, $Cs^+$) optimally stimulate Glc-6-P uptakes at higher salt concentrations than previously studied. The optimal salt stimulation occurs throughout a concentration range from 140 mM to 500 mM. The fact that increasing concentrations of $tris^+$ and $Me_4N^+$ ions produce a pattern of stimulation of Glc-6-P transport which is different from that resulting from other ions may indicate that a different mechanism of stimulation is involved. Since neither of these ions are readily taken up by the cells, it is suggested that their activity is independent of ion gradients.

The cation experiments designed to determine the relationship between ion size and stimulation of Glc-6-P uptake have provided evidence that the cation is interacting with a binding site whose highest affinity is for ions with a radius of 1.7Å. Ammonium ion appears not to conform too well to the scheme described above, since its specificity is difficult to explain on the basis of size. Its radius (1.48 Å) is the same as that of rubidium ion which is very effective in stimulating transport. Evidence has been reported which indicates that possibly two energy dependent ammonium transport systems function to transport ammonium in E. coli cells (111, 112). One of these systems

is quite sensitive to the uncoupler, CCCP, at pH 7.0. This finding may indicate that a ΔpH across the membrane can be coupled to this transport. Assuming that this transport system is coupled to the ΔpH, or possibly the Δp, then it might be that ammonium transport occurring during the period of the Glc-6-P uptakes is partially depleting the ΔpH. This partial deplation may result in a slightly reduced level of stimulation of Glc-6-P uptake by ammonium ion.

This pattern of salt stimulation seems to be unique compared to other salt-stimulated systems. In other cases, only sodium ion stimulates transport, in particular, when the transport systems are coupled to sodium gradients. However, there are reports of lithium ion stimulating thiomethylgalactoside transport in both S. typhimurium (113) and E. coli (68), and proline transport in E. coli (114). For these transport systems it is possible that the binding sites would be optimal for the smaller ions with radii near 1.0 Å, and would exclude potassium (1.33 Å). Glutamate transport in E. coli requires not only sodium, but also potassium (63). This system is apparently coupled to the sodium gradient, and shows an intracellular potassium requirement. The potassium requirement can only be detected in cells that are depleted of internal potassium. Also, it is reported that marine pseudomonads have an internal requirement for potassium in the transport of α-amino isobutyric acid (α-AIB) (73). Sodium ion also stimulates this transport, but apparently sodium and potassium gradients are not required, since the $K_m$ and $V_m$ for α-AIB influx was unchanged when the standard suspension medium was replaced by one containing $Na^+$ and $K^+$ concentrations which were present in respiring cells (74). Sodium or potassium stimulated transport of all amino

acids studied in H. halobium, but only glutamate transport appeared to couple to a $Na^+$ gradient (66).

It seems unlikely that potassium and/or sodium ion gradients would both stimulate Glc-6-P uptake by the same mechanism, since E. coli cells normally maintain oppositely directed gradients of these ions (59). The sodium gradient is directed inward, and the potassium gradient is directed outward even at very high external $K^+$ concentrations ($\sim$ 200 mM) (115).

In contrast to the stimulation of Glc-6-P uptakes by the alkali ions (excluding $Li^+$), the pattern of stimulation by $MgCl_2$ is quite different. This may indicate that $Mg^{2+}$ stimulated by a different mechanism from that of the alkali ions. The physiological importance of this divalent cation in ATP synthesis and other metabolic processes might suggest that the stimulation of Glc-6-P uptake may result from the stimulation of the energy producing processes.

Recent studies by Roomans and Borst-Pauwels (116, 117) on the effect of divalent and univalent cations on two separate phosphate transport systems in Saccharomyces cerevisiae have provided evidence that may explain the $Mg^{2+}$ stimulation and the univalent cations stimulation for anion transport systems. Studies on the $Na^+$ dependent phosphate system at pH 7.2 show that bivalent cations at low concentrations (4-20mM) stimulate phosphate uptake. In the $Na^+$-independent system one phosphate is transported with the influx of $2H^+$ and the efflux of $1K^+$. Results from kinetic studies show that bivalent cations increase the apparent affinity of phosphate for the transport system and decrease that for the $Na^+$. In the $Na^+$-independent system they

find that bivalents stimulate at pH 4.5, but inhibit at pH 7.2. The diverse effects of the divalent cations can be explained by a decrease in the surface potential. These investigators have offered theoretical evidence which shows when an anion is cotransported with cations both the magnitude of the effect of a decrease in surface potential and its modulation (stimulation, or inhibition) depends on the concentration of the cation. At high pH the decreased $H^+$ concentration near the membrane resulting from the decrease of the surface potential may cause cotranport inhibition as is the case with the $Na^+$ independent system. At low pHs the proton-binding sites are protonated and may not be significantly affected by the cations. Thus, the increase in phosphate concentration near the membrane (due to a decrease in the surface potential) results in stimulation of phosphate transport (116).

Other divalent cation studies have shown that the addition of optimum concentrations of $MgCl_2$ to cells in the presence of 140 mM KCl produces no additional stimulation of Glc-6-P uptakes above the levels obtained with KCl alone. These studies employed many other divalent cations ($Ca^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Zn^{2+}$, $Sr^{2+}$, $Ba^{2+}$, $Fe^{2+}$, $Be^{2+}$, $Sn^{2+}$, and $Ni^{2+}$) which were extensively tested throughout a range of 50 μM to 5 mM. In all cases there resulted no appreciable effect on Glc-6-P uptake (102). The results of these studies may indicate that $Mg^{2+}$ and the monovalent cations are stimulating Glc-6-P uptakes by the same mechanism. It is difficult to justify the $Mg^{2+}$ stimulated Glc-6-P uptakes based on the same monovalent cation model which requires the ion to interact with a site favoring 1.7 Å radius, since monovalent

ions with radii as small as that of $Mg^{2+}$ (0.65 Å) do not stimulate very well. However, monovalent cations at higher concentrations may also cause a decrease in the surface potential and produce a stimulatory effect. Studies on the effect of univalent cations on the $Na^+$ independent phosphate transport system in S. cerevisiae show that $K^+$, $Rb^+$, and $Cs^+$ stimulate phosphate uptake. These alkali ions were less effective than the divalent ions at low concentrations. However, 30 mM $K^+$ produced a stimulation about 40% as large as that obtained with the divalents at lower concentrations. The monovalent cation stimulation is thought to have resulted from a decrease in the surface potential (118). The fact that both monovalent and divalent ions may decrease the surface potential, indicates that their effects of stimulation of Glc-6-P uptakes may occur by the same mechanism even though the degree to which each stimulates is quite different depending on the ion concentrations. This conclusion is supported by the evidence from the divalent cation studies showing that $Mg^{2+}$ did not stimulate above the level of stimulation obtained with 140 mM KCl.

The Effect of Anions on Glc-6-P Uptake

As stated above, the anions effected only small differences in Glc-6-P transport, except for fluoride ion and perhaps $HCO_3^-$ which showed considerable inhibition at times. Fluoride ion has been shown to inhibit several enzymes involved in glycolysis and gluconeogensis such as enolase and phosphatases (119). Fluroide ion may adversely affect either energy production for driving transport, or proteins involved directly in the transport process. The inhibition caused by

$HCO_3^-$ may occur because Glc-6-P uptake proceeds by a countertransport mechanism. This inhibition would be expected to occur when the counter ion ($HCO_3^-$) was at high concentration in the external medium. The high external concentration would oppose the efflux of the counter ion, resulting in an inhibition of Glc-6-P uptake.

Acetate and ammonia have been shown to diffuse freely through membranes and collapse the pH gradient depending on the orientation of the gradient (120, 121). However, the electrical potential is not affected. They are thought to pass through the membrane in their uncharged forms. Acetate is expected to break down a pH gradient which is alkaline inside, while ammonia should break down that which is acid inside. Since K-acetate did not stimulate as well as KCl, perhaps acetate did partially deplete the $\Delta$pH across the membrane.

The results of the experiments comparing the KCl, $K_2SO_4$, and $K_3$citrate at different concentrations indicate that the correlation of Glc-6-P uptake with anion concentration, osmolarity, ionic strength, or potassium concentration was not good. This fact may suggest that the stimulation of Glc-6-P uptake activity is more complex than that which can be accounted for by a single property of an ionic solution.

## Speculations Concerning the Possible Mechanisms of Cation Stimulation

Since little is known about the molecular mechanisms by which substrates are transported, it is not too surprising to find that even less is known about mechanisms by which cations stimulate the transport of substrates. Results from the ion specificity studies suggest

a number of possible mechanisms by which Glc-6-P transport might be stimulated. The relationship established between ion size and stimulation of transport suggests that a specific binding is necessary for optimal activity. This implies that the monovalent cations may be stimulating by binding to the carrier in either a cotransport mechanism, or perhaps, as a cofactor. The fact that $Mg^{2+}$ stimulates may imply that the energetics of the cells are involved, since $Mg^{2+}$ is known to be involved in the energy producing metabolic processes.

Of all of the possible mechanisms by which the ions might be stimulating Glc-6-P transport, it seems likely that the cotransport mechanism is the one most readily testable. The cofactor and energy coupling mechanisms may be difficult to test directly. The cofactor mechanism can be supported by negative results obtained from studies on the other possible mechanisms. Studies on the stimulation resulting from stimulation of the energetics of the cell might involve measurements of the magnitudes of the energy parameters of the cells and correlating these results with the amount of stimulation of Glc-6-P uptake. This may be an extremely difficult study if at all possible. The methods for studying the stimulation of uptakes by a cotransport mechanism have been discussed above. Direct ion flux studies with either labeled cations (e.g., $^{22}Na$) or specific ion electrodes are necessary in this study. If the ions are coupled to Glc-6-P uptake, it might be expected that a stoichiometric amount of the cation would be taken up along with Glc-6-P. Thus, it may be possible to quantify the amount of cation taken up with the amount of Glc-6-P uptake such that the cation-Glc-6-P stoichiometry can be determined. Also, it may

be possible to use specific ionophores (e.g., monensin) in order to deplete suspected cation gradients (122). Depleting the ion gradient which is coupled to the uptake system should at least reduce the amount of Glc-6-P transport.

There appears to be many other possible mechanisms which may be involved in cation stimulation of Glc-6-P uptakes, but they seem not so readily testable. For example, the cations at high concentration might be affecting the water structure of the hydrated Glc-6-P molecules such that they are more capable of being transported across a water-lipid-protein interface. If this were true, one might expect the ions to stimulate according to the Hofmeister ion series. However, there is poor correlation between the series of anions and cations ranked in order of most to least stimulating and the Hofmeister series of ions. The latter series is ranked from most to least effective in salting-out of euglobulins (123). This fact indicates that the ions may not be affecting the solubility of the proteins, making them more accessible to their substrates. Also, cations might be interacting with the negative charges on the membrane phospholipids (i.e., membrane surface charges) which may facilitate the transport of the negatively charged Glc-6-P molecules. This type of interaction results in a decrease in the surface potential. The results cited above by Roomans and Borst-Pauwels strongly suggest that this mechanism of ion stimulation may account for that which occurred in the Glc-6-P studies. Studies concerning the role of these and other possible mechanisms of cation stimulation of Glc-6-P transport might require special methods which certainly go beyond the original scope and objectives of this research project.

Hopefully, the results of the studies presented here have provided a sufficient characterization of the ion specificity such that subsequent studies based on this work can provide additional answers to some of the important questions concerning the identification and description of the mechanism(s) of cation stimulation of Glc-6-P transport in E. coli.

## Summary of the Ion Specificity of the Hexose-6-Phosphate Transport System

The observed pattern of stimulation of the monovalent cations indicates a higher degree of specificity for stimulating Glc-6-P transport than that observed for the anions, since the anions exhibit only small differences. Studies with a series of monovalent cations show that ions with crystallographic radii near 1.7 Å ($Cs^+$, $Rb^+$, $K^+$, and $NH_4^+$) stimulate Glc-6-P transport optimally throughout a concentration range from 140 to 500 mM. Cations with radii smaller ($Na^+$, $Li^+$) or larger ($Me_4N^+$, choline$^+$) than 1.7 Å are less effective or ineffective in stimulating Glc-6-P transport. Since tris$^+$ and $Me_4N^+$ produce a unique pattern of stimulation which is not as large as that of the alkali ions, it is suggested that this mechanism of stimulation may be quite different. $Mg^{2+}$-stimulated Glc-6-P transport also appears to be unique and may be effected by the indirect stimulation of the energetics of the cells. $Mg^{2+}$ stimulates optimally over a narrow range from 10 to 20 mM. Based on evidence which indicates that monovalent and divalent cations stimulate anion cotransport systems by effectively decreasing the surface potential, it is suggested that this effect may also account for the cation stimulation of Glc-6-P transport.

Fluoride ion and perhaps $HCO_3^-$ inhibit Glc-6-P uptakes when compared to the uptakes with 140 mM KCl. The complexity of the nature of the stimulation of Glc-6-P uptakes is suggested based on results from studies comparing KCl, $K_2SO_4$, and $K_3$citrate at different concentrations, indicating that a poor correlation exists between this stimulation and anion concentration, potassium concentration, osmolarity, or ionic strength. There appear to be many possible mechanisms by which cation might stimulate Glc-6-P transport. It is suggested that the cotransport mechanism of cation stimulation is the most readily testable.

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